Population Structure and Dynamics of *Campylobacter* Populations Carried by Wild Birds and Chickens Reared in a Free-Range Woodland Environment.

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Thesis Submitted in Partial Fulfillment for the Degree of Doctor of Philosophy

September 2006

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Frances M. Colles, St Catherine's College D.Phil thesis, Michaelmas Term 2006

Abstract.

Ingestion of contaminated chicken meat is a major cause of *Campylobacteriosis* in Europe and the USA. The environment, including wild birds, is considered to be an important reservoir for chicken colonization. The aims of this study were to determine the population structure of *Campylobacter* amongst chicken and wild bird sources on a single farm, and to establish the extent to which genotypes flow between them and ultimately infect humans, using MLST and antigen sequence typing.

A pilot study amongst farm animals and wild birds in Lancashire demonstrated that *Campylobacter* genotypes from human disease were common on the farm and could be isolated from more than one animal source. Between 30-50% of wild geese and Starlings were shedding *Campylobacter*, with a seasonal peak in shedding rate in Spring. Genotypes were divergent from those previously isolated from human disease, retail meat and farm animal sources, with the majority being restricted to the host source. The carriage rate of *Campylobacter* was between 70-100% amongst 78 free-range poultry flocks tested at 56 days of age. Up to seven genotypes were found to co-exist within a flock, and genotypes varied throughout the year on a random basis.

Some *Campylobacter* strains were isolated from one farm site only, but a small percentage of them had spread nationally and were stable over a period of a decade. A total of 23% of *Campylobacter* isolates from free-range chickens were indistinguishable to those from human disease, and 5% were indistinguishable from wild birds. A total of 6% of genotypes isolated from wild birds were indistinguishable from those isolated from human disease. Wild birds could not be completely disregarded as a potential reservoir of *Campylobacter* for both humans and poultry, but their role is likely to be limited.

Preface.

The work included in this thesis is my own with the exception of the nucleotide sequence data for *Campylobacter* isolates from human disease and retail chicken meat that are held on the *Campylobacter* MLST database. In addition nucleotide sequence data for *Campylobacter* isolates from Oxfordshire cattle were provided by Anna Harnmeijer, and *Campylobacter* isolates from animals in Lancashire were provided by Dr Keith Jones. The databases used for storing data collected from all animal species were constructed by Dr Keith Jolly. Julian Howe collected faecal samples from Starlings and recorded data relating to Starling age, sex, weight and wing length. Julian Howe and Phil Smith collected faecal samples from chickens during the second year of the study.

Some of the work in this thesis has been published and a copy of the paper can be found in the appendices; Colles, F.M., K. Jones, R.M. Harding and M.C. Maiden, 2003. Genetic diversity of *Campylobacter* isolates from farm animals and the farm environment. Applied and Environmental Microbiology 69:7409-13. No part of this work has been submitted for any other degree.

F.M. Colles
September 2006

Acknowledgements.

I would like to thank Martin Maiden my supervisor for his great enthusiasm and support, and for allowing me to follow my research interests in the area of zoonotic disease. My thanks also, to Angela Mclean who helped to design the chicken study at the beginning. I am very grateful for the support and friendship of colleagues past and present, and in particular Dr Keith Jolley for his endless patience with computer 'problems', no matter how trivial, Dr Noel McCarthy for his valuable advice with statistics, being able to see the wood for the trees, to Roisin Ure for providing backup with the microbiology and to Lynne Richardson and Becky Busby for operating the automated sequencers. Thanks must also go to Ruth Layton at the Food Animal Initiative based at the Oxford University Farm in Wytham for her help, advice and eagerness in providing animals for sampling of *Campylobacter*. I am extremely grateful to Phil Smith and Julian Howe from the Department of Zoology who helped me collect samples from chickens and to Julian, Dr Andy Gosler and Dr Claire Devereux from the Edward Grey Institute, Department of Zoology for collecting samples from Starlings. I would also like to thank Rosalind Harding from the Department for Zoology for her advice with Arlequin and F_{ST} analysis, to Anna Harnmeijer, a student in the Department of Zoology, for allowing me to use her data from cattle, and to Dr Keith Jones from Lancaster University for kindly providing *Campylobacter* isolates from animals in Lancashire. All of the work was funded by the UK Department of Environment, Food, and Rural Affairs (DEFRA) to whom I am grateful. And finally I would like to thank my friends and family and most especially Nick Webb for their continued support and encouragement.

Contents

List of figures.

List of tables.

Abbreviations.

aspA	aspartase A locus
BURST	'Based upon related sequence types' software package.
C	Central genotype
DLV	Double locus variant
flaA	flagellin A locus
F_{ST}	An estimate of gene flow in combination with standardized variance of allele frequency.
GBS	Guillain Barré Syndrome
glnA	glutamine synthetase locus
gltA	citrate synthetase locus
MLST	Multilocus sequence typing
MLEE	Multilocus enzyme electrophoresis
MFS	Miller Fisher Syndrome
PFGE	Pulsed field gel electrophoresis
Pgm	phosphoglucomutase locus
SLV	Single locus variant
ST	Sequence type
SVR	Short variable region
tkt	transketolase locus
TLV	Triple locus variant
uncA	ATP synthase subunit locus

Abstract

Chapter 1. Introduction.

This chapter reviews previous studies regarding *Campylobacter*. The organism was first described as a veterinary pathogen and pathogenicity for humans was discovered only in the 1970's, largely in association with improved detection methods. It is currently the most common bacterial cause of gastroenteritis world-wide infecting more than 400,000 people a year in the UK and 2.1-2.4 million people per year in the U.S. In Europe and the U.S. *Campylobacter* induced gastroenteritis is typically sporadic and self limiting. Complications such as Guillain Barré syndrome, an acute peripheral neuropathy, may occur in 1-2 persons per 100,000 population following infection.

Campylobacter can be isolated from many sources including mammals, birds, insects, soil and water. The bacterium is unable to multiply outside animal hosts and its survival is largely influenced by factors such as temperature, drying and UV light. In contrast to humans, gastroenteritis is not typically induced in animals and indeed *Campylobacter* may form part of the normal gut flora. The most common source of infection of humans in Europe and the U.S. is thought to be contaminated chicken meat which may partly reflect the automated slaughter process used.

It has proven difficult to determine epidemiological routes of infection, largely due to the inadequacy of typing systems. Previously studies have relied on serotyping and enzyme digest methods, which are difficult to perform and unreliable. Recently a nucleotide sequencing based multilocus sequence typing (MLST) method has been described for *Campylobacter* whereby data can be readily compared between different host sources and are perfectly amenable to further genetic and evolutionary analyses.

The aims of the study were to test the MLST system on isolates from livestock and wild birds and to determine whether genotypes isolated from human disease originate on the farm. In particular *Campylobacter* isolates from wild birds and free-range chickens on the same farm site in Oxfordshire were studied to determine whether transmission of genotypes occurred between the host sources and ultimately reach the human food chain.

Chapter 1: Introduction.

1.1 *General introduction.*

Campylobacter is the most common bacterial cause of gastroenteritis worldwide. It has a significant economic impact and, although usually self-limiting, 1-2 cases per 100,000 population in the UK and US develop more serious symptoms such as motor paralysis (Nachamkin *et al.* 1998). The organism can be isolated from a wide range of sources including food, animals, water and soil, although contaminated chicken meat is thought to be a particularly common source of human infection in Europe and the US (Allos 2001; Kessel *et al.* 2001). Detailed epidemiology has been difficult since typing methods have not been reliably reproducible among laboratories (Dingle *et al.* 2001a). The aim of this study was to use the recently developed multilocus sequence typing (MLST) method to investigate the population biology of *Campylobacter* and its potential transmission cycle amongst poultry and wild birds (Dingle *et al.* 2001a). Such knowledge is essential if the pathogenicity and spread of the organism to humans is to be fully understood, and preventative measures be identified in the future.

1.2 The discovery and classification of *Campylobacter* species.

The pathogenicity of *Campylobacter* species for humans has only been recognized since the 1970's (Nachamkin and Skirrow 2000). The organisms were previously known as 'micro-aerophillic vibrios' and were first isolated from aborted

material from sheep and cattle in 1906 by McFadyean and Stockman (Skirrow 1994). From that time they were primarily known as veterinary pathogens. It is thought the first documentation of *Campylobacter jejuni* (or *Campylobacter coli*) was made by Theodor Escherich in 1886, who discovered vibrios in the large intestinal mucus of infants who had died of 'cholera infantum'. He was unable to grow the organism and thought it to be non-pathogenic. The first successful culture of *Campylobacter* from human stools occurred in Australia in 1971 (Cooper and Slee 1971) and in Belgium in 1972 (Dekeyser *et al.* 1972). Butzler demonstrated the common occurrence of the organism amongst stool samples from children with diarrhoea, but the work was largely unrecognized until Skirrow confirmed and extended the findings in the UK in 1977 (Skirrow 1994).

Campylobacter and *Arcobacter* are members of the *Campylobactereacae* group of micro-organisms proposed in 1991 by Vandamme and De Ley, based largely on 16S rRNA sequencing (Nachamkin and Skirrow 2000). There are currently 15 species of *Campylobacter* and four species of *Arcobacter* in the group. The complete genome sequence of *C. jejuni* was determined in 2000 (Parkhill *et al.* 2000) providing new insights into the organism's biology. The genome is relatively small, approximately a third of the size of *Escherichia coli,* consisting of 1,641,481 base pairs, 94.3% of which encodes 1,654 genes, of average length 948bp. There is a lack of repetitive DNA sequences in the sequenced genome, and apart from the predicted coding sequence Cj0752, which is similar to part of an insertion element in *Helicobacter pylori*, there is no evidence of functional insertion sequence elements, transposons, retrons or prophages. The *C. jejuni* chromosome is AT rich with a GC ratio of approximately 30%.

1.3 Pathogenesis and disease in humans.

Campylobacter is the most common bacterial cause of gastroenteritis in Europe and the U.S. There were 42,679 laboratory confirmed cases in the UK in 2005 (HPA 2006) and 2.1-2.4 million cases confirmed annually in the U.S. However the true incidence is likely to be much higher, since only the most severe cases will be seen by doctors. In the UK it is estimated that for every case that is laboratory confirmed, there are another 7.6 unreported cases in the community (Frost 2001). Incidence in developing countries is unlikely to be accurately recorded, but case-control studies estimate it to be 40,000-60,000/100,000 children under five years of age (Coker *et al.* 2002). *C. jejuni* is the most common subspecies but *C. coli* also causes gastroenteritis and is responsible for 1200 GP presentations a year in the US (Tam *et al.* 2003). Infection may be mixed with both species being present (Richardson *et al.* 2001; Tam *et al.* 2003).

Infection arises through consumption of contaminated food and water. In the developed world contaminated poultry is thought to be an important, but not the only, source of infection (Allos 2001; Kessel *et al.* 2001). Risk factors in the developing world include inadequately treated water and contact with farm animals (Butzler 2004). In one study in Peru where humans often shared their homes with chickens, toddlers were observed to contact poultry faeces with their hands on average 2.9 times in 12 hours (Oberhelman *et al.* 2003). Campylobacteriosis is typically a self limiting disease but it can contribute significantly to malnutrition in developing countries (Butzler 2004). Deaths are rare, but they can occur amongst the young, elderly, or immunocompromised (Altekruse *et al.* 1999). The infectious dose for humans is thought to be 500 organisms or fewer, and the incubation period is one to seven days (Ketley 1997).

1.3.1 Gastroenteritis.

Symptoms in developed countries are typically 2-3 days acute inflammatory diarrhoea, abdominal cramps and fever which may last for a week (Ketley 1997; Altekruse *et al.* 1999; Allos 2001). Leucocytes and red blood cells can be seen in the faeces of 75% of infected people, and bacteraemia can sometimes be detected, although blood cultures are not typically taken (Ketley 1997). Infection is generally self-limiting and resolves within a week, although relapsing diarrhoea can sometimes occur over the following months, and some patients may enter a carrier state for several weeks post infection (Ketley 1997; Allos 2001). Infection is most frequent amongst people aged 45 years or under, and is more common amongst males (Ketley 1997; Louis *et al.* 2005). The majority of cases are sporadic with relatively few outbreaks reported, although they are also the least likely of infectious agents to be followed up by local health authorities (Frost 2001). The incidence of gastroenteritis is seasonal, with a peak of incidence of infection occurring in the spring and summer months in temperate climates.

There are differences in the disease spectrum in developing countries in comparison to industrialized countries (Ketley 1997). Infection in developing countries is most common amongst children under the age of two years where prevalence may be in the region of 0.89 episodes/child/year, and also in early adulthood (Allos 2001; Oberhelman *et al.* 2003). Disease is typically non inflammatory, with no mucosal damage or blood or leucocytes seen in the faeces. There is no seasonality, and there appears to be a much higher rate of exposure and infection, and also asymptomatic carriage (Ketley 1997). There is little evidence of the differences being linked to different strains of *Campylobacter*, with a large variety being prevalent around the world.

1.3.2 Other sequelae.

Complications may occasionally occur following *Campylobacter* gastroenteritis. Musculoskeletal and neuropathic problems are the most common, but there are occasional reports of meningitis, carditis, pancreatitis and skin and urinary problems (Zia *et al.* 2003). Guillian Barré Syndrome (GBS) consists of at least four subtypes of acute peripheral neuropathy, a condition which was first reported in 1982 and affects 1-2 persons per 100,000 population in the US and Europe (Nachamkin *et al.* 1998; Hughes and Cornblath 2005). At least 30% of cases are proceeded by *C. jejuni* infection, perhaps more, since symptoms generally occur 1-3 weeks post infection making diagnosis difficult to confirm. The incidence of GBS is 1.5 times more common in males than females, and in Europe and North America the incidence increases with advancing age from less than one per 100,000 under 30 years to 4 per 100,000 over 75 years (Hughes and Cornblath 2005).

GBS associated with *C. jejuni* infection usually results in more severe paralysis and greater need for ventilation compared to other causes. However GBS is not associated with particularly severe bouts of *C. jejuni* infection, and patients may often be asymptomatic beforehand. GBS and the Miller-Fisher Syndrome (MFS) subtype are thought to be autoimmune diseases, with specific antibodies raised against the LPS of *C. jejuni* mimicking human gangliosides, which in turn activate inflammatory and T cells, and cause macrophages to invade the axon at the Nodes of Ranvier (Ang *et al.* 2001; Hughes and Cornblath 2005; Usuki *et al.* 2005; Wokke and van den Berg 2005). In particular anti GM1 antibodies are associated with motor neuropathy and anti Q-o16 are associated with osculomotor symptoms. MFS is thought to be associated with Anti-GQ1b antibodies in particular, which may cause

damage of the motor nerve terminal through a complement mediated mechanism (Hughes and Cornblath 2005).

An over-representation of *Campylobacter* strains with Penner serotype 0,19 (see section 1.5.2) has been reported in the US and Japan in association with GBS, and similarly *Campylobacter* strains with Penner serotype 0,41 have been reported in South Africa (Endtz *et al.* 2000). Studies using MLST report that strains are diverse, although there may be a slight over representation of ST-22 complex and under representation of ST-45 complex (Endtz *et al.* 2000; Dingle *et al.* 2002). A particular *flaA* SVR marker had been reported, but again it is not totally representative of all the strains associated with GBS or MFS. Patients may recover completely from the syndromes, although 20% are left with some form of disability, and death may occur in 5% of cases (Altekruse *et al.* 1999). Reiters syndrome is a chronic reactive atrophy which affects 1% of *C. jejuni* infected patients. It usually occurs 7-10 days post infection and affects multiple joints, especially the knee. Together with Reactive Arthritis, people who have HLA-B27 are most susceptible.

1.3.3 Pathogenesis of disease in humans.

Innate immunity mechanisms such as gastric acidity and intestinal transit time, are thought to be an important defense against *Campylobacter* infection, although they have been poorly defined to date (Zilbauer *et al.* 2005; Vuckovic *et al.* 2006). It is thought that the gastrointestinal epithelium plays a major role both as a physical barrier between the intestinal lumen and underlying mucosa, and also to signal infection, since it is able to up-regulate production of cytokines, chemokines and anti-microbial peptides such as β-defensins (Zilbauer *et al.* 2005). A strong local and serum antibody response has been observed following *Campylobacter* infection, and

$CD8^+$ and $CD4^+$ T cells are also thought to contribute to host protection, although the precise mechanisms are unknown (Vuckovic *et al.* 2006).

Few virulence factors have been described amongst *C. jejuni*, and little is known in comparison to other gastrointestinal pathogens such as *E. coli* or *Shigella* species. The bacteria enter the body via contaminated food or water and cross the acidic environment of the stomach before colonizing the distal ileum and colon (Ketley 1997). Survival mechanisms for both the stomach and serine protease enzymes in the small intestine have not yet been discovered (Pei *et al.* 1991). Both the flagellum and cell shape are thought to aid motility and invasion of the mucus layer above the gut epithelium, and motility has been found to be an essential virulence factor (Morooka *et al.* 1985). In addition, chemotaxis appears to play an important role in pre-colonization; a possible regulatory protein *cheY* was identified from the genome sequence (Parkhill *et al.* 2000), and various chemoattractants such as mucin, L-serine and L-fructose as well as chemorepellants such as bile salts have been recognized.

C. jejuni appears to adhere to cell junctions of cells in the gastrointestinal epithelia before invading them, and several potential adhesins have been identified (Kopecko *et al.* 2001). These include the PEB1 and PEB4 proteins, a 43KDa major outer membrane protein (MOMP), a 37kDa outer membrane protein CadF, LPS and JlpA (Ketley 1997; Jin *et al.* 2001; Monteville and Konkel 2002; Mamelli *et al.* 2006). The PEB proteins were thought to be important in cell binding, although their role is still in question as they also show amino acid sequence homology with amino acid and protein transporter systems (Pei and Blaser 1993; Ketley 1997). The MOMP protein, product of the *porA* gene, is a pore forming and multifunction surface protein which may play a role in adherence (De *et al.* 2000; Zhang *et al.* 2000; Jin *et al.*

2001). The role of lipopolysaccharide (LPS) in adherence is still to be fully resolved (Ketley 1997). The CadF outer membrane protein is thought to play an important role in binding to fibronectin enabling invasion at the basal pole of epithelial cells, and promotes the phosphorylation of another adhesion protein paxillin (Monteville and Konkel 2002; Mamelli *et al.* 2006). Jejuni phospholipase A, (JlpA), the product of the *jlpA* gene has also been found to mediate attachment to host epithelial cells (Jin *et al.* 2001). The use of fimbrae, the expression of which are enhanced in the presence of bile salts is still in question, but they appear to have a minor role in adhesion (Ketley 1997; Jin *et al.* 2001). Other proteins proposed to be involved in the colonization process include docA (thought to be a periplasmic cytochrome C peroxidase), docB and docC (thought to be methyl accepting chemotaxis proteins (MCPs), and cgtB and wlaN (thought to be involved in lipo-oligosaccharide (LOS) biosynthesis (Muller *et al.* 2005).

C. jejuni is believed to invade the gastrointestinal epithelium either by endocytosis or by microtubule or microfilament dependant mechanisms to enter epithelial cells or by translocating across M cells and entering the lymphoid tissue below (Ketley 1997; Kopecko *et al.* 2001; Biswas *et al.* 2003). The accumulation of actin and tubulin may also protect the bacteria from incoming lysosomes. In contrast to infections by *Shigella* species and Enteroinvasive *E. Coli* (EIEC), *C. jejuni* undergoes limited replication once inside the host cell and instead exits by exocytosis where upon basolateral re-invasion is possible. Cell death is mediated by a toxin, cytolethal distending toxin (CDT) and IL-8, released from intestinal epithelial cells. CDT is a multi subunit protein encoded by the *cdtA*, *cdtB* and *cdtC* genes. It is thought to cause the death of host cells by directly damaging the DNA, causing the cells to arrest in the G2 phase of the cell cycle (Hassane *et al.* 2003). IL-8 is a

cytokine responsible for many localised inflammatory responses and is a strong chemoattractant for immune effector cells (Hickey *et al.* 2000). *Campylobacter* organisms appear to be readily killed by polymorphonuclear leucocytes if they are opsonised by antibodies and complement (Ketley 1997). However they are able to survive in macrophages for up to seven days, perhaps due to a capsular polysaccharide, although the bacterial cells may become coccoid in morphology (Zilbauer *et al.* 2005). The roles of catalase and LPS in counteracting nitric oxide synthesis and the respiratory burst are thought to be essential (Ketley 1997; Day *et al.* 2000).

Other important factors in *Campylobacter* pathogenesis include molecular mimicry, perhaps helping to evade the host immune system, for example the flagella shows post translational modification by glycosylation and unusually, sialylation (Guerry 1997). In addition a group of proteins, *Campylobacter* invasion antigens (Cia proteins) such as ciaB and iamA have been identified *in vitro* in response to environmental changes such as presence of bile salts, but their precise functions are unknown at present. (Rivera-Amill *et al.* 2001; Muller *et al.* 2005).

1.4 Pathogenesis and disease in farm animals.

Much less is known about the extent of pathogenesis and disease amongst animals. *Campylobacter* species can be isolated from the intestines of many animals (Stanley *et al.* 1998a; Stanley *et al.* 1998b; Wallace *et al.* 1998), including most mammals and birds (Waldenstrom *et al.* 2002) which often appear to be asymptomatic. In chickens, there is evidence to suggest that the crypts in the intestine may become colonized, with little pathological change (Evans and Sayers 2000). However *C. jejuni* may cause clinical symptoms, such as scouring, particularly

amongst young animals (Pearson *et al.* 1996). It is also a cause of abortion amongst cattle and sheep. During the 1950-60's a disease called 'vibrionic' hepatitis with 10-15% mortality was prevalent amongst laying flocks over the age of eight weeks in North America and Europe, and was thought to be caused by *C. jejuni* (Corry and Atabay 2001). A subsequent decline in the disease is probably due to keeping laying hens in cages.

Many investigations suggest that chickens naturally become infected between two and three weeks of age, although experimentally, chicks as young as one day old may become colonized (Corry and Atabay 2001). The time lag in natural infection may be due to maternal antibodies which are present at birth but decline at about 14 days of age (Sahin *et al.* 2003). In addition it may be possible that caecal microflora are inhibitive to *Campylobacter* species at this stage, perhaps a reflection of the conditions in which they are kept (Evans and Sayers 2000). Even upon natural infection, some birds may produce watery diarrhoea and there is experimental evidence suggesting the liver and spleen may become infected (Wallace *et al.* 1998; Corry and Atabay 2001). Circumstantial evidence suggests that small or weak birds in particular may be affected (Berndtson *et al*, 1996b). Differences in *Campylobacter* strain can also result in different levels of colonization or rate of shedding (Wallace *et al.* 1998; Ahmed *et al.* 2002). Little is known about *Campylobacter* populations in older birds since the production life cycle is often between four and six weeks in age. It is presumed that they enter a chronic state of infection, with studies suggesting that shedding rate decreases with age to levels of 20-30% (Willis *et al.* 2002).

1.4.1 Epidemiology of infection amongst poultry.

Campylobacter colonization of poultry flocks ranges from 0-100% of flocks on a farm. Once *C. jejuni* has been detected in a flock, 98-100% of the birds within it become colonized within a few days (Evans and Sayers 2000; Newell and Fearnley 2003). However, with conventional broiler systems it is also possible that with strict hygiene barriers some flocks may be kept negative for *Campylobacter*, despite other flocks with positive birds being kept on the same site (Berndtson *et al.* 1996). There is evidence that the carriage rate and numbers of *Campylobacter* organisms colonizing chickens are seasonal, with lower rates occurring during the cooler months and highest rates occurring during the early spring and summer months (Wallace *et al.* 1997; Hanninen *et al.* 1998; Hudson *et al.* 1999; Willis *et al.* 2002; Logue *et al.* 2003). The exact timing may relate to geographical region, and is usually similar to the timing of peaks in incidence of human infection.

Flocks are colonized by one or two genotypes of *Campylobacter*, which can be sporadic, farm specific, or more widespread types, which again, may be seasonal (Hanninen *et al.* 1998; Hudson *et al.* 1999; Manning *et al.* 2001; Petersen and Wedderkopp 2001; Shreeve *et al.* 2002; Bull *et al.* 2006). Mixed genotypes have been found in 24-40% of flocks and may occur in succession or co-exist within a flock (Wallace *et al.* 1998; Petersen *et al.* 2001b; Shreeve *et al.* 2002; Hein *et al.* 2003). There are commonly mixed infections of *C. jejuni* and *C. coli*, this may suggest that there are multiple origins of infection (Rivoal *et al.* 1999). There is some evidence that *Campylobacter* strains with Penner serotypes 0,2 or 0,4 (see section 1.5.2) are particularly common amongst isolates from broiler chickens and human disease (Petersen *et al.* 2001a; Dingle *et al.* 2002). Less is known about *Campylobacter* populations amongst poultry kept in less intensive systems such as

organic or free range, although generally all of the flocks can be expected to be positive for *Campylobacter* (Heuer *et al.* 2001; Newell and Fearnley 2003). There are significant differences in the way organic flocks are kept compared to conventional systems. Organic birds are allowed unlimited access to the outside environment, and are slaughtered at an older age, generally around 81 days (Heuer *et al.* 2001).

The source of infection in poultry is still unknown, but a number of risk factors have been identified. There is evidence that flocks become infected by 'horizontal' transfer of bacteria from other infected flocks or from environmental sources. In particular, poor house maintenance, breakdown of hygiene barriers, use of untreated or contaminated water supplies, poor ventilation where oxygen levels may be reduced and temperature increased, and large numbers of farm staff are thought to be largely responsible for flocks becoming positive (Pearson *et al.* 1996; Newell and Fearnley 2003; Zimmer *et al.* 2003). The process of 'thinning', whereby larger birds are removed for slaughter a week or so before the remainder of the flock, also carries a significant risk since teams of people often travel between farms with equipment that is usually contaminated with *Campylobacter*, and birds become stressed which could make them more susceptible to infection (Evans and Sayers 2000). One study found carriage rates (but not numbers) of *C. jejuni* to be positively correlated with minimum temperature and hours of sunshine, negatively correlated with maximum temperature and no correlation with amount of rainfall (Wallace *et al.* 1998). It is possible also that temperature may be indirectly related to other factors such as migratory birds or insects (Jacobs-Reitsma *et al.* 1994; Wallace *et al.* 1998). *C. jejuni* can often be isolated from wild birds, rodents, litter beetles and even flies caught in or around poultry houses and it is possible that they may transfer *Campylobacter* around the farm (Petersen *et al.* 2001a; Bates *et al.* 2004; Nichols 2005). However

serotyping with the Penner scheme (section 1.5.2) suggests that there is limited sharing of *Campylobacter* strains between poultry and wildlife. In addition, it appears strains are carried for shorter periods by wildlife compared to domestic animals, which is perhaps due to their different living conditions (Petersen *et al.* 2001a).

Contaminated housing could cause *Campylobacter* infection to carry over to as many as 15% of flocks, but cleansing and disinfection of houses is generally deemed adequate to resolve the problem since *C. jejuni* cannot be detected afterwards (Shreeve *et al.* 2002; Newell and Fearnley 2003). It is recommended that houses are left empty for a period of 14 days between flocks as an additional preventative measure, although 5 days would be more typical on the commercial farm (T. Humphrey personal. communication., (Anonymous 2006). Litter is generally thought to have little influence and some countries outside the UK do not dispose of it between flock cycles. For example, a study into the microbiological quality of litter found no *Campylobacter*, but in the region of 10^9 aerobic bacteria; mostly *Staphylococci*, enterococci and Gram negative enteric bacteria (Lu *et al.* 2003). It is presumed that the environment of litter is too harsh for survival of *Campylobacter* unless, perhaps, it becomes damp (Willis *et al.* 2002; Newell and Fearnley 2003). Similarly feed is thought to have little influence, with *Campylobacter* species dying off rapidly, although one study identified bought in wheat rather than home grown wheat to be a risk factor (Pattison 2001).

The role of 'vertical' transmission (transfer of infection from parent flocks to their offspring) is more controversial and mostly disregarded. Parent flocks are often highly contaminated, and *C. jejuni* can be isolated from genital tracts and semen of birds, thus providing a route by which a fertile egg may become infected (Cox *et al.* 2002a; Cox *et al.* 2002b; Hiett *et al.* 2002). Egg shells are permeable to

Campylobacter, and in the case of *Salmonellae*, infection appears to vary according to genetic strain and age of the parent bird, as well as location and strain of the bacteria (Jones *et al.* 2002). However the extent to which eggs are colonized by *C. jejuni* is unknown, and experimental inoculation of the chorioallantoic membrane has proven lethal to chicks (Newell and Fearnley 2003). There are investigations where *Campylobacter* diversity can not be explained by vertical transmission since flocks colonized with different strains have come from the same hatchery. But in other studies flocks have restricted *Campylobacter* types, thought to have originated in parent flocks (Petersen *et al.* 2001b). One study demonstrated identical genotypes by ribotyping and *flaA* SVR typing in both parent flocks and their offspring, despite different farm personnel and a distance of 20 miles between them (Cox *et al.* 2002a). Researchers remain sceptical due to the time lag of infection, although it may perhaps be explained by low levels of vertical transmission followed by rapid amplification of *Campylobacter* numbers amongst those flocks that have been 'seeded' (Corry and Atabay 2001).

1.4.2 Contamination of poultry meat during the slaughter process.

Statistics logged by DEFRA reveal human consumption of chicken meat is increasing: in 1998 over 700 million broiler chickens were slaughtered and in 2005 over 800 million broiler chickens were slaughtered in the UK (Harrison *et al.* 2001). Just over a million of the chickens were produced in organic systems in 2005. The percentage of poultry meat products contaminated by *Campylobacter* is generally found to be large, in the region of 49.5-94% in countries such as England, Northern Ireland and Spain, and organisms are usually present in high numbers (Harrison *et al.* 2001; Dominguez *et al.* 2002; Moore *et al.* 2002). Flocks that were previously

negative for *Campylobacter* often become contaminated during the slaughtering process, and flocks that were positive for *Campylobacter* may be contaminated by serotypes that have never been isolated from the flock previously (Rivoal *et al.* 1999; Slader *et al.* 2002). Thus the way in which chickens are slaughtered and processed appears to exacerbate the problem of *Campylobacter* contamination of poultry meat.

Many investigations have determined *Campylobacter* incidence at various stages of processing: including transport of chickens in crates, with journey times up to 18 hours in the US; stunning of birds by electric shock and killing by bleeding; loosening of feathers by submerging in 'scald' water held at 50-53°C; feather plucking by machine that uses copious amounts of water spray; mechanical evisceration without opening the carcase (unlike other food animals) together with washing the carcase inside and out; and finally chilling and packaging (Corry and Atabay 2001). Transport crates are often found to be contaminated with *Campylobacter* due to inadequate cleaning and disinfection procedures. Often organic material protects the bacteria, but the temperature of the washing water or concentration of the disinfectant may be inappropriate (Slader *et al.* 2002; Hansson *et al.* 2005). It is thought the stress of transport may increase faecal shedding of *Campylobacter* up to a thousand fold (Corry and Atabay 2001; Logue *et al.* 2003; Northcutt *et al.* 2003). In addition, there is evidence that subtypes found on crates may contaminate carcasses of flocks that tested negative for *Campylobacter* immediately before their departure: feathers of negative flocks can become contaminated even within a couple of hours (Newell *et al.* 2001). Feed withdrawal may help to reduce the amount of intestinal contents and therefore faecal contamination, but the effects are unclear, and the extent of *Campylobacter* shedding may actually increase (Corry and Atabay 2001).

The majority of contamination during processing is thought to arise initially from leakage of intestinal contents and by cross contamination from the large amounts of water and aerosols present (Logue *et al.* 2003). *Campylobacter* organisms appear to survive well on chicken skin which is relatively moist, by becoming trapped within crevices and pores, and by specific and non-specific interactions, such as forming biofilms with other microorganisms or water-skin interfaces, and also perhaps some protection is offered by fatty acids and oils (Mandrell and Wachtel 1999; Park *et al.* 2002). It is possible that some of the water sprays drive them even deeper into the skin. Whilst most contamination is considered to be on external surfaces, with the actual meat being sterile, one investigation found *Campylobacter* in the respiratory tract of birds before processing, presumably due to inhalation of aerosols during rearing or transport (Berrang *et al.* 2003). Thus the thoraco-abdominal cavity could become contaminated during evisceration of infected birds.

There is evidence that both chilling and scalding procedures reduce numbers of bacteria on the carcass; in one study, the mean numbers of *Campylobacter* organisms contaminating the carcass skin was in the region of 10^3 colony forming units (CFU) during bleeding, but fell to between 10^1 and 10^2 CFU after scalding, and also after chilling in water (Corry and Atabay 2001). Chilling in air appeared to be less effective in reducing the bacterial load. However, numbers still seem to increase after these stages and during plucking and evisceration. In particular, the plucking machine has been observed to push faecal material out of the carcass (Corry and Atabay 2001). Separate surveys have shown that even when equipment and scald water have been shown to be negative prior to processing, or there is flawless evisceration by hand, contamination of chicken meat still occurs (Berrang *et al.* 2003; Miwa *et al.* 2003).

The final packaging process may also influence the level of *Campylobacter* contamination. One study noted that a higher percentage of supermarket poultry products were contaminated compared to those sold at a butcher (Harrison *et al.* 2001). This may be due to packaging, where pre-packed supermarket products contained trapped moisture which would aid survival of bacteria. In addition, a greater percentage of whole chickens were contaminated compared to chicken pieces, presumably due to the removal of skin from the latter (Corry and Atabay 2001; Harrison *et al.* 2001). An additional concern for consumers is that *Campylobacter* species were isolated from 3% of external and 34% of packaging material, leading to potential cross contamination to other products in the shopping basket or at home (Harrison *et al.* 2001).

There is little known about how *Campylobacter* species survive the harsh conditions experienced in the processing plant, for example low osmolarity encountered in the scald, rinse and chill baths, chemical disinfectants such as chlorine, rapid temperature fluctuations from 8 to 60°C and the sealed packaging atmosphere (Mandrell and Wachtel 1999; Newell *et al.* 2001). It is possible that survival is strain specific and some studies have reported restricted numbers of strains surviving the whole process, or even contaminating the next days flocks (Slader *et al.* 2002; Berrang *et al.* 2003). More detailed subtyping procedures are required to investigate this further.

1.4.3 Epidemiology of infection amongst farm animals other than poultry.

Genotyping using RAPD (section 1.5.1) revealed that *Campylobacter* isolates from human disease closely resemble those isolated from retail chicken meat (Hein *et al.* 2003; Workman *et al.* 2005). But there are still other significant sources of human

Campylobacter infection, for example ST-61 complex commonly found amongst human disease isolates is associated with cattle and sheep sources (Dingle *et al.* 2002; Kemp *et al.* 2005). The carriage rate of *Campylobacter* amongst cattle has been reported from 23-79% positive faecal samples in dairy cattle to 89.4% positive intestinal contents of beef cattle at slaughter (Stanley *et al.* 1998a; Nielsen 2002). Calves appear to be born *Campylobacter* free, but begin to excrete the organism in high numbers shortly afterwards. The cycle of infection is probably maintained through consistently contaminated deep litter bedding and in turn contaminated hides of individual animals. There is some evidence suggesting that cattle kept in feed lots have higher rates of contamination in comparison to cattle kept at pasture. In addition an under developed rumen in younger animals may allow higher numbers of *C. jejuni* to infect the small intestine in comparison to older cattle. One study noted a predominance of Penner serotype 0,2 (see section 1.5.2) amongst calves and it is possible the strains associated with the serotype may be adapted to infect young animals (Nielsen 2002). In addition two serotypes were identified in 8% of animals indicating that infection had been introduced into the herd on separate occasions, or by different routes. There may also be mixed *Campylobacter* species infection with 1-6% *C. coli* and 0-3% *C. lari* isolated in one study and *C. hyointestinalis* isolated in another. There appears to be significant seasonality in the numbers of *C. jejuni* shed in faeces, with peaks occurring in spring and summer. It is not clear whether they relate to recrudesence of infection or whether there is seasonal reinfection of *Campylobacter* types. The peaks roughly correlate with changes in management and diet but show no relation to minimum or maximum air temperature, hours of sunshine or amount of rainfall (Stanley *et al.* 1998a). It is possible that indirect environmental factors such as migratory birds, insects or rodents may be involved.

Sheep often show higher levels of contamination than cattle, with 91.7% of intestinal contents from lambs at slaughter being positive year round, although there is pronounced seasonal variation in faecal shedding (Stanley *et al.* 1998b). Greatest levels of faecal shedding, often 100% of animals, are reached during lambing, weaning and movement to different pastures, although this is not directly associated with increased stress since procedures such as dipping were not associated with increased shedding. In addition, increased levels of shedding were associated with poorer grazing giving higher levels of indigestibility and therefore more plant material movement through the intestines, and also with outbreaks of other infections amongst lambs caused by Rotavirus, *Salmonellae* or *Cryptosporidia*. Reduced or no shedding was associated with a hay or silage diet, gestation and vaccination against other bacterial pathogens such as *Pasteurella* and *Clostridia*. As with cattle, there appears to be no correlation between seasonality and minimum and maximum temperature, amount of rainfall and hours of sunlight. Instead, it is thought that contaminated ground water may be a significant source of infection, and the presence of *C. jejuni* in the rumen of slaughtered lambs suggests a recent and constant source of infection. Lambs were born *Campylobacter* free, but became infected 1-5 days post lambing, those with mothers shedding *C. jejuni* becoming positive most rapidly (Jones *et al.* 1999).

C. jejuni have been isolated from pigs, and a pig associated clone identified using MLST (see section 1.5.3) has been proposed (Manning *et al.* 2003). However the majority of isolates tend to be *C. coli* (Weijtens *et al.* 1999; Boes *et al.* 2005). During one study, pigs were found to be colonized by up to eight different strains of *C. coli* during a raising period, with infection beginning within the first ten weeks of

life. Some strains were identified in common with sows and their offspring, but others were found in either the sows or offspring only.

Despite the high numbers of bacteria in both cattle and lamb intestines, contamination of carcases and packaged meat are lower than poultry meat, which may reflect the different and more hygienic slaughtering practices (Madden *et al.* 1998). Animal waste may be a large source of contamination of water courses, either directly, or by application of slurry to land (Mawdsley *et al.* 1995). A flock of 2000 sheep grazing on salt marsh were calculated to excrete 1.1-2.2 x 10^{11} *Campylobacter* organisms per day, a dairy cow calculated to produce 50 litres of liquid faeces per day and a 100 cow dairy herd was calculated to produce 750m^3 slurry in six months (Stanley *et al.* 1998c; Jones 2001a). Traditional and less intensive farming would have composted animal waste and bedding, and temperatures as high as 70°C would kill *Campylobacter* species (Mawdsley *et al.* 1995). Modern farming methods store liquid waste as slurry and guidelines from the UK Department for Environment, Food and Rural Affairs (DEFRA) govern the conditions on which is put back onto the land. Long storage time and aeration is considered to reduce the microbial pathogen load, however the guidelines are not always strictly followed, and despite slurry being *Campylobacter* negative prior to land application, one study was able to retrieve *Campylobacter* from the land after slurry application.

1.5. Wildlife and environmental sources of *Campylobacter.*

1.5.1 Wild birds.

In addition to domestic animals, wildlife are often implicated as reservoirs of infection. Wild birds have been identified as a source of human disease on occasions,

causing contamination of milk by pecking bottle tops (Riordan *et al.* 1993). They are also thought to be the predominant source of contamination of some water sources (Jones 2001a). It is thought that *C. jejuni* may be adapted as a commensal of birds since they have a higher body temperature compared to other animal species, and the organism can be isolated in high numbers from apparently healthy individuals (Waldenstrom *et al.* 2002).

One investigation in Sweden studied 1794 migratory birds from 107 different species between March and November, and on average, isolated 5% *C. jejuni*, 5.6% *C. lari*, 0.9% *C. coli* and 10.7% unidentified *Campylobacter* species. Prevalence of infection appeared to be linked to habitat and type of bird, and was associated in particular with different feeding habits. Shoreline birds feeding on invertebrates had highest levels of infection at 76.8%, followed by opportunistic feeders with 50%. Other ground foraging birds had high prevalence, with 11% of invertebrate feeders, 20.3% insectivores and 18.8% plant eating species infected. Lowest rates of infection were found amongst ground foraging granivores (2.3%), arboreal (tree inhabiting) insectivores (0.6%), aerial insectivores (0%) or reed and herbaceous plant foraging insectivores (3.5%). During the autumn migration, high levels of juveniles were found to be positive indicating that infection must have occurred on the breeding grounds or during early migration, and within one to four months of life. In addition prevalence was associated with increasing body mass, which is also associated with longevity in passerines. A separate study of black headed gulls over a two year period identified a pronounced seasonality, with lowest prevalence in winter, increasing prevalence in spring and summer and highest prevalence in the autumn (Broman *et al.* 2002).

Comparison of macro-restriction profiles (section 1.5.2) isolated from humans, poultry and wild birds revealed high levels of similarity between human and poultry isolates, and little similarity between either source with wild bird isolates. A total of 48 birds were recaptured between one and four times and were found to have different *C. jejuni* strains on most occasions. Another study investigating isolates from varied wildlife sources found *Campylobacter* isolates to be diverse, with little clonal relation to human and poultry strains, although some isolates within 0,4 serotyping complexes were identified from both (Petersen *et al.* 2001a). In addition O,12 was common amongst the wildlife strains in comparison to human and poultry sources. Evidence suggested that a wildlife reservoir of infection was of limited importance, which raises questions as to whether strains vary in virulence and colonization potential. There are obvious differences in the way in which wildlife and domestic animals live, with wildlife being more dispersed, eating a more varied diet and coprophagy occurring infrequently, which may be important factors with respect to colonization.

1.5.2 Other wildlife.

Campylobacter has been isolated from other wild animals living in the vicinity of farms, such as mice, rats, badgers, rabbits, insects and even protozoa (Petersen *et al.* 2001a; Hiett *et al.* 2002; Maugeri *et al.* 2004; Axelsson-Olsson *et al.* 2005; French *et al.* 2005; Meerburg *et al.* 2006; Snelling *et al.* 2006). Results from two large studies suggest that *Campylobacter* genotypes isolated from wild mammals tend to be different to those from human disease, but this is not always the case and their role in transmission remains controversial (Petersen *et al.* 2001a; French *et al.* 2005).

Flies can act both as mechanical vectors carrying *Campylobacter* on hairs on their body or feet as well as biological vectors carrying the organism in the alimentary

canal (Ekdahl *et al.* 2005). They are considered by some authors to be strongly associated with seasonal variation in Campylobacteriosis in temperate countries since fly numbers increase dramatically in spring (Nichols 2005). In contrast fly numbers are relatively constant in tropical countries where seasonal variation in Campylobacteriosis is absent. Cross contamination of food by flies could also explain the lack of clusters of disease amongst families, which would be expected if ingestion of contaminated food or chicken meat were solely to blame. Estimates of the number of flies carrying *Campylobacter* were one in 40 in one study and 8.2% in another suggesting they may be more important than previously realized (Hald *et al.* 2004; Nichols 2005).

A study in New Zealand concluded that a subset of *Campylobacter* genotypes could be isolated from poultry and Darkling beetles (*Alphitobus diaperinus*) that inhabit the litter of poultry houses (Bates *et al.* 2004). The beetles may have a significant impact in the poultry industry since litter infestation is very difficult to control. *Campylobacter* have also been isolated from the lumen of protozoa such as *Acanthamoeba* species that are commonly found in high density amongst natural and man made water systems (Axelsson-Olsson *et al.* 2005; Snelling *et al.* 2006). Internalization may help to protect *Campylobacter* from unfavourable environmental conditions and reversal of the viable-non culturable status has been demonstrated in experimental conditions.

1.5.3 Soil and water contamination.

Although there are few identified outbreaks of *Campylobacter* infection, the ones that do occur are often associated with contaminated water, usually private untreated water supplies (Frost 2001). Contaminated water and soil have also been

implicated as sources of infection in broiler houses (Newell and Fearnley 2003). *Campylobacter* species are not able to grow outside of their host, although physiological activity can be detected at temperatures below 37°C. Contamination of soil and water supplies are probably inter related and arise largely from pollution by animal waste or sewage. *Campylobacter* species in soil may be protected from UV light and desiccation, and also by a low redox potential, absence of molecular oxygen and year round low temperatures. Movement of bacteria within soil is related to soil type and surface properties, water content and flow, PH, temperature, aspect, plant content, and type of waste (Mawdsley *et al.* 1995; Kemp *et al.* 2005). Lateral movement of organisms is greatest in soils with impermeable substrata or in waterlogged conditions and is also affected by the amount of rainfall and topography of the land. *Campylobacters* are thought to be better able to survive in soil with a north facing aspect since there tends to be more shading and less UV light (Kemp *et al.* 2005).

Extensive surveying revealed that water sources may vary with respect to *Campylobacter* load and also source of contamination (Jones 2001a). Ground water is generally considered to be free of *Campylobacter* species as it rises from the ground; however, contamination can occur if there is a point source within the hydrological catchment area, and is more common in lowland rather than upland areas. Drinking water treated with chorine, is rarely, if ever, contaminated with *Campylobacter*. Water troughs providing drinking water for livestock have been found to be contaminated, but at relatively low levels (11% of samples), perhaps due to chlorination of the water (Kemp *et al.* 2005).

Rivers are frequently contaminated but show significant seasonality with high levels of *Campylobacter* in winter and low levels of *Campylobacter* in summer;

changes that are positively correlated with UV light and temperature (Jones 2001a; Savill *et al.* 2001). Contamination is most commonly associated with abattoir or poultry processing plant effluent rather than residential sources and seasonal peaks are coincident with human infection peaks. In addition significant amounts of agricultural run off may occur, particularly during very high rainfall, which could also add to the seasonal effect. Such contamination is identified by the high proportion of *C. jejuni* and *C. coli,* whereas contamination by wild birds is recognized by high proportions of *C. lari* and urease-positive thermophilic Campylobacters (UPTCs). River sediments are considered to be a better indicator of river contamination since they show little seasonal variation in contrast to water samples and thus also reveal constant sources of contamination (Obiri-Danso and Jones 1999).

Ponds are typically of poor microbiological quality with *Campylobacter* profiles matching ducks, geese and other wild birds (Jones 2001a). A study of a reservoir similarly revealed avian sources of contamination together with that from a nearby flock of sheep. Coastal bathing water in Morecambe Bay, Lancashire, was also contaminated by birds despite there being a sewage outlet a few miles out to sea. Similarly, a study of sand bathing beaches revealed that typically 45% of sand samples were positive for *Campylobacter* with those of EEC standard being contaminated by birds and those of non-EEC standard being contaminated by sewage (Bolton *et al.* 1999). Further studies revealed that *Campylobacter* organisms were able to survive in water samples for between six and 25 days, with survival greatest at 4°C and in the dark (Obiri-Danso *et al.* 2001). *Campylobacter* have also been isolated from plankton which may also help to protect the bacteria from the harsh environment (Maugeri *et al.* 2004). Survival was not strain specific, although *C. lari* did appear to survive better in sea water and is perhaps more halotolerant compared to the other

species. Thus, predominance of *C. jejuni* within water samples probably reflects large amounts of contamination whereas predominance of *C. lari* may be in part explained by its prolonged survival in sea water.

1.6. Culture and identification of *Campylobacter* species.

1.6.1 Microbiology.

Campylobacter species are best isolated using specific complex media since they lack many of the genes to degrade carbohydrates or amino acids and are unable to use them as carbon or energy sources (Parkhill *et al.* 2000). Many different basal media types are available, although Nutrient Agar No 2 is commonly used and contains less thymidine, an antagonist of the antibiotic trimethoprim, than most (Corry *et al.* 1995). Lysed or defribinated blood, charcoal, FBP (ferrous sulphate, sodium metabisulphate and sodium pyruvate) or haemin are used to scavenge potentially toxic super oxides and free radicals. Specific antibiotics, to which Campylobacters are resistant, are added in order to suppress the growth of contaminant bacteria. Different media contain different combinations of antibiotics, but those commonly used include polymixin B, active against Gram negative bacteria; trimethoprim, active against Gram negative bacteria including *Proteus* species; rifampicin, active against Gram positive bacteria and also some Gram negative bacteria; cefoperazone, active against Gram positive bacteria; and cyclohexamide or amphotericin B, active against yeasts and moulds (Corry *et al.* 1995). The media, whilst successful, are not perfect since some strains of *C. jejuni* and *C. coli* may be inhibited by cephalothin, colistin and polymyxin B (Goossens *et al.* 1986; Arzate Barbosa *et al.* 1999). *Campylobacter* species need a microaerophilic atmosphere

consisting of 5% oxygen, 10% carbon dioxide and 85% nitrogen to grow in the laboratory (Collins *et al.* 1995). Unlike most *Campylobacter* species, *C. jejuni*, *C. coli* and *Campylobacter lari* show optimum growth at 42°C and are often called 'thermophillic' *Campylobacters*. There is no definitive method of isolation for *Campylobacter* and the approach taken is usually determined by sample type and *Campylobacter* strains required – not all species grow on all media (Baylis *et al.* 2000).

At least six types of selective media are used for direct culture of *C. jejuni* from faeces, but modified charcoal cefaperazone desoxycholate agar (mCCDA) was the most successful in terms of recovery rates of *C. jejuni* and suppression of other faecal flora when they were compared (Gun-Munro *et al.* 1987). Other sample types, such as food, where *Campylobacter* numbers may be sparse or cells sub-lethally injured, may require a pre-enrichment stage in liquid broth to improve recovery rates. Preston and Exeter enrichment broths are commonly used for isolation of *C. jejuni* and *C. coli* and contain the same components as solid media, although the antibiotic content differs, with the Preston broth containing polymyxin B, cyclohexamide, rifampicin and trimethoprim and the Exeter broth containing amphoteracin B, cefoperazone, polymyxin B, trimethoprim and rifampicin. The Exeter broth was developed to aid recovery of stressed *Campylobacter* cells that were found to have increased sensitivity to rifampicin (Mason *et al.* 1999). Sample types such as environmental waters, which provide an even harsher environment for *Campylobacter*, may require additional steps to achieve optimal recovery. These include pre-incubation at 37°C for four hours, gradual addition of antibiotics, or filtration onto non-selective media (Humphrey 1989; Mason *et al.* 1999).

Members of the genus *Campylobacter* are Gram negative curved rods, and are relatively small at 0.2-0.5μm wide and 1.5-6.0μm long (Ketley 1997). They are characteristically seen as 'seagull' shapes under the microscope, but may become coccoid in old cultures or on exposure to air (Nachamkin and Skirrow 2000). They are motile by a polar or bipolar flagellae and are cytochrome oxidase and catalase positive. Identification to subspecies level traditionally uses tests such as hippurate hydrolysis, H_2S production, nitrate reduction, growth in the presence of sodium chloride and at different temperatures. *C. jejuni* is unique in its ability to hydrolyze hippurate, but this is the only phenotypic test that distinguishes it from *C. coli.* However some *C. jejuni* isolates may appear to be hippurate negative, and results are sometimes difficult to interpret (Rautelin *et al.* 1999; On and Jordan 2003). A number of molecular techniques, such as multiplex PCRs, have been developed for identification (Wang *et al.* 2002).

1.6.2 Methods for typing *Campylobacter.*

1.6.2.1 Serotyping and phage typing.

Following on from the success of the Kauffman and White scheme for *Salmonella enterica*, serotyping has been routinely used for epidemiological typing of *Campylobacter* species. Two methods have been in use since the 1980's; the Lior scheme based on heat-labile (HL) antigens, and the Penner scheme based on heat-stable (HS or O) antigens (Chart *et al.* 1996). The Penner scheme has been more widely used in Europe, and was further modified for use in the UK in 1998, using direct bacterial agglutination rather than passive haemagglutination, and with absorbed rather than unabsorbed antisera (Frost *et al.* 1998; Wassenaar and Newell

2000; Frost 2001). The modified Penner scheme is able to detect 44 *C. jejuni* serotypes and 17 *C. coli* serotypes. Originally there was some confusion over which antigens the antisera were binding with, but there is now evidence that the antisera bind with capsular polysaccharides (Karlyshev *et al.* 2000). Disadvantages of the method are the high numbers of untypeable isolates, agglutination with one or more antisera, and the fact that it is time consuming and technically demanding, together with costly reagents that are difficult to produce. Phage typing using the Salama scheme has been routinely used in the UK in combination with serotying, in order to resolve some of the untypeability issues, and provide further discrimination amongst prevalent serotypes (Frost *et al.* 1999; Frost 2001). A total of 76 phage types have been identified. However, drawbacks of the method are similar to serotyping.

1.6.2.2 Enzyme digest methods.

There are a plethora of molecular typing methods for *C. jejuni*, with new ones frequently being developed (de Boer *et al.* 2000; Nielsen *et al.* 2000; Wassenaar and Newell 2000; Fitzgerald *et al.* 2001a; Frost 2001; Duim *et al.* 2003b). The most commonly used include pulsed field gel electrophoresis (PFGE) (Hanninen *et al.* 1998; Fitzgerald *et al.* 2001; Karenlampi *et al.* 2003; Broman *et al.* 2004; Connerton *et al.* 2004), amplified fragment length polymorphism (AFLP) (Duim *et al.* 2000; Manning *et al.* 2001; Schouls *et al.* 2003), flagellin gene restriction fragment length polymorphism (fla typing), (Stern *et al.* 1997; Camarda *et al.* 2000; Newell *et al.* 2001), ribotyping (Wassenaar and Newell 2000) and random amplified polymorphic DNA (RAPD) methods (Carvalho *et al.* 2001).

PFGE consists of bacterial cells being lysed within an agarose gel using a 'rare cutting' enzyme, commonly *Sma*I. The DNA fragments are then separated on a gel to

give a profile - also known as macro-restriction profiling. AFLP is similar, but two restriction enzymes are used, and specific primers amplify a subset of the DNA fragments by PCR. The advantage of these methods is that they can be adapted to different bacterial species, and the whole genome is sampled. However, they are very difficult to reproduce and interpret among laboratories, and require specialized equipment. PCR-RFLP is most commonly used in fla typing, whereby PCR is used to amplify the *fla* gene, the products of which are then subjected to restriction enzyme digest, usually with *Dde*I or *Hinf*I (Harrington *et al.* 1997). The main advantage of this over the other enzyme digest methods is that it is easier to perform, with less DNA and equipment required (Cardarelli-Leite *et al.* 1996). The main disadvantage is that it is less discriminatory than methods such as PFGE (Fitzgerald *et al.* 2001). In addition it is not recommended to be used as a sole method for epidemiological typing since intergenomic and interspecies recombination is thought to occur within the *flaA* gene, and the occurrence of conserved fla types has been noted across different PFGE and ribotypes (Harrington *et al.* 1997; Dingle *et al.* 2005). Ribotyping consists of agarose gel electrophoresis of digested genomic DNA followed by Southern blot hybridization with probes specific for rRNA genes, the most discriminatory of which is specific for 16S and 23S in combination (Wassenaar and Newell 2000). There are highly conserved regions amongst the ribosomal genes which make them ideal targets for typing. Disadvantages include the limited discriminatory power, high costs and low throughput. RAPD uses arbitrarily selected primers to amplify random DNA products under conditions of low stringency, which then give a band pattern on an agarose gel. The results are difficult to interpret, particularly as amplicons may give strong or weak bands, and reproducibility is poor.

1.6.2.3 Nucleotide sequencing methods.

Direct nucleotide sequencing has been used in multilocus sequence typing (MLST) and for *flaA* SVR typing (Meinersmann *et al.* 1997; Maiden *et al.* 1998; Dingle *et al.* 2001a; Dingle *et al.* 2001b; Suerbaum *et al.* 2001; Dingle *et al.* 2002; Sails *et al.* 2003b; Sails *et al.* 2003a; Schouls *et al.* 2003). Advantages of nucleotide sequencing over serotyping and enzyme digest methods are that data can be generated for any organism, the data are definitive, comparable and easily reproducible amongst laboratories, and the technology and equipment required are relatively simple. Unlike serotyping the number of isolates that can not be characterized is minimal. Nucleotide sequencing data can be readily shared amongst research groups via the internet, removing the need to ship live culture collections around the world, and is directly applicable and amenable to evolutionary analyses.

MLST was first proposed in 1998 as a standardized and universal method for characterization of bacteria, analyzing *Neisseria meningitidis* in the first instance (Maiden *et al.* 1998; Maiden 2006). It is based on the multi-locus enzyme electrophoresis (MLEE) method that indexes slowly occurring genetic variation by comparing the electrophoretic mobility of gene products on a gel, but instead, portions of genes are directly sequenced and a greater level of variation identified (Selander *et al.* 1986; Maiden *et al.* 1998). MLST encompasses a number of requirements for characterization schemes that had not been previously possible, and incorporates numerous advances in the knowledge of bacterial populations, nucleotide sequencing and computing technology. The scheme is suitable for a range of bacterial population structures, particularly those that are non-clonal, where information from more than one locus is essential for accurate analysis. As with MLEE, MLST indexes variation in housekeeping genes (those encoding fundamental metabolic functions) which

provides sufficient levels of discrimination without being subject to diversifying selection. The capacity for the number of isolates that can be characterized is continually increasing as costs reduce and the dideoxy nucleotide chemistry, automated DNA analyzers and computerized data collection improve. The MLST data are freely available as 'virtual isolate collections' to the research community on sophisticated databases that are curated to ensure accuracy.

The MLST scheme for *Campylobacter* designed by Dingle *et al* (2001a) determines the nucleotide sequence of a 500bp region at each of seven housekeeping loci. The allele lengths were chosen as the length of nucleotide sequence that could be accurately read on an automated DNA sequencer, and seven was the minimal number of loci required to give sufficient resolution (Maiden 2006). Each allele at each of the loci is assigned a number in order of discovery using the *Campylobacter* MLST database. The combination of the seven allele numbers gives a sequence type (ST), which acts as a convenient bar code indexing the genetic information (Urwin and Maiden 2003). Every novel allele and ST is stored on the database to give a 'dictionary' of genotypes. Sequencing of the *flaA* SVR similarly identifies alleles, but since the nucleotide sequence is so diverse a peptide allele is also given. Meinersmann *et al* (Meinersmann *et al.* 1997) concluded that there could be as much as 30% difference in the *flaA* gene from one isolate to another, but that the degree of discrimination detected by sequencing the SVR region was similar to that of the whole gene. Sequencing of the *flaA* gene on its own is not sufficient to investigate evolutionary relationships as it is under diversifying selection.

MLST was the method of choice for this study due to the ability to directly compare *Campylobacter* isolates from different sources for the first time, the high throughput required for the large number of samples, the high degree of typeablitiy,

the unequivocal results and their suitability for evolutionary analyses. The *flaA* SVR was sequenced to give greater discrimination between isolates with identical STs, and to investigate rapidly evolving variation which could be occurring within herds or flocks of animals. Both MLST, and MLST and *flaA* SVR typing in combination, have been successfully used for epidemiological investigation for example investigating strains of *Campylobacter* associated with GBS, host associations and environmental sources of *Campylobacter* on farms (Dingle *et al.* 2001b; Siemer *et al.* 2005; Bull *et al.* 2006).

1.7 Population biology of *Campylobacter.*

Early studies investigating the population structure of *Campylobacter* were based on MLEE, but recent advances in high throughput sequencing have allowed large collections of isolates to be characterized, providing data that lends itself to evolutionary analysis. Many isolate collections are biased with isolates from human disease, but it is important, especially with an organism such as *Campylobacter* which is not a strict human pathogen, to include isolates from other niches it occupies and to address epidemiology and population questions from the pathogen perspective rather than the host (Gupta and Maiden 2001; Maiden 2002). Bacterial populations can be scaled from 'clonal', where bacteria are highly uniform, eg *Mycobacterium tuberculosis*, *Yersinia* species, or *Salmonella enterica* to 'non clonal' where nearly every isolate is different, eg *Helicobacter pylori* (Maiden 2002). The extent of clonality is dependent on the frequency of horizontal transfer of DNA by recombination or mutation. In a clonal population alleles at different loci are congruent, with the signal or phylogeny identical at each locus. Clonal populations may arise due to recent emergence of a desirable characteristic, periodic selection or

bottle necking. Alleles at different loci are not congruent in non-clonal populations, which is indicative that recombination has occurred (Jolley and Urwin 2001). Many bacterial species such as *Neisseria meningitidis* and *Streptococcus pneumoniae* fall in between the 'clonal' and 'non-clonal' ends of the scale and are often described as being 'partially clonal'.

Analysis using MLST of housekeeping genes has shown that relatively high levels of recombination occur in *Campylobacter,* falling between the clonal structure of *Salmonella enterica* and fully diverse *Helicobacter pylori*, and that the population structures of both *C. jejuni* and *C. coli* are partially clonal and diverse (Dingle *et al.* 2001b; Suerbaum *et al.* 2001; De Boer *et al.* 2002; Dingle *et al.* 2002; Fearnhead *et al.* 2005; French *et al.* 2005; Meinersmann *et al.* 2005; Thakur and Gebreyes 2005). Clustering of genotypes into clonal complexes is best analysed at present using the BURST or more recent eBURST programmes developed by Feil *et al* (Feil and Chan 1999; Feil *et al.* 2004), although results are a theoretical model of the population and biological fit should be confirmed. The central genotype is thought to become predominant in a population perhaps due to a fitness advantage. As it increases in number it diversifies giving rise to variants differing at one and eventually two or three loci. Some of the variants themselves may also become predominant within a population forming a sub-group within the clonal complex giving rise to variants of their own.

The eBURST algorithm firstly clusters STs into groups that have a user-defined number of alleles in common (usually four to six), and then identifies the ST with the largest number of single locus variants (SLVs) as the central, and most likely ancestral genotype. The confidence that the assigned central genotype is correct can be calculated using bootstrap values. The values are usually high, but may be low if

two STs with an identical number of SLVs are identified, in which case the ST with the largest number of double locus variants (DLVs) is taken to be the central genotype. Frequent recombination and rapid clonal diversification has resulted in the population structure of *Campylobacter* being more complicated in comparison to some other bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus* (Feil *et al.* 2004). Dropping the number of alleles in common to zero on the eBURST programme gives a 'snap shot' of the *Campylobacter* population, and demonstrates the clustering of some STs, amongst many others that are not (Figure 1.1). The ST-21 complex has numerous subgroups, many of which have historically been labeled as clonal complexes in their own right. The structure of the ST-45 clonal complex, including its subgroups is shown in greater detail in Figure 1.2.

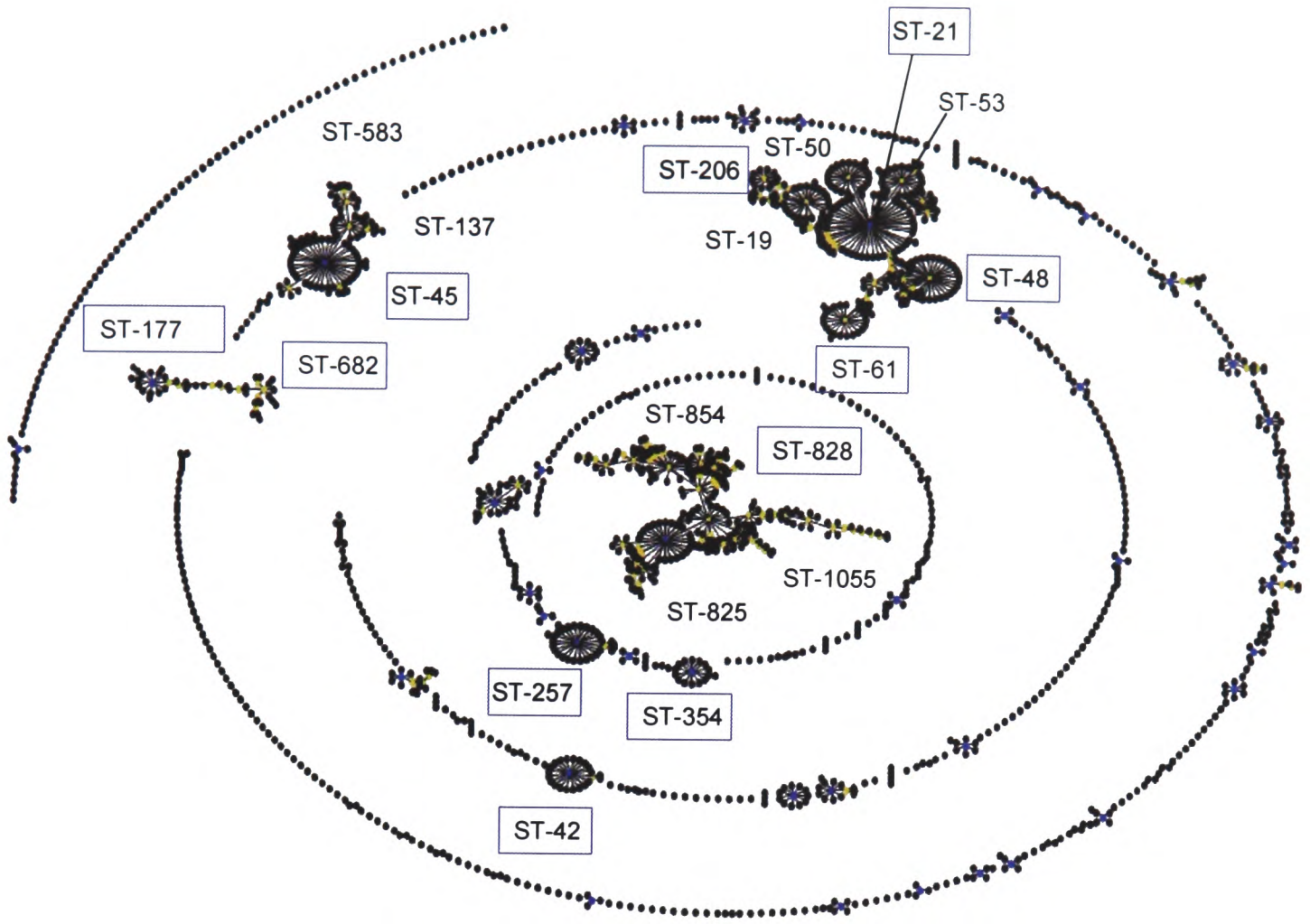
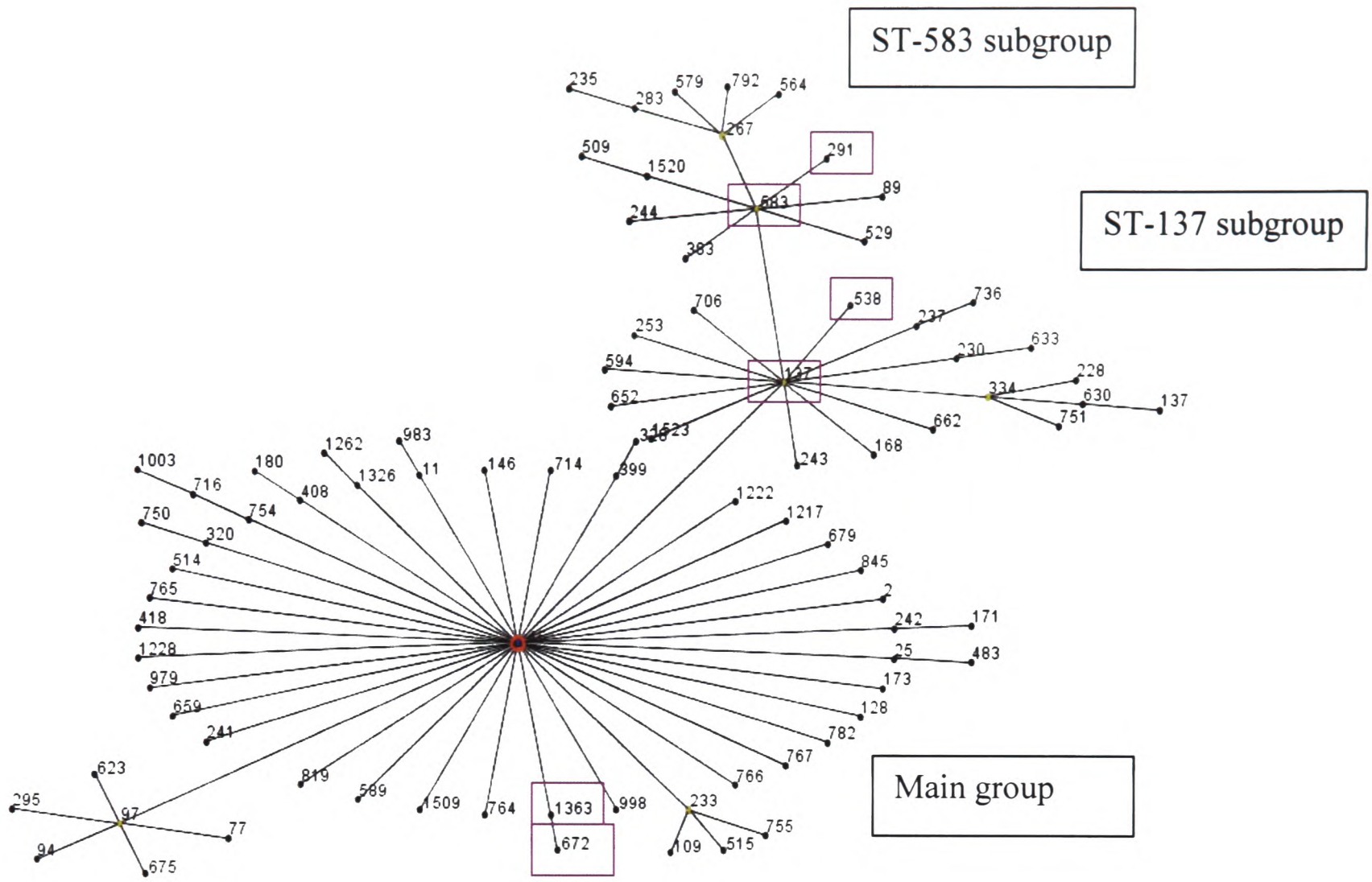


Figure 1.1. The partially clonal population structure of *Campylobacter* represented using eBURST. Some STs cluster into clonal complexes, whilst many more do not. The central STs of the larger clonal complexes are highlighted with a blue box. STs relating to subgroups are labeled but not highlighted.



Group	ST	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*unc*	Relationship to central genotype (ST-45)
Main group	45	4	7	10	4	1	7	1	
	1363	4	7	10	4	1	20	1	Single locus variant
	672	4	7	10	4	2	20	1	Double locus variant
ST-137	137	4	7	10	4	42	7	1	Single locus variant
subgroup	538	4	7	10	4	42	25	1	Double locus variant
ST-583	583	4	7	10	4	42	51	1	Double locus variant
subgroup	291	8	7	10	4	42	51	1	Triple locus variant

Figure 1.2. The clustering of STs within the *C. jejuni* ST-45 clonal complex, represented using eBURST. The central genotype ST-45 is highlighted by a red circle. STs, such as ST-137 giving rise to subgroups are highlighted with yellow circles. The number of lines between the central genotype ST-45 and another represents the number of different loci between them. The relationships between the STs highlighted in purple boxes are shown in greater detail in the table.

There is evidence that both interspecies and intragenomic transfer of DNA can occur and occasional 'disparate' alleles or STs arising from a combination of *C. jejuni* and *C. coli* alleles have been found (Dingle *et al.* 2005; Meinersmann *et al.* 2005). Recombination frequently occurs within the *flaA* SVR, both between the *flaA* and *flaB* genes as well as between different *Campylobacter* sub species (Harrington *et al.* 1997). Analysis of gene flow (F_{ST}) gave evidence that the *flaA* SVR genotypes of both *C. jejuni* and *C. coli* were derived from the same gene pool. Suerbaum *et al* (2001) noted that extensive diversity of allelic profiles was generated from a relatively small number of polymorphic nucleotides amongst the housekeeping genes. There are a number of factors that can cause a partially clonal structure, such as a) the frequency of recombination is not large enough to give a totally random structure, b) some subsets of the population may not be recombining, perhaps due to geographical separation c) some sub-populations may be more successful than others, e.g. niche adaptation or expansion of so called 'epidemic clones' and d) some portions of the genome may not recombine resulting in linkage islands within the genome that evolve clonally (Maynard Smith *et al.* 1993; Spratt and Maiden 1999; Dingle *et al.* 2002; Meinersmann *et al.* 2005).

Some *Campylobacter* genotypes may be host associated, for example amongst *C. jejuni*, ST-45 and ST-257 complexes were commonly identified amongst poultry isolates, ST-61 and ST-42 complexes were commonly identified amongst cattle and sheep and ST-177 and ST-179 complexes were isolated only from sand by Dingle *et al* (2002). Other studies have identified clones adapted to pig and wildlife hosts or survival in water, with isolates from the same host being less diverse than those from different hosts (Manning *et al.* 2003; French *et al.* 2005; Kemp *et al.* 2005). This may reflect niche adaptation or influences of host immune responses. It is not known

whether *Campylobacter* genotypes from one host are pathogenic to all, or whether other hosts are just not exposed to all potential genotypes. Meinersmann *et al* (2005) concluded that local environmental factors were most important in restricting the migration and thus mixing and recombination of genotypes. Similarly French *et al* (2005) found *Campylobacter* genotypes separated by less than 1KM were more similar than those separated by greater differences, independent of other clustering effects.

1.8 Objectives of the study.

The aims of the study were firstly to test the MLST method on *Campylobacter* isolates from live animals and the environment to see if it was viable and in order that the previous investigations amongst human and meat isolates may be expanded. The typing method was chosen to enable direct comparison of *Campylobacter* genotypes isolated from environmental and animal sources for the first time, and to provide data suitable for detailed genetic and population analysis. A number of *C. jejuni* isolates from farm animal species, slurry and wild birds in Lancashire were tested as a preliminary investigation and to examine whether identical genotypes could be isolated from more than one source. There was evidence that poultry were colonized by *Campylobacter* genotypes dominant amongst human disease. Wild birds were identified as a potential vector for transmission of *Campylobacter* genotypes amongst animals on the farm. The main focus of the study was then to determine the population dynamics of *Campylobacter* amongst free-range broiler chickens and temporally matched wild birds in Oxfordshire and to identify if transmission of genotypes occurred between them, and ultimately reach humans at the end of the food chain. In addition the effects of sharing arcs between flocks, the effects of rearing

chickens on plots of land that were initially fallow, or later contaminated with faeces from previous flocks, and the effects of high and low stocking density on *Campylobacter* colonization were considered. Wild geese and Starlings were present in large numbers on the farm and were identified as species that could interact with both farm animals and humans. The Starlings were identified by numbered leg rings and details such as age, weight and sex were recorded which enabled investigation into *Campylobacter* colonization amongst recaptured birds and nestlings, and correlation with age, weight and sex. Finally genotypes were compared amongst all animal sources and between two regions of the UK in order to examine the population biology of the organism on a national scale. The study utilized the recent advances in bacterial typing, population biology and genetic analyses to examine the *Campylobacter* population structures and transmission of genotypes amongst wild birds and domesticated poultry for the first time.

Abstract

Chapter 2. Materials and methods.

This chapter details the origin, type and sampling method for each of the *Campylobacter* isolates used in the study. In addition bacterial culture, speciation and MLST methods are described. Finally genetic and statistical analyses are presented.

Chapter 2: Materials and Methods.

2.1 *Campylobacter* isolates.

2.1.1 Farm animal and environmental samples from Lancashire, North-West England.

A total of 112 *C. jejuni* isolates collected in Lancashire during the 1990's were provided by Dr Keith Jones, Lancaster University. They comprised 16 isolates from the intestine of broiler birds from a poultry processing plant supplied by 35 farms located within a 150-mile radius (Wallace *et al.* 1998); 12 isolates from fresh faeces of turkey chicks kept on a dairy farm (Wallace *et al.* 1998); 14 isolates from the small intestines of adult beef cattle at an abattoir receiving cattle and sheep from north-west England, north Wales and south-west Scotland; nine isolates from fresh faeces of calves (Stanley *et al.* 1998a); nine isolates from the small intestines of lambs at the same abattoir as the beef cattle (Stanley *et al.* 1998b); 14 isolates from intestinal contents of sheep at slaughter; ten isolates from fresh faeces of sheep grazing on salt marsh, nine isolates from fresh faeces of sheep grazing on upland fell (Jones *et al.* 1999); ten isolates from slurry storage tanks (Stanley *et al.* 1998c); and nine isolates from starling faeces collected on the same dairy farm as the isolates from turkey chicks (Stanley and Jones 1998).

2.1.2 Wild geese isolates from Oxfordshire, Southern England.

A total of 331 fresh faeces samples were collected from areas were wild geese populations were grazing on the Oxford University farm at Wytham, Oxfordshire. Samples were collected on five occasions between August 2002 and February 2003 (40 samples on

18.08.2002, 84 samples on 11.09.2002, 82 samples on 20.09.2002, 43 samples on 3.02.03 and 82 samples on 26.02.03) and were immediately transported back to the laboratory for culture. Numbers of wild geese exceeded 150 on each occasion and consisted of mixed species, the majority being Canada (*Branta canadensis*) or Greylag (*Anser anser*) geese.

Figure 2.1 Mixed geese species grazing in the fields at the University farm, Wytham.

2.1.3 Lamb samples from Oxfordshire.

A total of 84 fresh faecal samples were collected by following lambs grazing on the same pasture as the wild geese, in October 2002. Samples were immediately transported back to the laboratory for culture.

2.1.4 Free-range broiler chicken samples from Oxfordshire.

The birds arrived at the Wytham farm site as day old chicks in batches of 1250. Mixed sex Sherwood White birds were reared during the first year, but health problems within the limited parent flocks available resulted in female Ross 308 birds being reared in the second year. The target weight was 2.28kg at 56 days of age and the male Ross birds were excluded through being too large. They were fed a commercial diet based on wheat without growth promoters, digestive enhancers and coccidiostats, and were vaccinated at day one with Paracox 5 Coccidiosis on feed, at day 13 with Infectious Bronchitis H120 spray and at day 20 with Grumbro Bursine in water. The chicks were raised in brooding sheds until 21 days of age. They were then moved to rearing arcs at either the Wytham or Northmoor Trust farm sites on a rotating cycle at a stocking density equivalent to free-range requirements ($1.2m^2$ per bird). Every third flock was divided and reared in two separate arcs under low stocking density conditions equivalent to that required for organic standards ($2.5m^2$ per bird). For reasons of efficiency the arcs were moved between two adjacent plots of land but were disinfected and left empty for five days between flocks. A total of eight separate flocks were raised within a set of arcs in the first year of the study. There were 16 fenced plots of land consisting of pasture or pasture and trees in combination onto which the flocks were placed in rotation and let out to roam (Figures 2.1a and b). Eight of the plots were at Wytham and were 45.2m x 36m = 1617m2 in size and eight were at Northmoor and were 50.8m x 32m = 1625.6m2 in size. A total of four flocks were raised on a plot of land in the first year of the study. Plots of land were left fallow for seven weeks before they were re-stocked. Birds were let onto the range from 23 days of age during daylight hours and depleted at 56 days of age.

The flocks were tested for *Campylobacter* in two stages. The first flock was sampled weekly from when the birds arrived on the farm as day old chicks up until 56 days of age

when the flock was unique in being partially depleted (Figure 2). Ten samples of fresh faeces were collected weekly from different areas within the brooding arcs. At 28 days of age or older, swabs were used to sample the anal area of individual chickens, ensuring the tip of the swab was still visible during sampling. A catching frame was used in separate areas of the arc to ensure that different birds were sampled. All samples were transported back to the laboratory for culture within a couple of hours. Approximately 100 chickens were retained at depletion due to their large size and moved into a barn where numbers were gradually reduced until they were finally all killed after 112 days of age. Sampling of these birds was carried out weekly from 77 to 112 days of age by collecting ten anal swabs from individual birds selected at random per week. The weekly sampling regime from 1 to 56 days of age was replicated later in the year with flock 43.

A total of 84 flocks in rolling production were all sampled as close to day 56 as possible and over a period of 18 months. Ten anal swabs were collected from randomly selected chickens and cultured individually. The number of swabs was increased to 25 per flock from flock 40 onwards to allow improved statistical analysis. Only isolates from the first 48 flocks were genotyped by MLST.

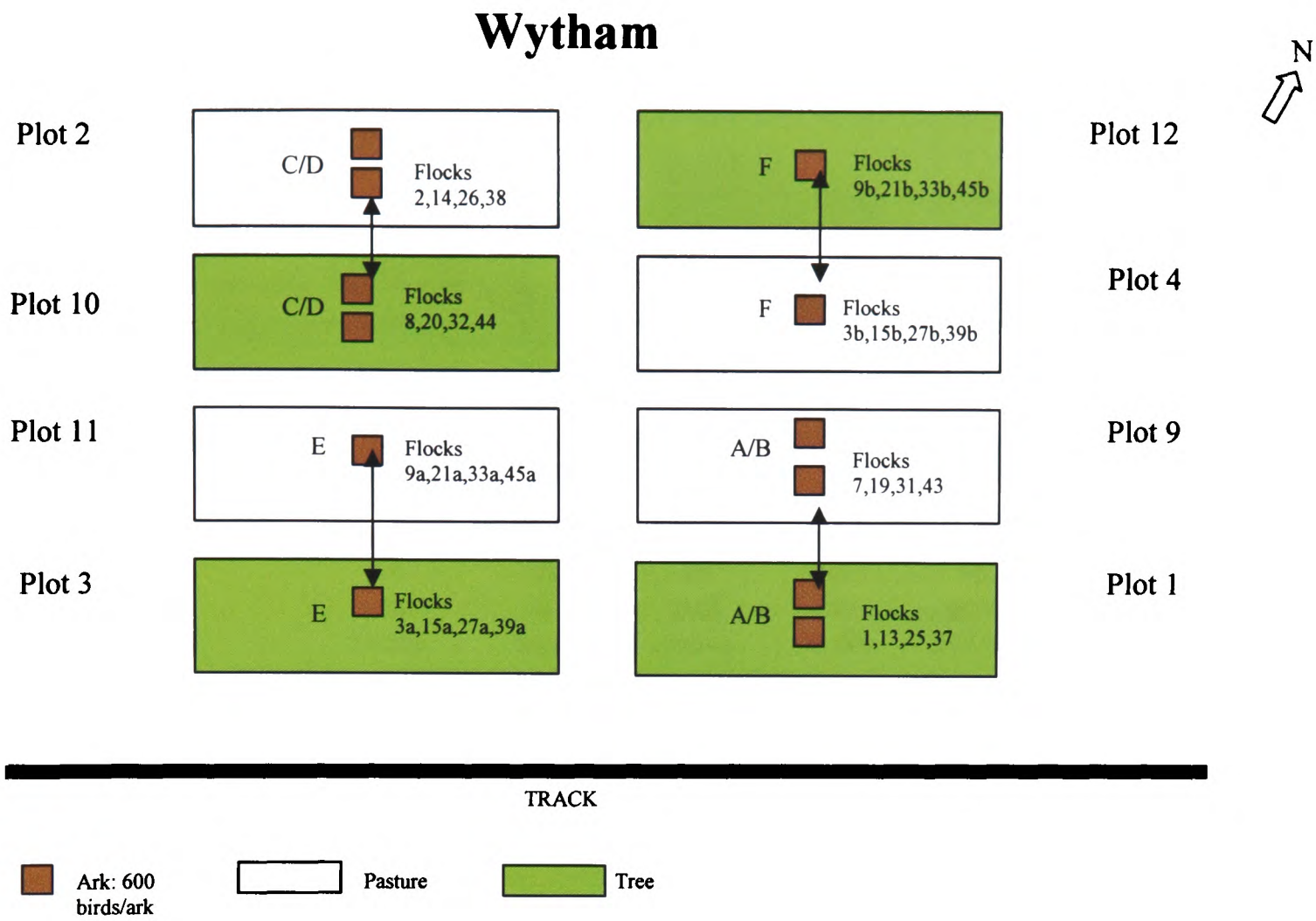


Figure 2.2a The free-range plots and flock rotations at the Wytham farm site.

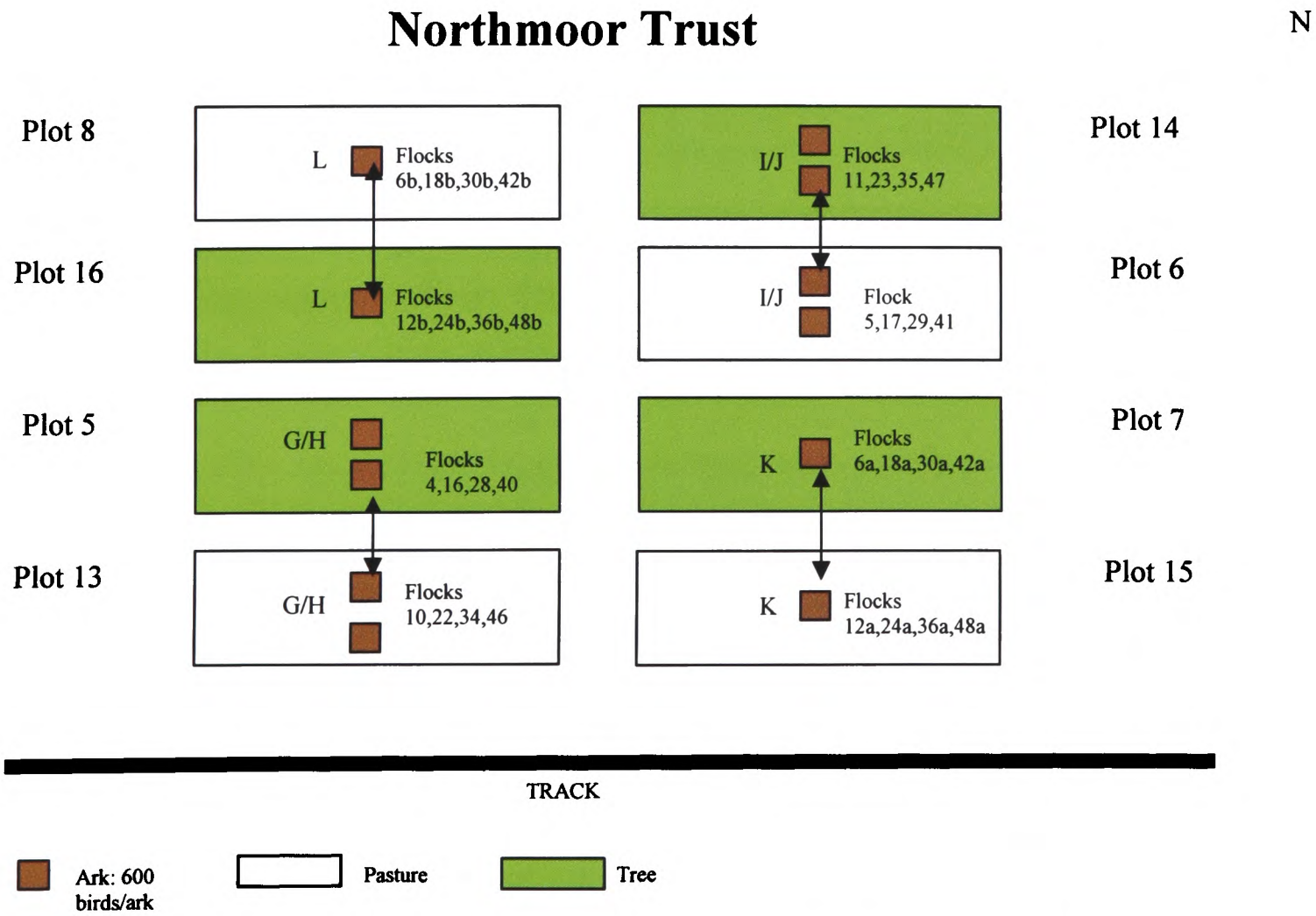


Figure 2.2b The free-range plots and flock rotations at the Northmoor Trust farm site.
A-K = arc identities.

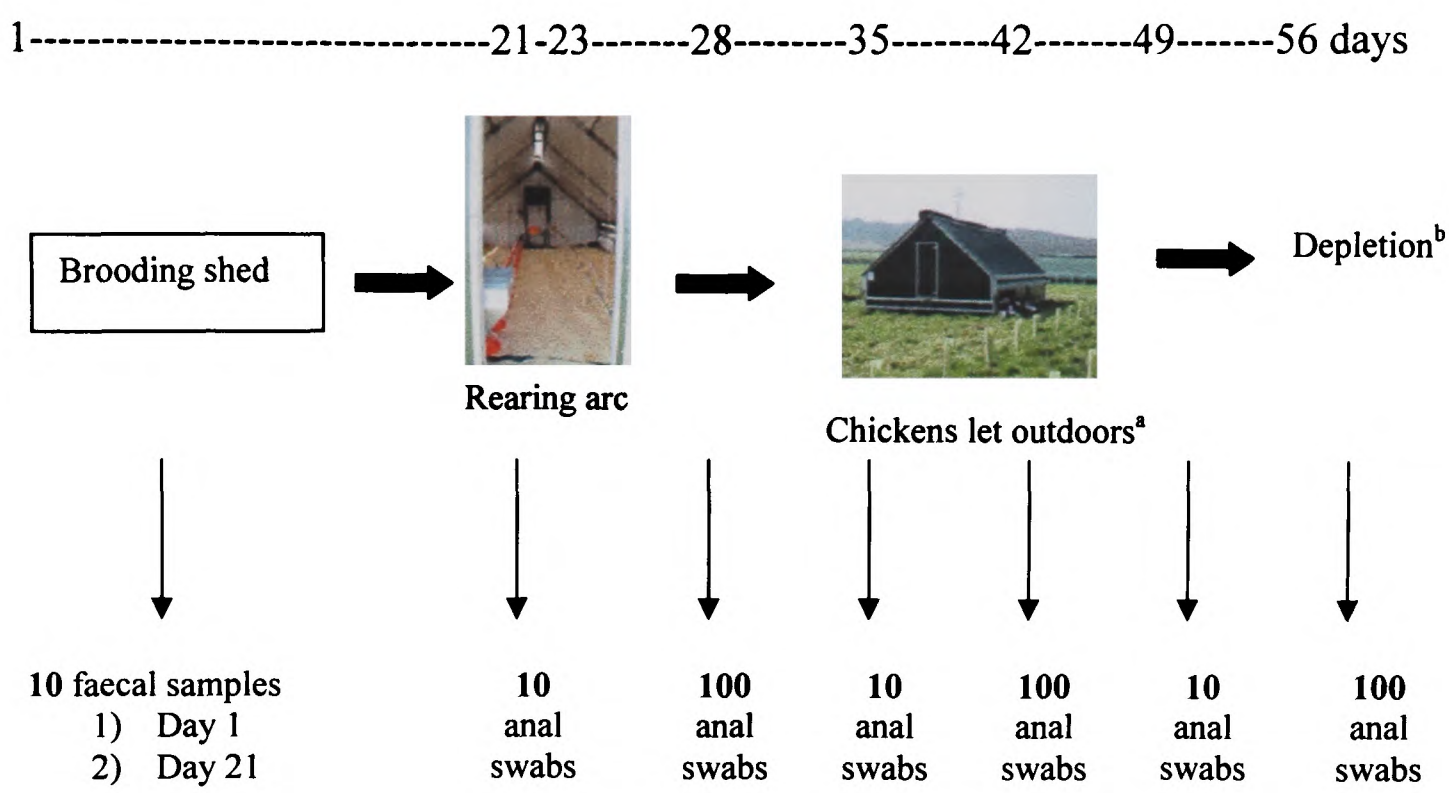


Figure 2.3 Sampling regime for flocks 1 and 43.

[a]Chickens from flock 1 were let outdoors at 35 days of age, chickens from flock 43 were let outdoors at 23 days of age.

[b]Flock one was partially depleted at 56 days, and ten of the remaining birds were sampled at weekly intervals between days 77 and 112 of age. Flock 43 was fully depleted at 56 days.

2.1.5 Wild Starling samples from Oxfordshire.

A total of 959 samples were collected from wild Starlings at Wytham between 16.7.2002 and 4.2.2005. Sampling was uneven throughout the study period as the number of birds on the farm was far greater during the winter months. The distribution of sampling is shown in Table 2.1. The birds from 2002 were caught using mist nets alongside the pig pens where they were feeding. During 2003 birds were baited in a field close to the farm buildings with chick feed and meal worms and caught using whoosh nets (Figure 2.3). In the latter part of the study birds were caught by baiting a trap on a site in amongst the buildings. The trap had a one way entry system whereby birds can enter and eat the food, but can not escape.

Nestlings, sampled at nine days of age, were located in numbered nest boxes forming three colonies, two in different areas at the University farm in Wytham and one at the Saw mill approximately half a mile away (Figure 2.4 and Figure 2.6). Each bird was identified with a British Trust for Ornithology (BTO) leg ring. The age, sex, weight and wing length were recorded where possible, according to BTO guidelines. The age was recorded with the following standardized code since the exact age could never be known: Nestling, Code 3=full grown bird born in the present breeding season, Code 5=a bird born last year and Code 6=a mature adult. Fresh faecal samples were collected from the bird bag using charcoal transport swabs and most samples were processed in the laboratory within two hours of collection, although occasionally they were stored at 4°C for 24 hours prior to processing with no obvious ill effects.

Table 2.1 The months in which faecal samples were collected from wild Starlings, 2002-2005.

	Year			
Month	**2002**	**2003**	**2004**	**2005**
January			70(11%)	41(61%)
February		35(15%)	32(5%)	26(39%)
March			12(2%)	
April				
May		21(9%)	94(14%)	
June		12(5%)	295(45%)	
July	12(100%)		4(1%)	
August				
September				
October				
November		60(26%)	84(13%)	
December		101(44%)	58(9%)	
Total	12(100%)	229(100%)	649(100%)	67(100%)

Figure 2.4 A woosh net set for trapping Starlings. The area was baited with chick crumb and meal worms. The net acted like a bungee and was poised to spring over the birds when the lengthy trigger cord was pulled.

Figure 2.5 One of the Starling nesting colonies at the University farm, Wytham. Nest boxes were numbered for ease of identification.

2.1.6 Human disease isolates for data comparison in Chapters 3, 4 and 5.

Data from 91 isolates from human disease, sampled in north-western England during 1999 were obtained by searching the *Campylobacter* MLST database with the following criteria; id<1316, country=UK, year=1999, source=human stool. The search criteria given ensured that the relevant data was chosen, and also that unpublished data belonging to other research groups was not included. In this particular collection every tenth isolate through the door of the reference laboratory that supplied them was included giving a representative population of *Campylobacter* genotypes infecting humans that year. A breakdown of the *Campylobacter* genotypes is given in the appendices.

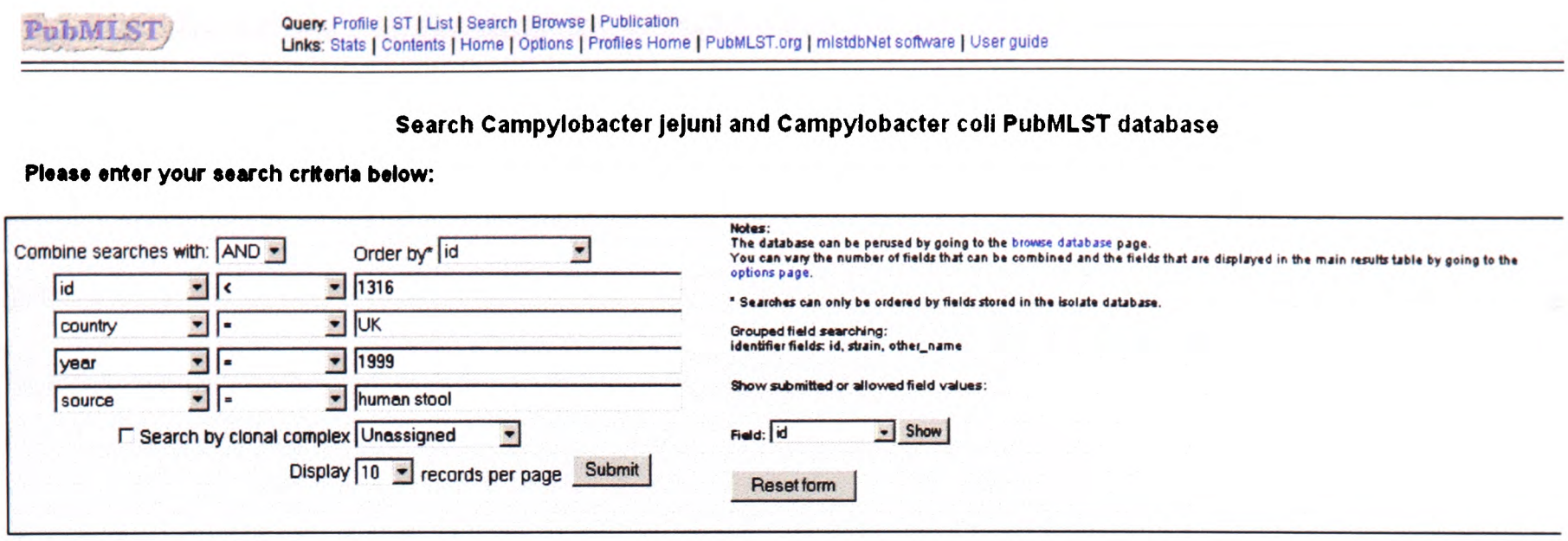


Figure 2.6 The *Campylobacter* MLST database showing the search criteria to select the human disease data used in Chapters 3, 4 and 5.

2.1.7 Oxfordshire cattle isolates for data comparison in Chapter 8.

A total of 268 faecal samples were collected from beef cattle kept at the University farm in Wytham on eight sampling occasions between 10.5.04 and 5.8.04 and cultured for *Campylobacter*. The cattle were of different age groups, although the majority (243 of 268, 90.7%) were adult, different sex and different breeds. Most of the cattle (205 of 268, 76.5%) were housed, with the remainder out at grass. *Campylobacter* was isolated from 33 samples, giving a shedding rate of 12.3%. Of these, 21 isolates (63.6%) were *C. coli* and 12 (36.3%) were *C. jejuni*. A breakdown of *Campylobacter* genotypes is given in the appendices.

2.1.8 Oxfordshire pig isolates for data comparison in Chapter 8.

A total of 10 faecal samples were collected from housed pigs that were approximately three months of age, on 2.2.04. *Campylobacter* was isolated from eight of the samples, giving a shedding rate of 80%. All of the *Campylobacter* isolates were *C. coli.* A breakdown of the *Campylobacter* genotypes is given in the appendices.

2.1.9 Human disease isolates for data comparison in Chapter 8.

Data from 722 isolates from human disease sampled in the UK between 1977 and 2004 were obtained by searching the *Campylobacter* MLST database with the following criteria; source=human stool, country=UK and id<2747. The search criteria ensured that all human disease isolates from stool samples collected in the UK were selected.

Some analyses in Chapter 8 used all of the 1358 human disease isolates collected from stools that were listed on the database. They were obtained by searching the database with the following criteria; source=human stool, id<3044. The isolates were collected between 1977 and 2005, from 19 different countries, including those in Europe, America, Asia, Africa and Australasia. A breakdown of the *Campylobacter* genotypes is given in the appendices.

2.1.10 Retail chicken meat isolates for data comparison in Chapter 8.

Data from 130 isolates from retail chicken meat sampled in the UK between 1982 and 2005 were obtained by searching the *Campylobacter* MLST database with the following criteria; source=chicken offal or meat, country=UK and id <2910. The search criteria ensured that all retail chicken meat isolates from the UK were selected.

Some analyses in Chapter 8 used all of the 144 isolates from chicken meat listed on the database. They were obtained by searching the database with the following criteria;

source=chicken offal or meat and id<2981. The isolates were collected between 1982 and 2005 from four different countries, the majority being from the UK(90%), with others from Senegal (5.6%) Denmark (2.8%) and the US (0.7%). A breakdown of the *Campylobacter* genotypes is given in the appendices.

2.1.11 Location of the sampling sites for animals in Oxfordshire.

The sampling sites for free-range broiler chickens (brooding and rearing arcs), wild geese and wild Starlings at the University farm in Wytham can be seen in Figure 2.6a. The additional sampling site for Starling nestlings at the Sawmill in Wytham woods is also given. Housed cattle and pigs, used for data comparison in Chapter 8 were kept in the farm buildings in the immediate vicinity of the chick rearing arcs. The free-range boiler chickens were sampled at a second site, the Northmoor Trust at Little Wittenham, which was 12 miles distant from Wytham. The locations of the two farm sites is given in Figure 2.6b.

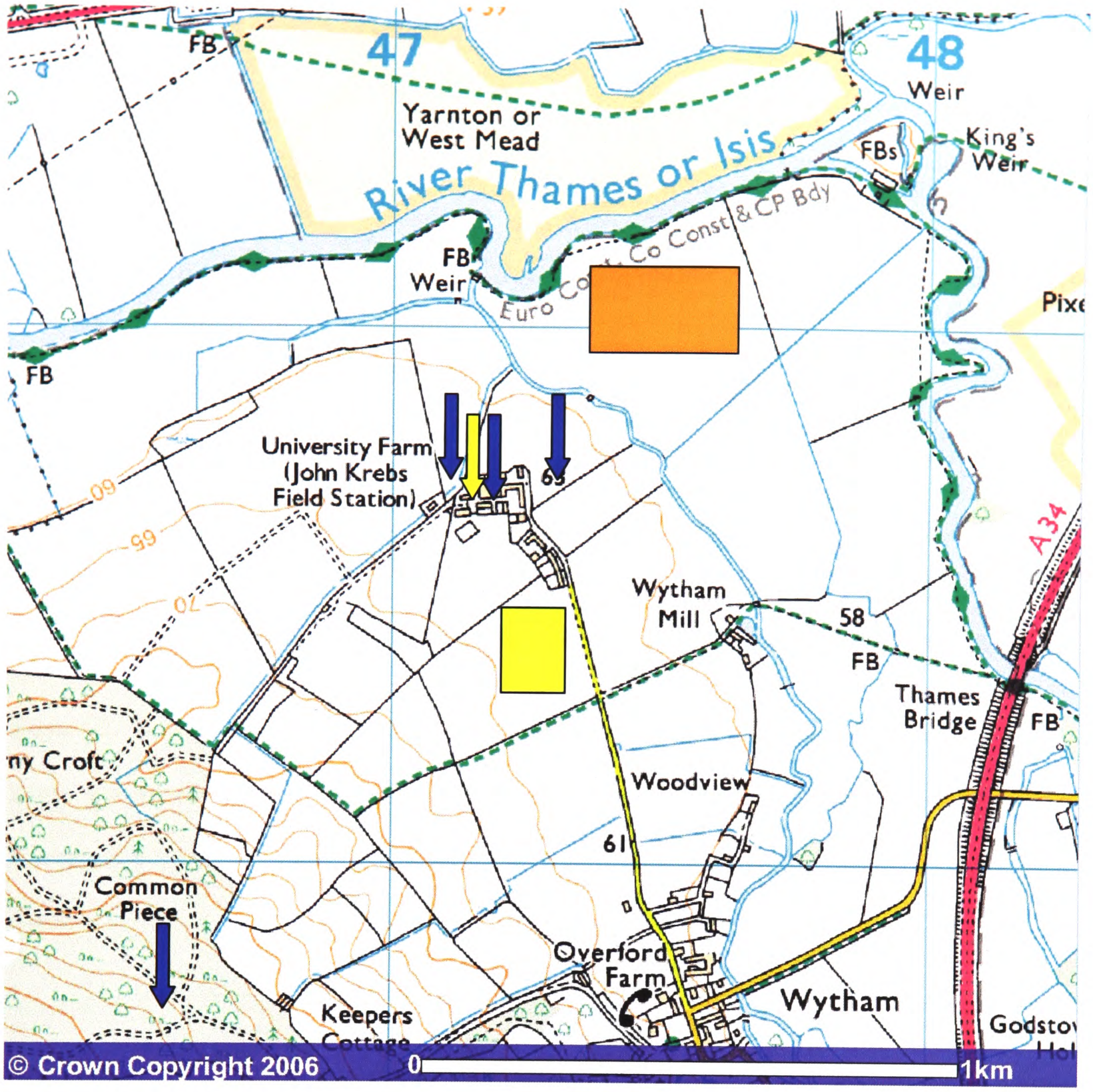


Figure 2.7a. Map of the Oxford University Farm site at Wytham, Oxfordshire showing the locations of sampling sites for free-range broiler chickens (yellow), geese (orange) and Starlings (blue). Yellow arrow, brooding arcs; yellow box, rearing arcs.

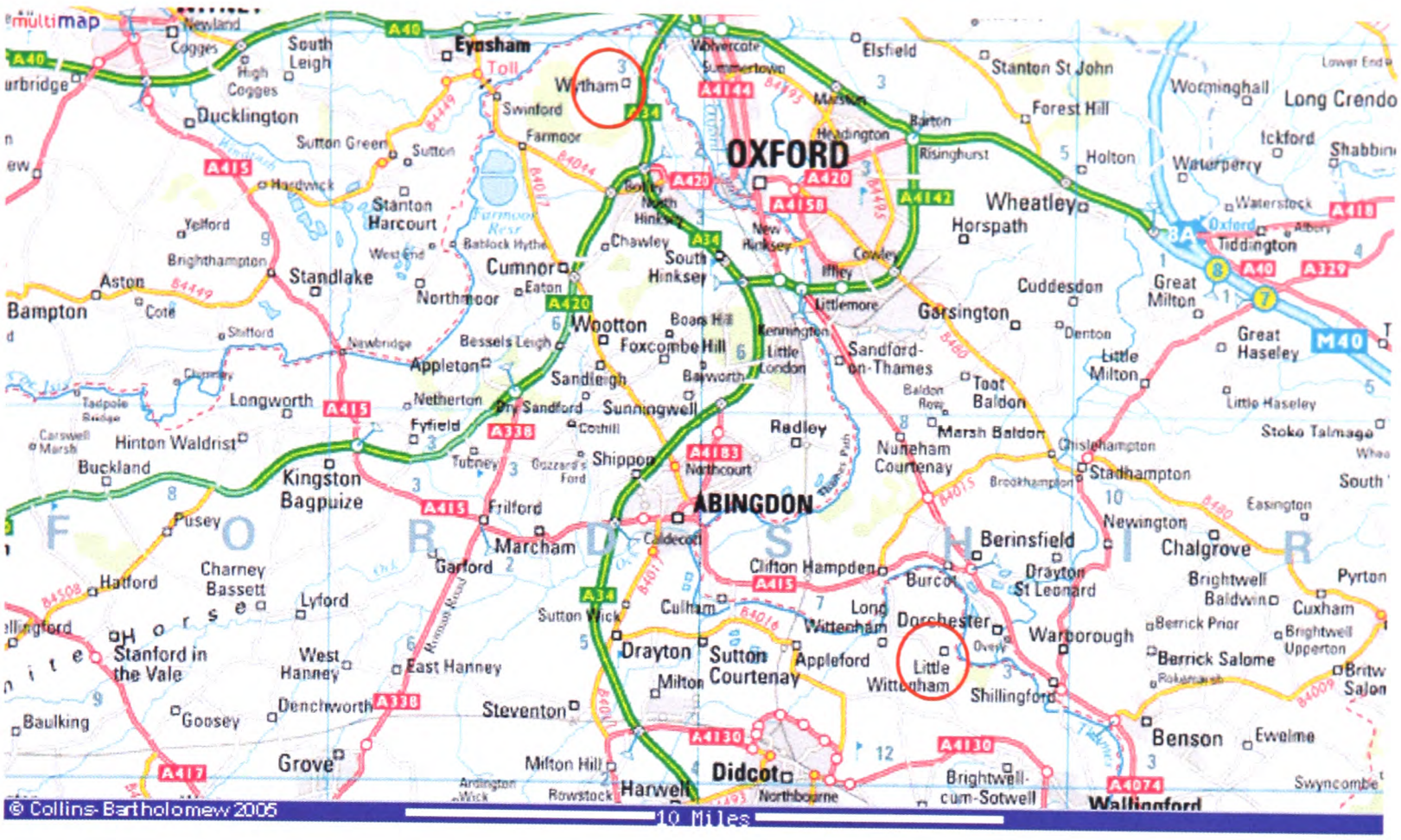


Figure 2.7b. Map showing the two Oxfordshire farm locations (circled) at which free-range broiler chickens were sampled; the University Farm at Wytham and The Northmoor Trust at Little Wittenham. The farm sites were separated by a distance of approximately 12 miles.

2.2 *Campylobacter* isolation and identification to genus level.

Isolates provided by Keith Jones were subcultured onto Columbia Horse Blood Agar (CBA) (agar base CM331 and 5% defibrinated horse blood SR50, Oxoid Ltd) and incubated in a microaerophillic atmosphere generated using the GenBox microaer system (96126 and 96128, bioMérieux UK Ltd, Basingstoke) at 37°C for four hours followed by 42°C for 44 hours. Cultures had already been identified as *Campylobacter* species.

Samples from geese and lambs, were incubated in 5ml Prestons Enrichment Broth (Selective supplement SR0117, Nutrient broth No 2 CM67, *Campylobacter* growth supplement SR084 and laked horse blood SR48, Oxoid Ltd, Basingstoke) at 42°C for 48

hours. They were incubated aerobically since a small air gap between the liquid level and the top of the bijoux is sufficient to provide microaerophillic conditions. Preston broths were subcultured onto mCCDA (CCDA selective supplement SR155E and blood free *Campylobacter* selective agar base CM739, Oxoid Ltd, Basingstoke) and incubated microaerobically for a further 48 hours at 42°C. Where possible single colonies were picked to CBA and incubated microaerobically for 48 hours at 42°C. *Campylobacter* colonies were identified by typical spreading and metallic appearance, characteristic Gram negative small curved rod morphology and presence of catalase (catalase test) and cytochrome oxidase (oxidase test).

Samples from the Oxfordshire free-range chickens were each inoculated onto separate mCCDA plates and incubated microaerobically at 42°C for 48 hours. Single colonies were sub-cultured onto CBA and incubated microaerobically for 48 hours at 42°C, after which *Campylobacter* colonies were identified as above.

Samples from the Oxfordshire Starlings were incubated aerobically in 5ml Exeter broth (Nutrient broth No 2 , CM67, *Campylobacter* growth supplement SR084E, lysed defibrinated blood SR48, Oxoid Ltd, Basingstoke, Exeter selective supplement SV59, MAST GROUP Ltd, Bootle) at 37°C for four hours and 42°C for 44 hours. They were sub-cultured onto mCCDA and incubated microaerobically for a further 48 hours at 42°C. Where possible single colonies were picked to CBA and incubated microaerobically for 48 hours at 42°C after which *Campylobacter* colonies were identified as above.

2.3 Storage and resuscitation of *Campylobacter* isolates.

Campylobacter isolates were stored in duplicate 0.5ml lots of brain heart infusion broth (CM225, Oxoid Ltd, Basingstoke) and 20% glycerol by using a cotton tipped swab

saturated in sterile distilled water to prepare a heavy suspension of bacteria. Freezer vials were labelled with the isolate identification using a permanent marker. Isolates were frozen at -80°C in duplicate freezers and the position noted.

Isolates were resuscitated by placing them in a freezer block and scraping some of the frozen suspension off the top with a sterile plastic loop and subculturing onto CBA plates which were incubated microaerobically at 42°C for 48 hours. Provided the original isolates remained frozen they were returned to their original position in the -80°C freezer. If they defrosted they were replaced with a fresh suspension, to avoid cell death by freeze-thawing.

2.4 DNA preparation.

Chromosomal DNA was extracted from pure cultures of the isolates grown on CBA using either IsoQuick nucleic acid extraction kits (ISC Bioexpress, Kaysville, U.S.), or by a boiling method. Briefly, with the kit method, a suspension of approximately 10^6 bacterial cells was prepared in sample buffer and the rapid DNA extraction protocol followed, which involved chemical disruption of the cells and inhibition of nucleases by addition of guanidine thiocyanate. Nucleic acid was then precipitated with sodium acetate and ethanol, and re-suspended in milliQ water.

The boiling method consisted of a thick cell suspension of the isolate in 150μl PBS (BR14, Oxoid Ltd, Basingstoke, UK) being heated on a heat block at 100°C for 10 minutes. The samples were sedimented at 13,200 rpm for 10 minutes and 120μl of the supernatant was transferred to fresh micro-centrifuge tubes, carefully avoiding the cellular debris. This method proved unreliable for isolates collected during this thesis, perhaps due to haem leaching from the CBA plates and inhibiting PCR reactions. The majority of isolates collected from the free-range poultry and Starlings were initially prepared using the boiling

method and then re-extracted using the Isoquick method, which appeared to resolve the difficulties with PCR amplification reactions.

2.5 Identification of *Campylobacter* isolates to species level.

Some of the isolates were screened by PCR of the hippicurase gene which is present in *C. jejuni* but absent in *C. coli* in order to differentiate between the species. Primers designed by Linton *et al* were used to amplify a 735bp amplicon of the *hipO* gene and were as follows; *hipO* F 5'-GAA GAG GGT TTG GGT-3' and *hipO* R 5'-AGC TAG CTT CGC ATA ATA ACT TG-3' (Linton *et al*, 1997). Each 50μl PCR contained 39μl milliQ water, 5μl 10x reaction buffer (Qiagen Ltd., Crawley, UK), 1μl of each primer, 1μl 10mM dNTPs (Applied Biosystems, Warrington, UK), 0.5μl *Taq* DNA polymerase (Qiagen Ltd.), 0.5μl 25mM $MgCl_2$ buffer (Qiagen Ltd) and 2μl chromosomal DNA. The reaction conditions were 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds (denaturation), 66°C for 30 seconds (primer annealing) and 72°C for 1 minute (extension), followed by a single cycle of 72°C for 10 minutes and storage at 4°C . In order to determine the outcome of the PCR, 5μl of each reaction, and 2μl of a 100bp DNA ladder (Promega, UK, Southampton, UK) together with 1μl 6X loading buffer were run out on a 100ml 1% agarose gel (agarose, Severn Biotech Ltd, Kidderminster UK, 10X TE buffer, National Diagnostics, Hull, UK) containing 5μl ethidium bromide (Aq 10mg/ml, Sigma, Poole) at 140V for 20 minutes. The gel was placed under UV light causing DNA within the PCR products combined with ethidium bromide to fluoresce. Isolates were identified as *C. jejuni* if there was a PCR product relating to presence of the *hipO* gene, or as *'Campylobacter* species' if there was no PCR product, relating to absence of the *hipO* gene.

Speciation using the *hipO* screening was not consistent with results from MLST and was later replaced by the muliplex PCR method described by Wang *et al* (Wang *et al.* 2002). The CJF 5'-ACTTCTTTATTGCTTGCTGC- 3' and CJR 5'-GCCACAACAAGTAAAGAAGC-3' primers specific for the *C. jejuni hipO* gene and the CCF 5'- GTAAAACCAAAGCTTATCGTG-3' and CCR 5'-TCCAGCAATGTGTGCAATG-3' primers specific for the *C. coli glyA* gene were used. Reaction sizes were reduced to 10µl with 9.6µl master mix (7.55µl milliQ water, 1µl 10X buffer, 0.8µl primer mix, 0.2µ dNTPs and 0.05µ hotstart taq (Qiagen Ltd)) and 0.4µl chromosomal DNA. The PCR conditions were 95°C for 15 minutes followed by 35 cycles of 94°C for 20 seconds, 55° C for 20 seconds and 72°C for 30 seconds, followed by a single cycle of 72°C for 5 minutes and storage at 4°C.

The multiplex PCR also proved to be inconsistent with MLST results on these isolates and so speciation on the majority of isolates from the free-range chickens and Oxfordshire Startlings was performed by sequencing the *aspA* locus using a single set of MLST primers (section 2.6.1), to give either *C. jejuni* or *C. coli* alleles. The *glyA* locus of Starling isolates that did not amplify with the *aspA* primers was sequenced using primers designed by Miller *et al*, to give *C. jejuni, C. coli* or *C. lari* alleles.

2.6 MLST.

2.6.1 PCR amplification reactions.

A fragment of each of seven housekeeping genes (aspartase A, *aspA*; glutamine synthetase, *glnA*; citrate synthetase, *gltA*; serine hydroxymethyl transferase, *glyA*, phosphoglucomutase, *pgm*; transketolase, *tkt*; and ATP synthase subunit, *uncA*) was amplified

by PCR using the primers listed in tables two and three. The previously published MLST protocol was used for all isolates but the primers varied according to source. The original primer scheme was used without modification for the Lancashire isolates and some of the Oxfordshire isolates including those from geese (Table 2.2.) (Dingle *et al.* 2001a). However the DNA from the Oxfordshire chickens and Starlings was not of the best quality having been extracted by the boiling method and then re-extracted using Isoquick kits. The best sequencing results for *C. jejuni* were achieved by using a combination of the original sequencing primers and new primers designed by Miller *et al*, 2005 many of which have a choice of bases in allowing for diversity in binding sites. Two primer schemes have been recently published for *C. coli* and the best results were obtained for the Oxfordshire isolates by using a combination of both (Table 2.3) (Dingle *et al.* 2001a; Miller *et al.* 2006). The revised primer schemes worked for all sources of *Campylobacter* with the exception of *aspA* in Starlings where subsititute *C. jejuni* primers A8 and A9 were used. Reactions were usually performed in 48 well micro titre plates unless there were insufficient numbers of samples. Each 50μl PCR contained 39.5μl milliQ water, 5μl 10x reaction buffer (Qiagen Ltd., Crawley, UK), 1μl of each primer, 1μl 10 mM dNTPs (Applied Biosytems, Warrington, UK) 0.5μ *Taq* DNA polymerase (Qiagen Ltd.), 0.5μl 25mM $MgCl_2$ buffer (Qiagen Ltd) and 2μl chromosomal DNA. PTC-200, Dyad^{TM} and Dyad Disciple^{TM} (MJ Research) and GeneAmp^{T} PCR system 9700 (PE Applied Biosystems) themal cyclers were used and the reaction conditions were 95°C for 3 minutes, followed by 39 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute, followed by a single cycle of 72°C for 10 minutes and storage at 4°C. PCR products were separated on a 1% agarose gel containing ethidium bromide as detailed previously (section 2.5) in order to determine the success of the reactions.

Table 2.2. Primers and reaction conditions designed by Dingle *et al* (2001a) and used for PCR and sequencing reactions with 112 *C. jejuni* isolates from Lancashire.

Locus	Sequence 5'-3' sequence	Annealing temp (°C)	No. of cycles
	MLST PCR		
aspA	F (A9) AGTACTAATGATGCTTATCC	50	40
	R (A10) ATTTCATCAATTTGTTCTTTGC	50	40
glnA	F (A1) TAGGAACTTGGCATCATATTACC	50	40
	R (A2) TTCGACGAGCTTCTACTGGC	50	40
gltA	F (A1) GGGCTTGACTTCTACAGCTACTTG	50	40
	R (A2) CCAAATAAAGTTGTCTTGGACGG	50	40
glyA	F (A1) GAGTTAGAGCGTCAATGTGAAGG	50	40
	R (A2) AAACCTCTGGCAGTAAGGGC	50	40
pgm	F (A7) TACTAATAATATCTTAGTAGG	50	40
	R (A8) CACAACATTTTTCATTTCTTTTTC	50	40
tkt	F (A5) TTTAAGTGCTGATATGGTGC	50	40
	R (A4) CATAGCGTGTTCTCTGATACC	50	40
uncA	F (A3) AAAGCTGATGAGATCACTTC	50	40
	R (A4) ATTCTTTGTCCACGTTCAAG	50	40
	MLST sequencing reactions		
aspA	F (S3) CCAACTGCAAGATGCTGTACC	50	30
	R (S6) TTCATTTGCGGTAATACCATC	50	30
glnA	F (S1) GCTCAATTCATGGATGGC	50	30
	R (S6) GCATACCATTGCCATTATCTCCG	50	30
gltA	F (S1) GTGGCTATCCTATAGAGTGGC	50	30
	R (S6) CCAAAGCGCACCAATACCTG	50	30
glyA	F (S3) AGCTAATCAAGGTGTTTATGCGG	50	30
	R (S4) AGGTGATTATCCGTTCCATCGC	50	30
pgm	F (S5) GGTTTTAGATGTGGCTCATG	50	30
	R (S2) TCCAGAATAGCGAAATAAGG	50	30
tkt	F (S5) GCTTAGCAGATATTTTAAGTG	50	30
	R (S4) AAGCCTGCTTGTTCTTTGGC	50	30
uncA	F (S3) AAAGTACAGTGGCACAAGTGG	50	30
	R (S4) TGCCTCATCTAAATCACTAGC	50	30

All primers were purchased from Oswel Oligos, now Eurogentec Ltd, Romsey, UK or Sigma Genosys, Pampisford, UK.

Table 2.3. The primers used for MLST of Oxfordshire isolates; a combination of schemes devised by Dingle *et al* (2001a), Dingle *et al* (2005) and Miller *et al* (2005).

Locus	Sequence 5'-3' sequence		Reference
	MLST *C. jejuni*		
aspA	F (MF)	GAGAGAAAAGCWGAAGAATTTAAAGAT	Miller *et al* 2005
	R (A6)	ATTATAGACAAAGTCGAAC	Unpublished
	[a]F(A9)	AGTACTAATGATGCTTATCC	Dingle *et al* 2001
	[a]R(A8)	CTTCCATGTGAGGATTTAGC	Unpublished
glnA	F (S1)	GCTCAATTCATGGATGGC	Dingle *et al* 2001
	R (S6)	GCATACCATTGCCATTATCTCCG	Dingle *et al* 2001
gltA	F (S1)	GTGGCTATCCTATAGAGTGGC	Dingle *et al* 2001
	R (S6)	CCAAAGCGCACCAATACCTG	Dingle *et al* 2001
glyA	F (MF)	ATTCAGGTTCTCAAGCTAATCAAGG	Miller *et al* 2005
	R (MR)	GCTAAATCYGCATCTTTKCCRCTAAA	Miller *et al* 2005
pgm	F (MF)	CATTGCGTGTDGTTTTAGATGTVGC	Miller *et al* 2005
	R (MR)	AATTTTCHGTBCCAGAATAGCGAAA	Miller *et al* 2005
tkt	F (S5)	GCTTAGCAGATATTTTAAGTG	Dingle *et al* 2001
	R (S6)	AAGCCTGCTTGTTCTTTGGC	Unpublished
uncA	F (MF)	TGAAAGAATTRTTTTTAATCATAGG	Miller *et al* 2005
(adk)	R (MR)	CTTTCATRTCWGCHACGATAGGTTC	Miller *et al* 2005
	MLST *C. coli*		
aspA	F (MF)	GAGAGAAAAGCWGAAGAATTTAAAGAT	Miller *et al* 2005
	R (S2)	ATCTGCTAAAGTATGCATTGC	Dingle *et al* 2005
glnA	F (MF)	TGATAGGMACTTGGCAYCATATYAC	Miller *et al* 2005
	F (MR)	ARRCTCATATGMACATGCATACCA	Miller *et al* 2005
gltA	F (MF)	GARTGGCTTGCKGAAAAYAARCTTT	Miller *et al* 2005
	R (S2)	AAGCGCTCCAATACCTGCTG	Dingle *et al* 2005
glyA	F (MF)	ATTCAGGTTCTCAAGCTAATCAAGG	Miller *et al* 2005
	R (MR)	GCTAAATCYGCATCTTTKCCRCTAAA	Miller *et al* 2005
pgm	F (MF)	CATTGCGTGTDGTTTTAGATGTVGC	Miller *et al* 2005
	R (MR)	AATTTTCHGTBCCAGAATAGCGAAA	Miller *et al* 2005
tkt	F (MF)	GCAAAYTCAGGMCAYCCAGGTGC	Miller *et al* 2005
	R (S2)	TGACTTCCTTCAAGCTCTCC	Dingle *et al* 2005
uncA	F (S1)	AAGCACAGTGGCTCAAGTTG	Dingle *et al* 2005
(adk)	R (MR)	CTTTCATRTCWGCHACGATAGGTTC	Miller *et al* 2005

[a]Primers used for Starling isolates.

Primers were used for both PCR and sequencing reactions. Annealing temperatures and number of cycles were unchanged from the original MLST method. Primers were purchased from Sigma Genosys, Pampisford, UK.

2.6.2 Precipitation of PCR amplification products.

Micro titre plates (high throughput); PCR amplification products were precipitated by adding 60μl of 20% polyethylene glycol (PEG_{8000} Sigma, Poole)-2.5M NaCl to each well. The microtitre plate was briefly vortexed and sedimented at 500×*g* for one minute before incubating at room temperature for 30 minutes, or at 4°C overnight, to give sufficient time for the products to precipitate. The microtitre plate, supported in a plate carrier was sedimented at 2750×*g* for an hour at 4°C using an eppendorf 5810R plate centrifuge in order to collect the precipitated PCR products at the bottom of the wells as a solid pellet. The supernatant was discarded by gently inverting the microtitre plate onto thick tissue paper and then spinning inverted on to a piece of thick tissue paper, at 500×*g* for one minute. The sedimented DNA was washed twice by adding 150µl of cold 70% ethanol and centrifuging at 2750×*g* for a further 10 min, in order to remove excess PEG and NaCl which could otherwise inhibit nucleotide sequencing reactions, with the supernatants discarded as before. The dried PCR products were resuspended in 30μl milliQ water.

Individual microcentrifuge tubes; PCR products were transferred into labelled 1.5ml microcentrifuge tubes and 60μl of 20% PEG_{8000}- 2.5M NaCl solution added to each tube. The tubes were briefly vortexed and incubated at room temperature for 30 minutes. The precipitated DNA was pelleted by centrifugation at 12,000rpm for 10 minutes in a micro centrifuge. The supernatant was removed by pipetting and the tube gently inverted onto thick tissue paper. The pellets were washed by adding 700μl 70% ethanol (stored at -20°C) to each well and centrifuging at 12,000rpm for a further five minutes. The supernatant was removed as before, and tubes dried in a vacuum drier on

medium heat for 5 minutes. The dried PCR pellets were re-suspended in 30μl MilliQ water.

2.6.3 Nucleotide sequence extension reactions.

Forward and reverse primers which were nested and internal to the PCR primers were used to initiate nucleotide sequence extension reactions for each strand of the PCR amplification product. Both sets of reactions were set up for the 48 PCR reactions using 96 well micro titre plates. The precise concentration of reagents making up the sequencing reactions were optimised over a period of time reflecting the increased sensitivity of the new models of sequencing analysers and differing versions of Big Dye reagent. Reactions a quarter the size of manufacturers recommendations were used for most of the Lancashire farm isolates. The remaining isolates were sequenced using 1/32 size reactions allowing a significant saving in costs. The reagents for a 1/32 reaction for 1 isolate was a follows; 1.875μl MilliQ water, 1.875μl 5X buffer (Applied Biosytems, Warrington, UK), 4μl forward or reverse primer, 0.25μl Big Dye Ready Reaction Mix Version 3.1 (Applied Biosytems, Warrington, UK) and 2μl PCR amplification product. PTC-200, Dyad^{TM} and Dyad Disciple^{TM} (MJ Research) and GeneAmp^{T} PCR system 9700 (PE Applied Biosystems) thermal cyclers were used as before and the reaction conditions were a cycle of 96 °C for 10 seconds, 50°C for 5 seconds and 60°C for 2 minutes, repeated 29 times, followed by storage at 4°C.

2.6.4 Precipitation of nucleotide extension reactions.

Micro titre plate method (high throughput); 15μl milliQ water was added to each well in order to dilute excess dye, followed by 52μl of a solution made up of 7ml 100% ethanol and 280μl of 3M NaOAc (Sodium Acetate) at pH5.2 (Anachem Ltd, Luton, UK) in order to lower the water activation potential causing all lengths of DNA to precipitate. The plate supported in a plate carrier was gently vortexed and incubated at room temperature for 45 minutes in the dark to ensure that the dyes did not become degraded by light. It was then centrifuged at 2750×*g* for an hour at 4°C using an eppendorf plate centrifuge to pellet sequencing products at the bottom of the wells. The supernatant was discarded by gently inverting and spinning inverted on to a piece of folded thick tissue paper, at 500×*g* for one minute. The plate was washed once by adding 150μl of cold 70% ethanol and centrifuging at 2750×*g* for a further ten minutes, in order to remove excess NaOAc (Sodium acetate) which could interfere with the functioning of the DNA analyser, with the supernatant discarded as before.

Individual microcentrifuge tubes; MilliQ water (10μl) was added to each tube before transferring the contents to1.5ml eppendorf tubes. Then 2μl of 3M NaOAc (pH 5.2) and 50μl of absolute ethanol were added to each tube, and incubated at room temperature for 15 minutes. The tubes were then centrifuged in a microcentrifuge at 13,000 rpm for 15 minutes. The supernatant was removed by pipetting and gently inverting the tubes onto thick tissue paper. The pellets were washed by adding 500μl of 70% ethanol to each tube and centrifuging at 13,000 rpm for a further ten minutes. The supernatant was removed as before and the tubes dried in a vacuum drier on medium heat for five minutes.

2.6.5 Separation of extension reaction products using automated DNA analysers.

The precipitated nucleotide extension products were transferred to the Zoology Department Sequencing Facility. Briefly, the products were resuspended in 10µl Hi-Di formamide loading buffer and sedimented at 2000×*g* for one minute to collect samples to the bottom of the microtitre plate wells prior to loading on automated DNA analysers. Some of the Lancashire farm samples were detected on an ABI Prism 377 DNA sequencer which separated products by polyacrylomide gel electrophoresis. The instrument was superseded by the ABI Prism 3700 DNA analyser and then the ABI Prism 3730 automated DNA analyser, both of which separate products using a polymer within 96 capillaries, making them more accurate and automated. The chain termination method of nucleotide sequencing was employed, whereby dNTPS were mixed with four types of dideoxynucleotide triphosphate 'terminators' labelled with dyes that fluoresce at specific wavelengths (Sanger 1992). The terminators were incorporated randomly in sequencing extension reactions by DNA polymerase, but they lack a 3' hydroxyl group meaning that no further nucleotides could be added, resulting in DNA fragments of many different lengths. The charged DNA fragments were separated in the automated analyser by electrophoresis, the smaller particles moving fastest. A laser excited the fluorescent end labels, causing them to emit energy at a wavelength particular to the dNTP fluorescent terminator, which was detected in the instrument and converted into an electropherogram by sequence analysis software.

2.6.6 DNA sequence assembly.

Raw data files from the automated DNA analyser were renamed using the Microsoft Excel macro 'PLATE.XLS', written by Dr Man-Suen Chan, to give a 'list file' consisting of forward and reverse sequence chromatograms for each isolate. The sequence pairs were assembled using Sequence Typing Analysis and Retrieval System (STARS) (http://neelix.molbiol.ox.ac.uk:8080/userweb/mchan/stars/), and where necessary manually edited for base mismatches or trimmed to the correct length. The consensus sequence files (.cons files) were saved and queried against the *Campylobacter* database which is semi automated in STARS. Sequences that could not be aligned using STARS (for example small batches of isolates) were assembled using Pregap and Gap4 in the STADEN software package (116). A list file was generated using 'PLATE-XLS' and a Unix script 'SORT-MLST' was used to place forward and reverse sequence chromatograms into folders for each isolate. Pregap and Gap4 were further automated using another Unix script 'SEQASS', allowing sequence alignments to be checked and edited in Gap4. The consensus files were saved and, using the GCG10 sequence analysis package (131), converted to rich sequence file (.rsf) format, and aligned in SEQLAB.

2.7 Antigenic characterisation; *flaA* short variable region (SVR).

A 321-bp (283-603 inclusive) region of the SVR of the *flaA* gene encoding the flagellum was sequenced for each of the isolates. The FLA4F 5'-GGA TTT CGT ATT AAC ACA AAT GGT GC-3' and FLA625RU 5'-CAA G[AT]C CTG TTC C[AT]A CTG AAG-3' primers were used for PCR reactions and FLA242FU 5'-CTA TGG ATG AGC AAT T[AT]A AAA T-3' and FLA625RU for sequencing reactions (Meinersmann *et al.*

1997). The reagents, reaction conditions and DNA precipitation procedures were exactly as described for the MLST described in sections 2.6.1 to 2.6.5. Sequence pairs were assembled using STARS and then aligned using the STADEN software package (Staden 1996).

2.8 Data analysis.

2.8.1 Allele and ST assignment.

Allele numbers and sequence types (STs) were assigned by copying and pasting text into the *Campylobacter* MLST database (http://pubmlst.org/campylobacter/). The database contains allele assignments for 3500 isolates, with distinct alleles for each locus being given an a unique number in order of discovery. Thus alleles corresponding to those previously numbered were immediately identified. Potential new alleles were checked by the website curator before being assigned the next allele number in sequence. The combination of allele numbers at the seven loci (in alphabetical order) gave the ST, which could similarly be assigned using the website.

2.8.2 Clonal complex assignment

Many of the STs identified amongst the isolates were automatically assigned to clonal complexes that had been previously defined, using an automated script incorporated into the *Campylobacter* MLST database. At the end of the study all of the unassigned isolates held on the database were analysed using BURST (section 1.7) to identify further central genotypes that may have become apparent amongst the additional

data sets. The clustering of related genotypes was confirmed by constructing a UPGMA tree (section 2.8.4.3) and manually checking that four or more alleles were shared with the central genotype. New clonal complexes were proposed if a central genotype had been identified, there were six or more related STs and they were biologically relevant. They were all agreed by a committee before being included on the database.

2.8.3 Nucleotide allele and peptide assignment for *flaA* SVR.

The 321-bp SVR nucleotide sequences were assigned allele numbers, and were also translated to give amino acid sequences and thus a peptide identifying number. As with the MLST allele assignment, SVR nucleotide and peptide assignment were given unique numbers in order of discovery. Copying and pasting the 321-bp nucleotide sequence into the *Campylobacter FlaA* Variable Region Database http://hercules.medawar.ox.ac.uk/flaA/ enabled those matching previously identified alleles and peptides to be recognised. Those with new nucleotide sequences and/or new amino acid sequences were checked by the website curator prior to assigning a new number.

2.8.4 Genetic analyses.

2.8.4.1 Analysis of sequence diversity and recombination.

The number of polymorphic sites, and thus percentage polymorphic sites amongst the nucleotide data at each locus and for concatenated sequence (STs) was calculated

using the sequence data explorer function in MEGA (Kumar *et al*, 2004). The success of a MLST scheme depends on an appropriate number of alleles being detectable to index the genetic diversity with sufficient selectivity and sensitivity. An approximation of recombination could be made if alleles differing at multiple nucleotide sites are assumed to have arisen by recombination and alleles differing at a single nucleotide site have arisen by point mutation (Feil *et* al, 2000). A point mutation is likely to be novel within a data set but a single nucleotide change arising from recombination may be present on more than one occasion.

2.8.4.2 Analysis of selection.

Loci chosen for an MLST scheme have strong house keeping functions and to ensure consistency, it is preferable that there are similar levels of nucleotide substitution at each (Jolley and Urwin 2001; Maiden, 2006). The d_N/d_S ratio of nonsynonymous (a nucleotide substitution that changes the encoded amino acid) to synonymous (a nucleotide substitution that does not change the encoded amino acid) is an indicator of selection and was calculated using the DNASP software package versions 3.53 and 4.0. (Rozas and Rozas, 1999; Jolley and Urwin, 2001). The Nei and Gojobori 1986 method of calculation was employed (Rozas and Rozas, 1999; Suzuki and Gojobori, 2003). It is a 'mutation fraction method' that computes the number of synonymous and nonsynonymous **sites** per sequence, the number of synonymous and nonsynonymous **differences** per sequence and corrects for multiple substitutions. Because there may be more than one pathway by which substitutions take place, the number of differences at a particular codon is calculated as the unweighted average for all pathways. A ratio of 1 indicates that sequences are evolving neutrally, and that synonymous and non-

synonymous substitutions have no effect on fitness (Jolley and Urwin, 2001). A ratio <1 is obtained for most genes with non synonymous changes usually being deleterious. A ratio >1 indicates positive selection for nonsynonymous changes, which would occur during selection for particular amino acids. Examples whereby positive or negative selection may be exerted include effects of the immune system or use of antibiotics.

2.8.4.3 Reconstructing the phylogeny.

Neighbour joining (NJ) trees were employed to reconstruct the phylogeny (evolutionary history) amongst the *Campylobacter* genotypes. Overlying the tree with the host source of the genotypes gave an insight into the evolution of *Campylobacter*-host associations. NJ trees were constructed with nucleotide data using MEGA Version 3.1 (Kumar *et al*, 2004; Saitou &Nei 1987). It is a distance model that firstly calculates the fraction of nucleotides that differ between two sequences (*p* distance) and constructs a matrix for the data set. A tree is fitted to the matrix in order that the branch lengths are minimised and the lengths of branches connecting operational taxonomic units (OTUs) are equal to the genetic distance between them (Van de Peer, 2003). The tree is un-rooted since it does not assume that evolution of different lineages occurs at the same rate.

It is possible that the *p* distances may be underestimated through multiple substitutions at the same sites and so the Kimura 2-parameter evolutionary model was employed at the time of phylogenetic reconstruction to estimate the number of substitutions that had occurred. It accounts for transitional (purine substituted by a purine, or pyrimidine substituted by a pyrimidine) and transvertional (purine substituted by a pyrimidine or vice versa) substitutional rates but assumes the four nucleotide

frequencies are the same and that the rate of substitution does not vary among sites. Bootstrap tests were used to test the reliability of the NJ trees in terms of goodness of fit (Jolley and Urwin, 2001). A new alignment of sequence data is randomly constructed from the original data set and used to construct another phylogeny. The branching patterns are stored and the process repeated 500 times. The bootstrap confidence level of a branch represents the percentage of times it was found amongst the replicate samples. Branches with a confidence level of 70% or more are considered to be well supported, whilst those with confidence levels below 70% should be treated with caution (Van de Peer, 2003).

Tree models such as the NJ tree described assume that once two lineages are formed there is no further interaction between them (Moulton, 2003). However the evolutionary events may be obscured by inter and intra specific recombination events between lineages and thus the relationships may be better represented by a network (Huson and Bryant, 2006). Splits-graphs were constructed using SplitsTree4 using nucleotide data in NEXUS format (Huson, 2006). A distance matrix is calculated using the split-decomposition theory to determine the genetic distance between pairs of sequences. The theory is based on the principles that a) a 'split' is a partition of the taxa into two subsets and b) the length of an edge in the network is proportional to the weight of the associated split (Huson, 2006). In effect the distances are calculated from the number of shared alleles (Jolley and Urwin, 2001).

If large data sets are analysed, or taxa are distantly related it may be necessary to deconstruct the data carefully in order that small networks at the centre may be deciphered (Jolley and Urwin, 2001). The process increases the scale and also the 'fit'

value which is an indication of how well the splits-graph represents the distance matrix from which it was built. Bootstrap values may be calculated to give the percentage of computed graphs in which a split corresponding to the labelled edge occurred (Moulton, 2003). Splits-graphs demonstrating networks indicate that recombination has taken place. The central genotypes within a lineage would often have other variants branching off them (Jolley and Urwin, 2001).

2.8.4.4 Population comparison methods.

The F_{ST} statistic, originating from Wright's F statistics for inbreeding coefficients, is a summary statistic which takes into account population size and frequency of alleles, to give a measure of gene flow, or genotypic similarity of two populations (Wright, 1951,Wilkinson-Herbots and Ettridge, 2004). The pairwise F_{ST} and test of significance calculations were performed using the ARLEQUIN software package, versions 2.000 and 3.0 (Schneider *et al.* 2000). Data input files were prepared using DNASP software package 4.0, with haplotype frequencies manually adjusted for each data set (Rosaz *et al*, 2003). For some of the analyses the gene sequences for each of the MLST loci were concatenated to give a single continuous nucleotide sequence of 3,309 nucleotide base pairs for each isolate. A definition of F_{ST} is given below;

$$F_{ST} := \frac{f_0 - f^1}{1 - f^1}$$

where f_0 is the probability that two genes sampled at random from a **single** subpopulation are identical (ie the same allele) and f_1 is the probability that two genes randomly chosen from the **collection** of subpopulations considered are identical (Winkinson-Herbots and Ettridge, 2004). The programme computes a distance matrix of F_{ST} values for all

sequence pairs and then calculates Nei's average number of pairwise differences within and between populations. The *p* value arising from the test of significance is the proportion of permuations leading to an F_{ST} value larger or equal to the observed value. The *p* value is significant if it is less than 0.05. An F_{ST} value of 0 suggests that the genotypes in two populations are identical, and a value of –1 or 1 suggests that they are completely different.

Shared polymorphisms and fixed differences were calculated to give an alternative descriptive measure of sequence divergence between subpopulations. The values were calculated using DNASP software package 4.0 using nucleotide data (Rosaz *et al*, 2003). Shared polymorphisms are those sites at which the sequence found in both subpopulations are identical. Fixed differences are those sites at which the sequence in one subpopulation is different to all of those found in a second sample and indicate divergence between the two subpopulations (Hey, 1991). The long term evolution of a species results from the successful fixation of particular alleles, which reflects fixation of mutations (Vandamme, 2003). It is likely that some population genetic forces may affect more than one locus within a genome simultaneously whilst others are more localised resulting in different parts of the genome having different phylogenies (Hudson, 1993).

2.8.5 Statistical analysis.

2.8.5.1 Logistic regression analysis.

Regression analysis estimates the components of a model that reflects the relationship between dependent and independent variables in a population (Hirsch and Riegelman, 1992). The relationship is assumed to be a straight line and the method of

least squares is used to estimate the slope and intercept of the line from the sample observations. Logistic regression was first introduced in the 1960's for analysis of individual and joint effects of a set of variables on the risk of disease (Schlesselman, 1982). It is used when the dependent variable is dichotomous (e.g. *Campylobacter* positive or negative) but the independent variables are continuous, discrete, or both, and are not affected by time (Hirsch and Riegelman, 1992; Elston and Johnson, 1994). The cumulative distributions of the dependent variable as a function of an independent variable tend to be S-shaped but the logarithmic equations used in the analysis convert the S-shape into a straight line (Elston and Johnson, 1994). A 'logit transformation' is performed on the dependent variable using the formula where p is the probability of an event;

$$\text{Logit} = \ln \frac{p}{(1-p)}$$

The logit is then regressed on the independent variable(s).

The logistical regression analyses and associated tests of significance performed in Chapters 5 and 7 were calculated using Stata (StataCorp LP, Texas, U.S.). The output gives the odds ratios, standard errors, the regression coefficient (or slope) (Z), confidence intervals and a chi squared test of significance. Comparison of *Campylobacter* shedding rates between years was considered to give a cyclic trend and thus sine and cosine lines were fitted as part of the analysis (Altman 1991). A χ^2 test was performed to test whether or not the sine or cosine models were a good fit.

2.8.5.2 Simpson's diversity index.

A modified version of Simpson's diversity index, adapted for situations where strains can not be placed into mutually exclusive groups, was used to determine the diversity of genotypes at a given point in time whilst taking into account different sample sizes (Hunter 1990). It was calculated manually using functions on a Microsoft Excel spreadsheet, using the formula below;

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} aj$$

Where D= index of discriminatory power, N is the size of the population and a_j is the number of strains in the population which are indistinguishable (*i.e.* identical) from the jth strain. A D value of 1.0 indicates that each member of a population can be distinguished from every other, and a D value of 0.0 indicates that all members of a population are identical. A D value of 0.5 indicates that if one member is chosen at random from the population, there would be a 50% probability that the next strain chosen at random would be indistinguishable from the first.

2.8.5.3 Tests of significance.

The Student's t test was used to compare the mean outcomes in two exposure groups, for example to determine whether the year affected the frequency of *Campylobacter* genotypes that were isolated from free-range chickens (Kirkwood and Sterne, 2003). The formula tests the difference between the means of the exposure groups and takes into account the variance or standard deviation amongst the

observations. The formula for an unpaired *t* test is given below, where s.e. is the standard error and the degrees of freedom were $n^1 + n^0 - 2$.;

$$t = \frac{\bar{x}^1 - \bar{x}^0}{\text{s.e.}}$$

The tests were performed using STATA (StataCorp LP, Texas, U.S.) using the command line "ttest variable1==variable0, unpaired" (the default option is a paired *t* test). The result was considered significant if the *p* value was <0.01 and the null hypothesis that there was difference in the exposure groups was rejected.

The paired *t* test was used to compare paired data, for example to test the shedding rate of *Campylobacter* during 10 months at Wytham and Northmoor. The formula combines the standard error for the two observations and was performed on Stata using the command line "ttest variable1==variable0".

The standard normal deviate or *z* test was performed to test the mean outcome between exposed and unexposed groups, for example the effect of nest size on Starlings that were shedding *Campylobacter* compared to those that were not (Kirkwood and Sterne, 2003). The formula was the same as the *t* test given above. The *p* values were determined using statistical tables. Confidence intervals giving the limits that with 95% probability that the mean was between, and odds ratios giving the odds of the outcome event in the exposed group compared to the odds in the unexposed group were also calculated (Kirkwood and Sterne, 2003).

2.8.5.4 Chi squared test.

The Chi squared test is used to determine whether the distribution of individuals among the categories of one variable is independent of their distribution among the categories of another (Kirkwood and Sterne, 2003). Contingency tables (2 rows x 2 columns) are shown for Starling data in Chapter 5, with the variables of male or female sex and *Campylobacter* shedding or non-shedding. The expected values were calculated using the formula;

$$E = \frac{(a + b)(a + c)}{n}$$

where for e.g. E = the expected number of male Starlings to be shedding *Campylobacter*, a = the number of male Starlings that were shedding *Campylobacter*, b = the number of male Starlings there were not shedding *Campylobacter* and c = the number of female Starlings that were shedding *Campylobacter*, n = the total number of observations (Hirsch and Riegelman). The value of Chi squared was calculated using the formula;

$$\chi^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

where O_i = observed frequency in a particular cell of the 2 x 2 table and E_i = expected frequency in a particular cell of the 2 x 2 table. The χ^2 value was compared that found in the χ^2 distribution tables at $p = 0.05$ with one degree of freedom. The null hypothesis that the sex did not significantly affect *Campylobacter* shedding would be rejected if the calculated value of χ^2 was larger than the value in the distribution tables.

Similarly the distribution of clonal complexes amongst animal sources was tested in Chapters 3, 4 and 8 using chi squared, with calculations performed using STATA (StataCorp LP, Texas, U.S.). The contingency tables of observed frequencies were

constructed manually, and Pearson's χ^2 calculated using the command line 'tabi n(column 1 row 1) n (column 1 row 2) \ n(column 2 row 1) n(column 2 row 2),all'. The distribution was significant (variables not independent) if the *p* value was greater than 0.05. The precise *p* value could be obtained using the command line 'dis r(p)'.

Abstract

Chapter 3: Genetic diversity of *C. jejuni* isolates from farm animals and their environment, in North-West England.

The aims of this chapter were to establish whether *Campylobacter* genotypes that have been isolated from human disease could be isolated from livestock or their local environment, and to test whether the MLST method that had been developed using human disease and retail meat isolates would be applicable. Isolates representing the widest possible selection of farm animal sources were chosen from a series of studies performed in Lancaster, UK.

There was evidence that 'human-disease like' *Campylobacter* genotypes were common amongst livestock. Wild Starlings were identified as a potential transmission vector between host sources since they carried the widely distributed ST-21 and ST-45 clonal complexes. In common with other studies, poultry were highlighted as a priority area for research having the highest number of isolates in common with those isolated from human disease. The MLST method proved to be successful in typing isolates from livestock with no modifications required.

Chapter 3: Genetic diversity of *C. jejuni* isolates from farm animals and their environment, in North-West England.

3.1 Introduction.

Campylobacteriosis has a significant health impact in the UK with approximately 50,000 cases of human disease per year. Cases are typically sporadic, and the potential sources of the organism vast, which together with the use of poorly reproducible typing systems has made epidemiological investigation and strategic intervention very difficult.

The recent introduction of multilocus sequence typing (MLST) has allowed detailed and reliable analysis of collections of *Campylobacter* isolates which to date have been dominated by isolates from human disease and retail meat. The aims of this chapter were to test the method and primers on a range of isolates from live animals and to design modifications if required. If successful, a survey of the genetic types colonizing common food animals and their environment would be undertaken and direct comparisons could be made with those isolated from human disease. In addition potential host/isolate associations amongst retail meat sources and links to genotypes isolated from human disease identified by Dingle *et al*, 2002 could be tested amongst isolates from live animals (Dingle *et al.* 2002).

3.2 Results.

It was possible to determine the nucleotide sequences at each of the MLST loci from all of the farm and environmental isolates with previously published methods and reagents. The level of discrimination was sufficient to compare isolates amongst these sources with those isolated from human disease, data from which are held on the *Campylobacter* MLST database.

3.2.1. Clonal complex distribution.

Virtually all of the isolates (110 of 112 isolates, 98%) had genotypes that could be assigned to ten clonal complexes previously identified amongst isolates from human disease and retail meat (Table 3.1) (Dingle *et al.* 2001). A further isolate was assigned to ST-692 complex which was only identified once wild geese had been sampled. One isolate remained unassigned. The number of clonal complexes identified in each isolation source varied from two for turkey chicks, broiler chicks and slurry to seven for adult beef cattle at slaughter. The ST-21 complex was the most widely distributed clonal complex, present in eight of the ten different isolation sources. The ST-52, ST-177, ST-206 and ST-692 complexes were the least widely distributed and were each identified in one isolation source only. The ST-45 complex was predominant among turkey and broiler chick sources and absent from ovine sources. Conversely, ST-42 and ST-61 complexes were predominant among ovine and bovine sources but were absent from avian sources. Two clonal complexes were identified in both turkey and broiler chicks, although only the ST-45 complex was common to both sources in this study. Adult cattle and calves shared the ST-21, ST-45 and ST-48 complexes, but each had further complexes. Sheep at slaughter, sheep grazing on salt marsh and sheep grazing on fell (hill) land shared three clonal

complexes, ST-21 complex, ST-45 complex, and ST-61 complex. Adult sheep and lambs shared the ST-61 and ST-48 complexes, but only the ST-61 complex, was found in all ovine groups. Isolates belonging to the ST-21 complex, ST-45 complex, and ST-257 complex were present in Starlings, which were the only source from which the ST-177 complex was recovered. The most common clonal complexes among the farm isolates were: ST-45 complex (30 isolates); ST-21 complex (26 isolates); ST-61 complex (14 isolates); and ST-42 complex (11 isolates). The remaining clonal complexes were represented by nine or fewer isolates with the ST-692 complex, represented by one isolate, being the smallest.

Table 3.1. The distribution and frequency of *C. jejuni* clonal complexes among farm and environmental sources.

	Clonal complex											
Source (n)	**45**	**21**	**61**	**42**	**48**	**206**	**257**	**22**	**177**	**52**	**692**	**U**
Turkey chicks (12)	11	-	-	-	-	-	1	-	-	-	-	-
Broiler chicks (16)	15	1	-	-	-	-	-	-	-	-	-	-
Calves (9)	2	3	-	-	2	-	1	-	-	-	-	1
Adult beef cattle at slaughter (14)	1	2	1	1	4	4	-	-	-	-	1	-
Slurry (10)	-	7	-	-	-	-	-	3	-	-	-	-
Lambs at slaughter (9)	-	-	6	-	2	-	-	1	-	-	-	-
Sheep at slaughter (14)	-	5	1	3	1	2	-	-	-	2	-	-
Sheep grazing in salt marsh (10)	-	5	2	3	-	-	-	-	-	-	-	-
Sheep grazing in fell (9)	-	1	4	4	-	-	-	-	-	-	-	-
Starlings (9)	1	2	-	-	-	-	3	-	3	-	-	-
Total (112)	30	26	14	11	9	6	5	4	3	2	1	1

U, STs that could not be assigned to a clonal complex.
-, no genotypes of this type were isolated.

3.2.2 ST distribution.

A total of 28 different STs were identified amongst the 112 isolates, of which 27 (99%) were grouped into 11 clonal complexes (Table 3.2). Nine STs were isolated from more than one animal source. ST-53 was the most widespread being isolated from all animal sources. ST-45 was isolated from cattle and poultry and ST-257 was isolated from poultry and starlings. Six STs were shared between cattle and sheep sources. Nineteen STs were isolated from a single source only. A total of eight STs were isolated from sheep sources and seven from cattle sources, but only one each were isolated from poultry and starling sources.

The clonal complexes were represented by between one isolate (ST-692 complex) and 30 isolates (ST-45 complex). The number of STs within each clonal complex ranged from one (ST-52, ST-177, ST-206 and ST-692 complexes) to eight (ST-21 complex). With the exception of the ST-21, ST-177 and ST-692 complexes, the previously assigned central genotype was the most predominant ST present in each of the clonal complexes identified in this sample. The most common ST was ST-45, which was represented by 26 isolates, with several STs, occurring only once in the data set. The four most predominant STs (ST-45, ST-42, ST-61, and ST-262) represented approximately half of the isolates (61 of 112 isolates, 54%). A total of 12 STs were novel to this study.

Table 3.2. Distribution of STs among 112 *C. jejuni* isolates from farm animals and their environment and their resolution into clonal complexes.

Clonal complex	Number of isolates	ST	Complex variant[a]	Frequency	Isolate source
21	26	21	C	1	sheep
		19	SLV	1	sheep
		53	SLV	7	cattle,poultry,sheep,starling
		262	SLV	13	cattle,sheep,slurry
		376	SLV	1	sheep
		518	SLV	1	sheep
		519	DLV	1	sheep
		520	DLV	1	sheep
22	4	22	C	3	sheep,slurry
		512	SLV	1	slurry
42	11	42	C	10	cattle,sheep
		517	SLV	1	sheep
45	30	45	C	26	cattle,poultry
		137	SLV	1	starling
		514	SLV	1	cattle
		515	DLV	1	cattle
		100	TLV	1	poultry
48	9	48	C	4	cattle,sheep
		205	SLV	1	cattle
		516	SLV	1	cattle
		38	DLV	3	cattle,sheep
52	2	52	C	2	sheep
61	14	61	C	12	cattle,sheep
		81	SLV	2	sheep
177	3	521	SLV	3	starling
206	6	206	C	6	cattle,sheep
257	5	257	C	4	poultry,starling
		513	DLV	1	cattle
692	1	690	TLV	1	cattle
Unassigned	1	688	N/A	1	cattle

C: central genotype, SLV: single locus variant, DLV: double locus variant, N/A: not applicable.

[a]The relationship to the central genotype.

3.2.3 Allelic diversity.

The number of unique sequences at each locus varied from five for the *aspA* locus to 12 for the *gltA* locus. The majority of alleles at each locus were shared between more than one source of isolation. The percentage of variable sites in this

data set ranged from 2.7% for the *aspA* locus to 14.5% at the *uncA* locus. The apparent high diversity of the *uncA* locus was caused by a single allele, allele 17, which occurred 18 times in the data set. Exclusion of this allele gave diversity for this locus of 0.8%. The ratio of non-synonymous to synonymous substitutions (d_N/d_S) observed ranged from 0.000 to 0.143 (Table 3.3). All except two of the MLST allele sequences found in this isolate collection had been described previously.

Table 3.3. Allelic diversity amongst the 112 isolates from farm animals and their environment.

Locus	Fragment size (bp)	No. of alleles	No. of variable sites	% variable sites	d_N/d_S ratio
aspA	477	5	13	2.7	0.045
glnA	477	8	17	3.6	0.093
gltA	402	12	16	4	0.048
glyA	507	11	28	5.5	0.045
pgm	498	11	34	6.8	0.026
tkt	459	9	26	5.7	0.008
uncA	489	6	71 (4)[a]	14.5 (0.8)[a]	0.143 (0.000)[a]
All loci	3309	30	207 (140)[a]	6.3 (4.2)[a]	0.030 (0.037)[a]

[a]Figures in parentheses exclude *uncA* allele 17 which may have come from a different *Campylobacter* species (11).

3.2.4 Antigenic diversity.

There were 11 *flaA* SVR peptide alleles amongst 104 typed *C. jejuni* isolates from the farm animals. Of these, peptide one was the most common, and isolated 59 times, followed by peptide 14 isolated 12 times, and peptide 12 isolated 11 times. The remainder were isolated six times or less (Table 3.4). A total of 19 *flaA* SVR nucleotide and peptide allele combinations were identified. Of these 22-1 was the

most common and was isolated 26 times, followed by 37-1 isolated 13 times, 42-14 and 239-9 isolated ten times, and 32-1 isolated nine times. The remainder were identified six times or fewer, with ten combinations being seen only once in the data set. Seven clonal complexes, namely ST-22, ST-42, ST-48, ST-52, ST-177, ST-257 and ST-692 clonal complexes containing between one and five isolates were each associated with only one antigen type. The remainder, ST-21, ST-45, ST-61 and ST-206 complexes each had more than one *flaA* SVR nucleotide-peptide allele, and contained between six and 30 isolates. ST-21 was most varied with eight different combinations. Within clonal complexes, the antigen, or even peptide type associated with the central genotype was not necessarily the same as that associated with the variant STs. A total of seven ST-*flaA* SVR strains were isolated from more than one host source (Table 3.5). Two were isolated from avian sources, four were isolated from ruminant sources and one, ST-53;32-1 was isolated from both avian and ruminant sources.

Table 3.4. The distribution of *flaA* SVR alleles amongst *C. jejuni* genotypes isolated from farm animals and their environment.

Clonal complex	ST	Complex variant[a]	Frequency	*flaA* SVR allele-peptide frequency
21	21	C	1	198-4(1)
	19	SLV	1	36-1(1)
	53	SLV	7	32-1(4), 8-1(1), 22-1(1), 23-1(1)
	262	SLV	13	37-1(12), NT(1)
	376	SLV	1	NT(1)
	518	SLV	1	8-1(1)
	519	DLV	1	37-1(1)
	520	DLV	1	34-1(1)
22	22	C	3	108-64(2)
	512	SLV	1	108-64(1)
42	42	C	10	239-9(9)
	517	SLV	1	NT(1)
45	45	C	26	239-9(1)
	137	SLV	1	16-12(1)
	514	SLV	1	70-5(1)
	515	DLV	1	70-5(1)
	100	TLV	1	165-85(1)
48	48	C	4	32-1(4)
	205	SLV	1	NT(1)
	516	SLV	1	32-1(1)
	38	DLV	3	41-4(2),227-4(1)
52	52	C	2	57-4(2)
61	61	C	12	42-14(10), 43-14(1), NT(1)
	81	SLV	2	108-64(2)
177	521	SLV	3	86-18(3)
206	206	C	6	14-11(1), 96-34(1), NT(4)
257	257	C	4	16-12(4)
	513	DLV	1	16-12(1)
692	690	TLV	1	66-1(1)
Unassigned	688	singleton	1	NT(1)

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant; NT, not tested.

[a]The relationship to the central genotype.

Table 3.5. ST and *flaA* SVR genotype combinations shared by more than one host source.

Clonal complex	ST	Number of isolates	*flaA* SVR allele-peptide	Source (frequency)
21	ST-53	4	32-1	starlings(2), calf(1), sheepSaltMarsh(1)
	ST-262	11	37-1	cattle(1), sheepFarm(3), slurry(7)
42	ST-42	9	139-9	cattle(1), sheepFarm(3), sheepSaltMarsh(2), sheepFell(3)
45	ST-45	25	22-1	chick(14), turkey chicks(11)
48	ST-48	4	32-1	calf(1), cattle(1), lamb(2)
61	ST-61	12	42-14	cattle(1), sheepFell(3), lamb(6)
257	ST-257	4	16-12	starlings(3), turkey chick(1)

3.2.5 Comparison of *C. jejuni* genotypes isolated from farm animals and human disease.

The 112 isolates from farm animals and their environment were compared with a temporally and geographically matched dataset of isolates from human disease, extracted from the *Campylobacter* MLST database (Figure 3.1). A total of 17 clonal complexes were identified amongst the isolates, 12 from human disease and 11 from the farm animal and environment isolates, with seven of the clonal complexes overlapping between the sources. Poultry, Starlings and cattle were associated with the three largest clonal complexes from human disease with ST-45 complex being particularly common amongst poultry. Additional links between isolates from human disease included cattle and sheep and ST-48 and ST-61 complexes. ST-177, ST-22, ST-48 and ST-206 complexes were identified amongst the animal and environmental

isolates but not from human isolates and ST-353, ST-443 and ST-354 complexes were identified from human disease but not amongst the animal and environmental isolates.

Chi squared analysis gave evidence that distribution of clonal complexes amongst host sources was non-random, (χ^2 71.67, p 0.000) and in particular for seven of the clonal complexes (Table 3.6). Results suggested that ST-45 and ST-257 complexes were significantly associated with poultry, ST-21, ST-42, ST-48 and ST-61 complexes were significantly associated with ruminants and ST-177 complex was significantly associated with Starlings.

Table 3.6. Chi squared analysis to establish whether or not distribution of clonal complexes amongst host sources was random.

Clonal compex	$\chi 2$	p value	Significant
21	7.93	0.005	Yes
22	1.54	0.214	No
42	6.11	0.013	Yes
45	59.41	0.000	Yes
48	4.90	0.027	Yes
52	1.02	0.313	No
61	7.89	0.005	Yes
177	6.17	0.013	Yes
206	3.17	0.075	No
257	4.63	0.031	Yes
692	0.50	0.478	No

A total of 70 STs were identified amongst the combination of isolates. Of these 40 (56.3%) were unique to isolates from human disease and 22 (31.0%) of isolates were unique to the farm and environmental isolates. Eight of the 70 STs (11.4%) were isolated from both human and farm and environmental sources.

ST-53 was the most widely distributed, being isolated from all animal sources. ST-45 was isolated from both poultry and ruminant sources. Two of the overlapping STs ST-137 and ST-257 were isolated from avian sources and three, ST-21, ST-48 and ST-52 were isolated from ruminant sources. The unassigned ST, ST-688 has been isolated from geese.

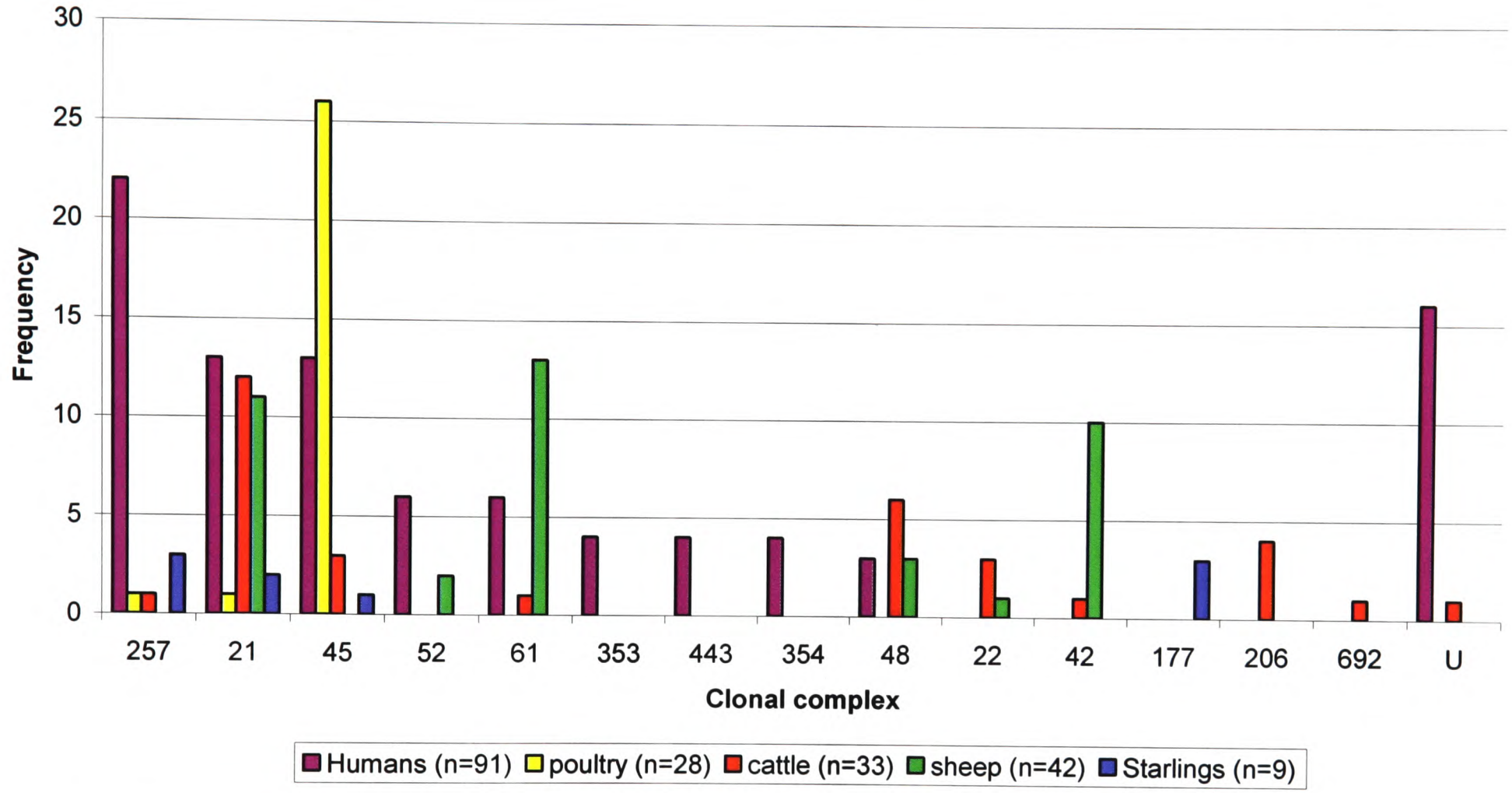


Figure 3.1. The distribution of *C. jejuni* isolates amongst farm animals and human disease[a].

[a], a collection of temporally and geographically matched isolates from human disease, data taken from the *Campylobacter* MLST database.

U, genotypes that could not be assigned to a clonal complex.

3.3 Discussion.

The results demonstrated for the first time that the MLST primers and method used by Dingle *et al* (Dingle *et al.* 2001) could be used without modification on *C. jejuni* isolates from live animal sources and their environment. The nucleotide diversity present in the farm derived isolates was similar to that described in human disease in terms of the number of alleles, nucleotide variability and d_N/d_S ratio at each locus, giving a good level of discrimination between isolates (Dingle *et al.* 2002). The largest degree of variability was seen amongst the farm data at the *uncA* locus with the inclusion of allele 17, which may have originated in a different *Campylobacter* species, such as *C. coli* (Dingle *et al.* 2001).

Despite the small number of samples from each host source, the *Campylobacter* populations were diverse in terms of STs, but 99% of the isolates had STs that grouped into clonal complexes revealing that the diversity was also structured. The distribution of *C. jejuni* clonal complexes appeared to be uneven and could be broadly divided into those that colonized ruminant species and those that colonized avian species (Table 3.1). Isolates belonging to the ST-45 clonal complex were dominant amongst turkey and broiler chick samples and absent from sheep and lamb samples. In contrast, ST-61 and ST-42 complexes were dominant amongst sheep isolates but were absent from poultry isolates: similar trends have been reported for retail food sources (Dingle *et al.* 2002).

The results from this investigation suggest that a range of *C. jejuni* genotypes may be prevalent within wild birds. Starlings were found to carry *C. jejuni* belonging to complexes associated with poultry and environment sources; however none of the complexes potentially associated with cattle or sheep were present. This could be a reflection of host adaptation by *C. jejuni*, behavioural patterns of the birds, or small

sample size and further work is required to determine which of these hypotheses are true. Wild birds have been implicated in spreading infection in the farm environment on numerous occasions, although the extent of their contribution is as yet unknown (Craven *et al.* 2000; Jones 2001; Jones 2001; Petersen *et al.* 2001; Broman *et al.* 2002; Waldenstrom *et al.* 2002).

The ST-21 complex appeared to have a wide distribution; this clonal complex probably corresponds to the large stable cluster of isolates capable of colonizing a wide range of hosts identified with other typing techniques (Fitzgerald *et al.* 2001; Schouls *et al.* 2003). Several other observations concerning the distribution of clonal complexes among isolation source, while based on smaller numbers of isolates, warrant further investigation. A larger number of clonal complexes were represented in animals at slaughter than in the other animal groups, perhaps reflecting different sampling sites or contamination within the slaughterhouse. Differences in clonal complex distribution between both adult cattle and calves, and adult sheep and lambs could be due to the different conditions and farming practices in which the animal age groups are kept, or host immunological maturity (Stanley *et al.* 1998; Stanley *et al.* 1998; Jones *et al.* 1999; Jones 2001). The lowest number of clonal complexes was seen amongst chicks and slurry perhaps resulting from bottle necks in *Campylobacter* distribution. Eight of 30 STs accounted for 73% (82 of 112) of the isolates, implying that these genotypes may be particularly stable. This was supported by the observation that there were only two genotypes isolated from slurry to which a potentially large number of different genotypes were added on a regular basis (Stanley *et al.* 1998).

The data were consistent with the idea that particular genotypes, indicated by clonal complex, are associated with given host sources, as suggested previously using

MLST, serotyping and PFGE (Fitzgerald *et al.* 2001; Dingle *et al.* 2002) (Schouls *et al.* 2003) (Wareing *et al.* 2002). In addition, some *Campylobacter* 'strains' with identical ST and antigenic type appeared to be particularly well adapted to colonize a a range of host sources, for example ST-53 *flaA* SVR 32-1 was isolated from Starlings, cattle and sheep. The other combinations isolated from more than one source were more limited being isolated from either avian or ruminant hosts.

Comparison of the farm animal isolates with a collection of human disease isolates from a similar time period and geographical area demonstrated that all but two of the alleles, 16 of the 30 STs, and all of the clonal complexes identified in the farm and environment isolates, had been previously described amongst the human disease and retail food isolates (Dingle *et al.* 2001; Dingle *et al.* 2002). Whilst many polymorphisms were shared between the farm animal and human disease isolates, there were no fixed differences between them. These observations are consistent with the farm populations of *C. jejuni* being a source for food contamination and human infection. The farm animal genotypes most frequently isolated from the representative human disease population collection were ST-257, ST-45 complexes, predominately isolated from avian sources and ST-21 complex which was common amongst all sources.

Campylobacter genotypes isolated from poultry and wild birds clustered with the three clonal complexes most commonly isolated amongst temporally and geographically matched isolates from human disease suggesting that, in common with other studies, future epidemiological studies should be prioritized into the investigation of avian sources of human disease (Hein *et al.* 2003; Workman *et al.* 2005). Wild birds should be investigated as likely candidates to transfer *Campylobacter* around the farm and amongst different animal hosts and as well as

being a potential source of infection for humans. Future work should include detailed investigation of the population structure and dynamics of *Campylobacter* amongst poultry flocks and wild birds in order that they may be more directly compared with the extensive collections of genotypes from human. It is necessary to extend the sample size, the number of flocks/species and sampling time in order to fully capture the *Campylobacter* diversity amongst host types. In addition large sample numbers from related farm sites are required to monitor the movement of ST-*flaA* SVR type 'strains' amongst host sources, which may occur at low frequency. Populations from live and slaughtered broilers could be compared to determine whether genotypes entering the food chain are present on the farm, and thus at which point in production intervention strategies are best employed.

Abstract

Chapter 4: Genetic diversity of *Campylobacter* isolates from wild geese and lambs grazing the same pasture at the Oxford University farm.

The aim of this chapter was to establish the population biology of *Campylobacter* amongst wild geese congregating on the University farm at Wytham. It is important to study population biology from the point of the pathogen since human disease collections may consist of evolutionary unusual isolates. Isolates were compared with those obtained from free-range broiler chickens reared in the same location (discussed in Chapters 6 and 7) in order to establish whether transmission of strains occurs between the host sources and ultimately reach the human food chain. Lambs grazing the pasture heavily contaminated with geese faeces were also sampled to test for transmission of Campylobacters from the geese.

The *Campylobacter* genotypes isolated from geese were very different to those previously sampled from livestock. Instead they clustered with genotypes isolated from Mallard ducks in Sweden suggesting the presence of 'aquatic bird' adapted clones of *Campylobacter*. The absence of *C. coli* amongst geese isolates was surprising since a water adapted clone of *C. coli* has recently been identified. Genotypes associated with human disease were present only at very low frequency. Although only four isolates were obtained from the lambs, the fact that none were genotypes typically associated with lambs, two were identical to strains isolated from geese, a third closely related to geese strains and a fourth closely related to other wild bird strains suggests that the *Campylobacters* may have transferred from the geese to the lambs albeit at low levels. The clonal complexes most commonly isolated from geese were ST-692, ST-702, ST-1034 and ST-1332 complexes.

Chapter 4: Genetic diversity of *Campylobacter* isolates from wild geese and lambs grazing the same pasture at the Oxford University Farm.

4.1 Introduction.

Besides domestic animal sources, the environment is considered to be an important source of *Campylobacter* in human disease. The organism has been isolated from soil and water, as well as many species of wildlife, including insects (Frost 2001; Petersen *et al.* 2001a; Ekdahl *et al.* 2005; Kemp *et al.* 2005). Little is known about the genetic diversity of *Campylobacter* amongst these sources, for instance, it is unknown whether genetic types circulate between humans, animals and the environmental, and the extent to which environmental sources contribute to human disease. It is debatable whether *Campylobacter* species can actively multiply amongst some environmental sources, however C. *jejuni* may have adapted as a commensal of birds since the organism can be isolated in high numbers from apparently healthy individuals (Waldenstrom *et al.* 2002). In addition *C. jejuni* is thermophillic with an optimal growing temperature of 42°C in the laboratory, which may reflect the higher body temperature of birds (Waldenstrom *et al.* 2002). Previous studies have linked bird pecked milk with sporadic cases of human disease and some authors consider migratory birds to be a contributing factor to the seasonal peak in human *Campylobacteriosis* during the late spring and early summer months in temperate countries (Riordan *et al.* 1993; Stanley and Jones 2003).

The aim of this chapter was to determine whether a second wild bird species other than Starlings may be a potential source of *Campylobacter* for other animals on a farm. A survey of the *Campylobacter* species (*C. jejuni* or *C. coli*), genotypes and population dynamics was carried out to allow comparison with genotypes previously isolated from farm animals and human disease and determine if there was any evidence of transmission between them. In addition, the work formed the foundation for future studies exploring *Campylobacter* populations colonising wild Starlings and free-range chickens on the same farm site. The geese were chosen due to their large numbers, close vicinity to and interaction with farm animals and their accessibility for sampling. Lambs that were grazing pasture heavily contaminated with geese faeces were tested to see if there was any evidence of direct and natural transfer of genotypes between the species.

4.2. Results.

A total of 166 *Campylobacter* isolates were cultured from 331 faecal samples using enrichment in Prestons broth with subculture onto mCCDA. It was possible to determine the nucleotide sequences at each of the MLST loci from all of the geese isolates with previously published methods and reagents. The level of discrimination was sufficient to compare isolates with those isolated from farm animals and human disease, data from which are held on the *Campylobacter* MLST database.

4.2.1 Shedding rate of *Campylobacter* species.

Of the 331 faecal samples collected from geese, 167 (50.5%) were positive for *Campylobacter* species, of which 166 (99.4%) were *C. jejuni* and 1 (0.6%) was *C. coli*.

The *C. coli* isolate was not included in further analysis. The isolation rates on each occasion were; 19 of 40 samples positive (47.5%) on 18.08.2002, 37 of 84 samples positive (43.4%) on 11.09.2002, 51 of 82 samples positive (62.2%) on 20.09.2002, 32 of 43 samples positive on 3.02.03 (74.4%) and 27 of 82 samples positive on 26.02.03 (32.9%). Of the 84 faecal samples collected from lambs on 11.10.2002, four (4.8%) were positive for *C. jejuni*. No other *Campylobacter* species were isolated.

4.2.2 Distribution of clonal complexes.

The 166 *C. jejuni* isolates clustered into six clonal complexes, with 19 STs remaining unassigned (Figure 4.1). The most commonly isolated clonal complexes were ST-1034 complex consisting of nine STs and 26 (15.7%) isolates, ST-702 complex consisting of three STs and 20 (12.0%) isolates, and ST-692 complex consisting of three STs and 15 (9.05%) isolates. The remaining three complexes each contained two or fewer STs and fewer than ten isolates. Each of the four lamb isolates was a different ST and only two, ST-137 (ST-45 complex) and ST-696 (ST-1332 complex) could be assigned to clonal complexes. The other two, ST-784 and ST-787 could not be assigned since they did not share four or more alleles with a central genotype.

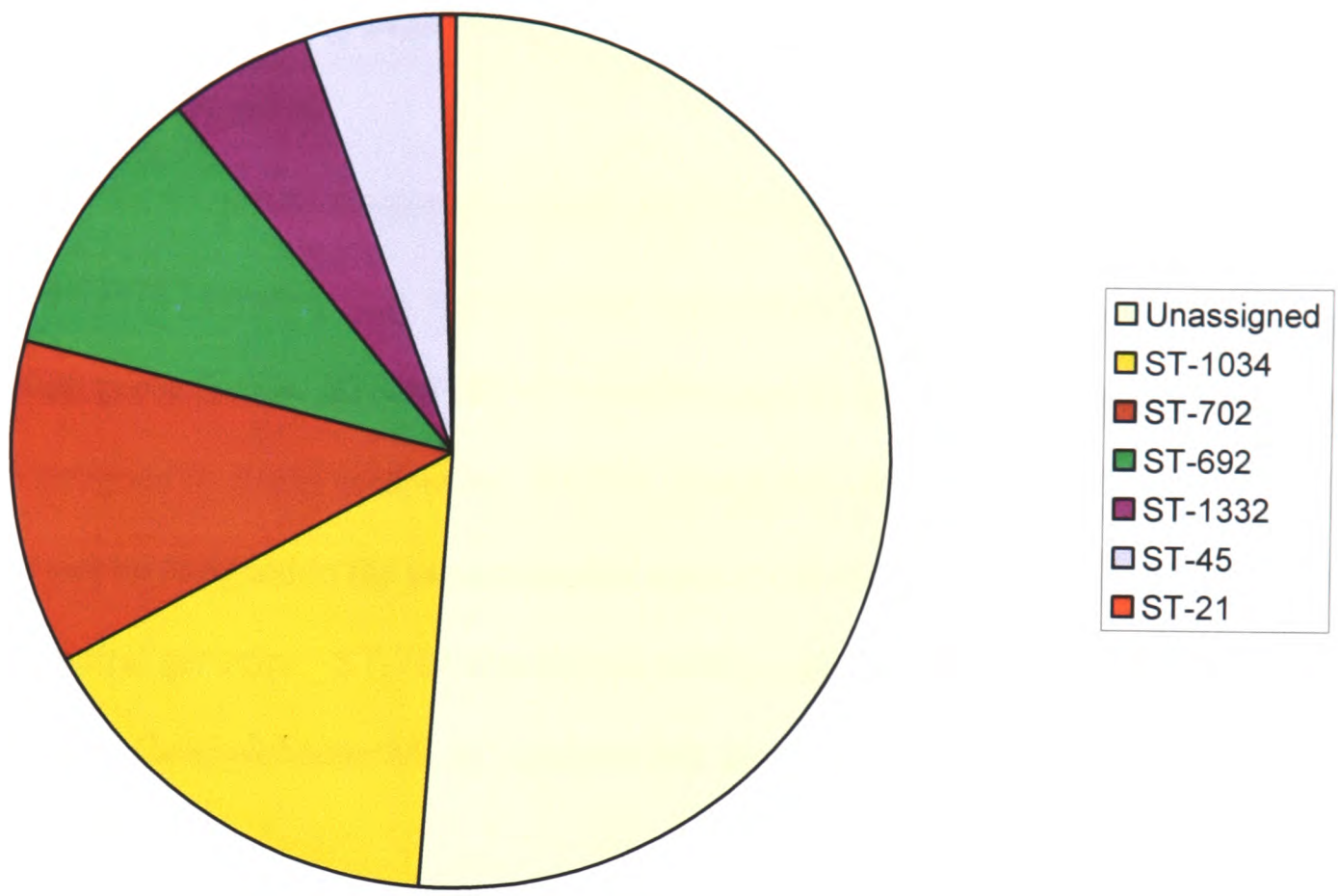


Figure 4.1 The clonal complexes, identified amongst 166 *C. jejuni* isolates from wild geese sampled between August 2002 and February 2003.

4.2.3 Distribution of STs.

A total of 38 STs were identified amongst 166 geese isolates, 35 of which had not been seen previously amongst over 3000 isolates on the *Campylobacter* MLST database (Table 4.1). Only three STs, ST-137, ST-193 and ST-977 had previously been recorded. The central genotypes for four of the six clonal complexes were not isolated during this study. In comparison to other variants within a complex, the central genotypes that were identified were isolated over the longest period of time during the study, followed by unassigned isolates. Although 19 STs could not be assigned to a clonal complex, 16 were

related to at least one other ST isolated from geese by sharing four or more alleles. Clustering amongst the unassigned isolates was confirmed using a neighbour joining tree (Figure 4.2).

Four STs, ST-137 (ST-45complex), ST-696 (ST-1332 complex), ST-784 (unassigned) and ST-787 (unassigned), were isolated from the lambs grazing the pasture contaminated with geese faeces. ST-137 (ST-45 complex) and ST-696 (ST-1332 complex) were assigned to clonal complexes. ST-784 shared four of seven alleles with ST-137 but was not be assigned to the same complex since it did not share four or more alleles with the central genotype. ST-787 was unique to this study, and there were no STs identified on the *Campylobacter* MLST database that shared four or more alleles.

Table 4.1 The composition of clonal complexes identified amongst 166 *C. jejuni* isolates from wild geese in terms of STs, genetic variant and time period over which they were isolated. The unassigned STs are shown in clusters that share four or more alleles.

Clonal complex	No. of isolates	ST	Complex variant[a]	Frequency	Isolation period (days)[b]
21	1	193	TLV	1	1
45	8	137	SLV	7	1
		706	DLV	1	1
692	17	692	C	4	192
		699	SLV	7	33
		707	SLV	6	1
1034	26	711	SLV	2	9
		977	SLV	1	1
		788	DLV	1	1
		1033	DLV	16	13
		694	TLV	2	1
		697	TLV	1	1
		698	TLV	1	1
		1029	TLV	1	1
		1608	TLV	1	1
1332	9	696	SLV	9	33
702	20	702	C	18	33
		703	SLV	1	1
		705	SLV	1	1
Unassigned	85	693	cluster I	9	169
		704	cluster I	3	159
		709	cluster I	6	159
		785	cluster I	1	1
		1028	cluster I	3	1
		1030	cluster I	4	23
		1031	cluster I	10	1
		1032	cluster I	2	1
		1606	cluster I	3	1
		1607	cluster I	1	1
		691	cluster II	5	33
		700	cluster II	8	33
		695	cluster III	5	33
		708	cluster III	1	1
		710	cluster IV	13	159
		786	cluster IV	1	1
		789	singleton	1	1
		701	singleton	8	9
		993	singleton	1	1

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant. [a]The relationship to the central genotype. [b]The greatest number of days between sampling occasions on which the ST was isolated.

Table 4.2. The summary statistics for the genetic variants within the clonal complexes, identified amongst 166 *C. jejuni* isolates from geese.

Complex variant[a]	Average period of isolation (days)	Longest period of isolation(days)	Number of STs	Frequency of isolates
C	112.5	192	2	22
SLV	10	33	8	34
DLV	5	13	3	18
TLV	1.2	1	6	7
U	41.4	169	19	85

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant.

[a]The relationship to the central genotype.

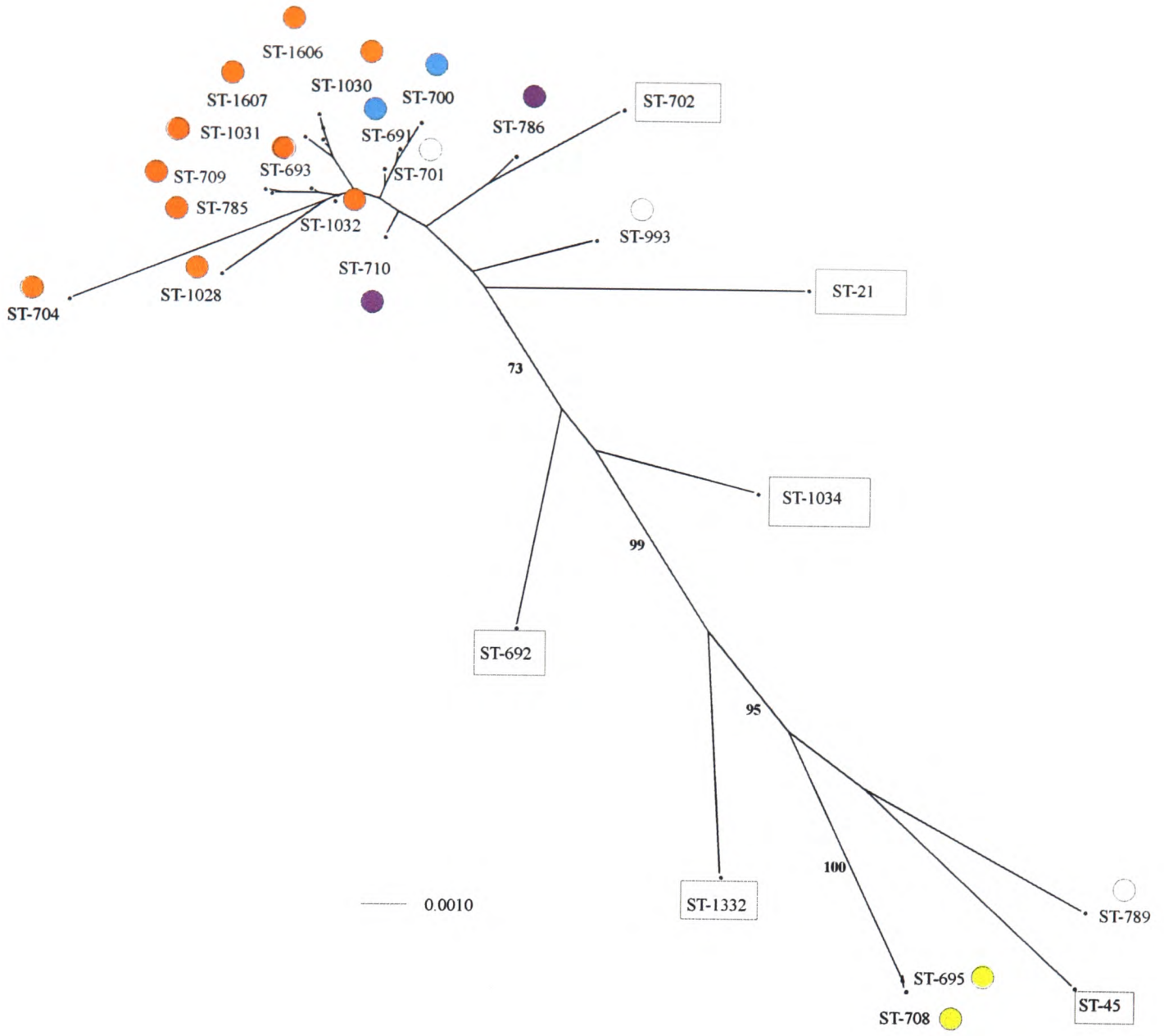


Figure 4.2. A Neighbour Joining tree constructed with concatenated sequence showing the relationship of unassigned STs with the central genotype of clonal complexes (shown in boxes), identified amongst *C. jejuni* isolates from wild geese.

STs sharing four or more alleles are marked with the same colour, singleton STs are colourless. Bootstrap values are given where they exceed 70%.

4.2.4 Distribution of *C. jejuni* genotypes over time.

Between one and five clonal complexes were identified on each sampling occasion, together with unassigned STs (Figure 4.3a). The distribution of the clonal complexes over time was not random (χ^2 71.32, p 0.000). Two complexes, ST-21 complex and ST-45 complex were only seen on one sampling occasion. ST-702 and ST-1332 complexes were identified only during 2002. ST-692 complex was seen on four of the five sampling occasions and ST-1034 complex was identified on all five sampling occasions. The proportion of ST-692 complex was similar on each occasion (χ^2 6.09, p 0.193) but the proportion of ST-1034 complex differed significantly between sampling occasions (χ^2 39.60, p 0.000) and ranged from 21% of isolates on 18.8.02 to 2% of isolates on 20.09.02. The distribution of ST-702 complex also appeared to be non-random (χ^2 18.25, p 0.001), but the distribution of ST-1332 complex was just deemed to be random (χ^2 9.30, p 0.054).

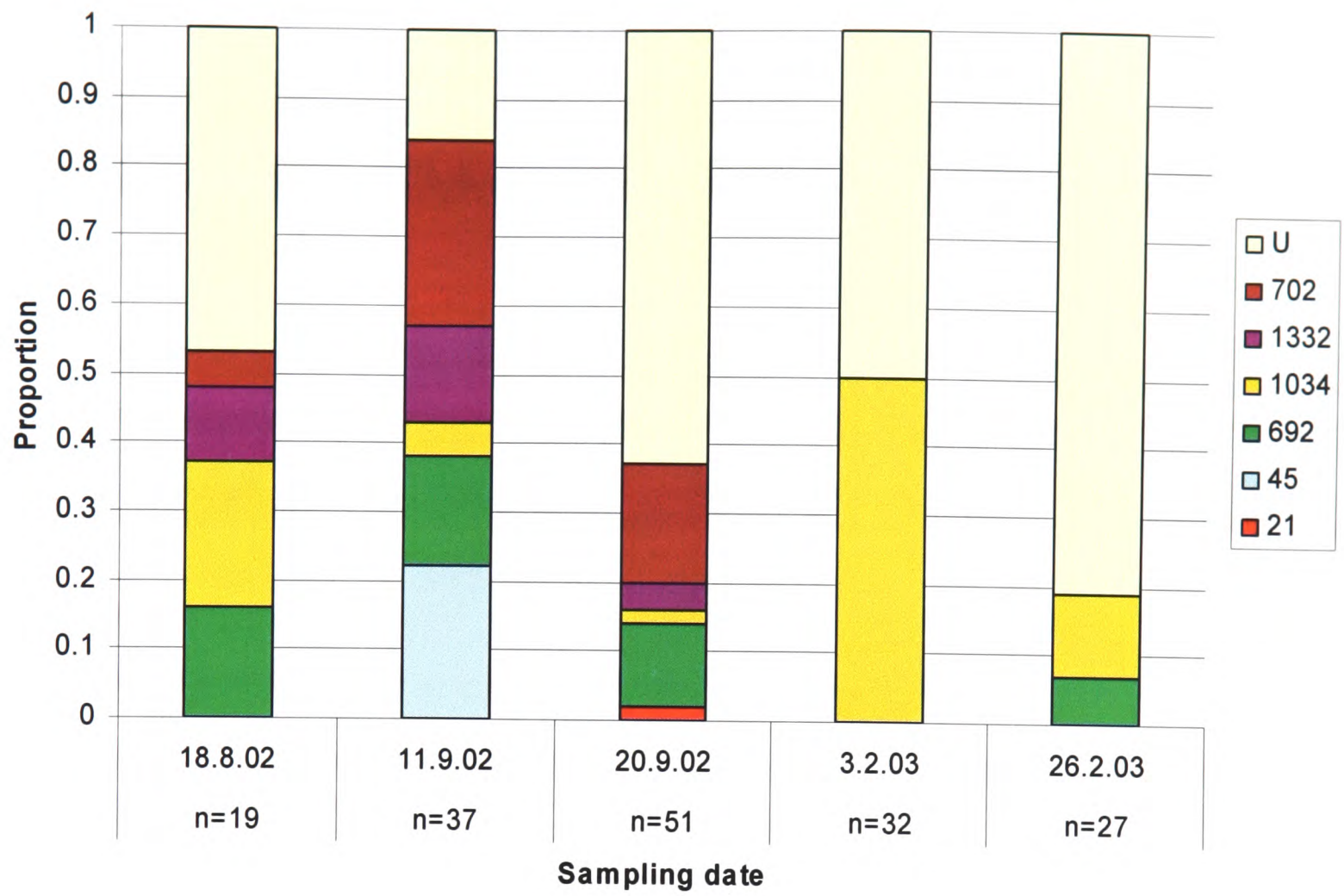


Figure 4.3a. Distribution of *C. jejuni* clonal complexes during the five sampling occasions in 2002-2003.

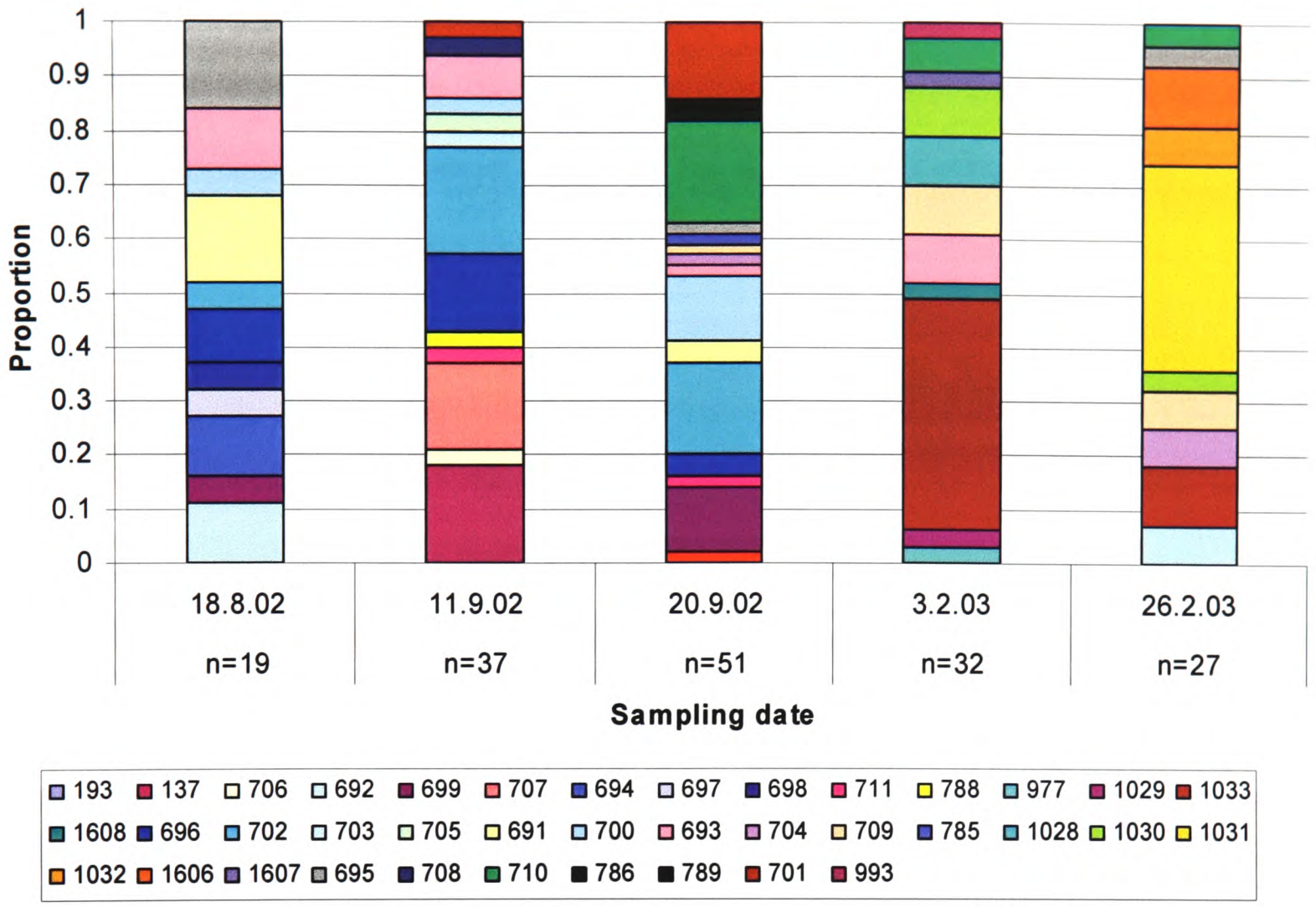


Figure 4.3b. Distribution of *C. jejuni* STs during the five sampling occasions in 2002-2003.

Between ten and 16 STs were identified on each sampling occasion (Figure 4.3b). The majority of STs, 23 of 38 (60%), were isolated on only one sampling occasion. ST-693 was the only ST seen on four of five sampling occasions and none of the STs were isolated on all five sampling occasions. The modified Simpson's Index of Diversity using ST data gave the highest measure of diversity for isolates collected in August 02 (0.94) followed by those collected in September 02 (11.9.02 = 0.88, 20.9.02 = 0.89), with the lowest measure of diversity seen in February 03 (3.2.03 = 0.82, 26.2.03 = 0.84).

4.2.5 Diversity of allele sequences.

Amongst the geese isolates, the number of alleles at each locus ranged from six at the *aspA* locus to 18 at the *pgm* locus (Table 4.3). The number of variable sites ranged from nine (1.9%) for the *aspA* locus to 40 (8.0%) for the *pgm* locus. The d_N/d_S ranged from 0.010 for the *aspA* and *uncA* loci to 0.026 for the *glyA* locus. Using concatenated sequence of all seven loci, the number of variable sites was 157 (4.7%) and the d_N/d_S was 0.012.

Amongst the lamb isolates there were three alleles at each locus with the exception of *gltA* which had four alleles (Table 4.4). The number of variable sites varied from six (1.3%) at the *glnA* locus to 23 (4.5%) at the *glyA* locus. The d_N/d_S ratio ranged from 0.000 for the *gltA* and *uncA* loci to 0.274 for the *glnA* locus.

Table 4.3 Diversity of allele sequences amongst geese isolates (n=166).

Locus	Fragment size (bp)	No. of alleles	No. of variable sites	% variable sites	d_N/d_S ratio
aspA	477	6	9	1.9	0.010
glnA	477	10	18	3.8	0.013
gltA	402	9	15	3.7	0.014
glyA	507	12	33	6.5	0.026
pgm	498	18	40	8.0	0.024
tkt	459	16	27	5.9	0.017
uncA	489	10	13	2.7	0.010
All loci	3309	38	157	4.7	0.012

Table 4.4 Diversity of allele sequences amongst lamb isolates (n=4).

Locus	Fragment size (bp)	No. of alleles	No. of variable sites	% variable sites	d_N/d_S ratio
aspA	477	3	11	2.3	0.031
glnA	477	3	6	1.3	0.008
gltA	402	4	10	2.5	0.000
glyA	507	3	23	4.5	0.042
pgm	498	3	16	3.2	0.041
tkt	459	3	15	3.3	0.020
uncA	489	3	18	3.7	0.000
All loci	3309	4	100	3	0.032

4.2.6 Antigenic diversity.

A total of nine *flaA* peptide sequences, and 23 combinations of *flaA* nucleotide sequence-peptide sequence were identified amongst the 166 geese isolates (Table 4.5). Peptide one was the most common (61 isolates), and was associated with all of the clonal complexes except ST-45 and ST-1332 complexes. Peptide 15 was the next most common (41 isolates) and was present amongst ST-1034 complex and unassigned isolates, and peptide 35 was the next most common (16 isolates), and was present amongst ST-692 complex and unassigned isolates. Peptide 5 was isolated only from ST-1332 complex and peptide 24 was isolated only from ST-45 complex. Four clonal complexes, ST-21, ST-45, ST-702 and ST-1332 complexes, were consistently associated with one nucleotide-peptide sequence type (Figure 4.4). Amongst the four lamb isolates, three peptide alleles, and three nucleotide/peptide combination alleles were identified. The *flaA* SVR allele 85-24 was associated with both ST-137 (ST-45 complex) and ST-784 (unassigned) (Table 4.6).

Table 4.5. The distribution of *flaA* SVR alleles amongst *C. jejuni* genotypes isolated from wild geese.

Clonal complex	ST	Complex variant[a]	Frequency	flaA SVR allele/peptide (proportion)
21	193	TLV	1	64-1(1)
45	137	SLV	7	85-24(1)
	706	DLV	1	85-24(1)
692	692	C	4	225-11(0.5), 224-35(0.25), 275-27(0.25)
	699	SLV	7	391-24(1)
	707	SLV	6	22-1(1)
1034	711	SLV	2	352-1(1)
	977	SLV	1	89-1(1)
	788	DLV	1	22-1(1)
	1033	DLV	16	199-15(0.94), 225-11(0.06)
	694	TLV	2	22-1(1)
	697	TLV	1	64-1(1)
	698	TLV	1	225-11(1)
	1029	TLV	1	199-15(1)
	1608	TLV	1	56-1(1)
1332	696	SLV	9	15-5(1)
702	702	C	18	355-1(1)
	703	SLV	1	355-1(1)
	705	SLV	1	355-1(1)
Unassigned	693	cluster I	9	64-1(0.77), 213-15(0.23)
	704	cluster I	3	56-1(1)
	709	cluster I	6	209-1(1)
	785	cluster I	1	224-35(1)
	1028	cluster I	3	56-1(1)
	1030	cluster I	4	206-53(0.75), 224-35(0.25)
	1031	cluster I	10	224-45(0.8), 225-11(0.2)
	1032	cluster I	2	225-11(1)
	1606	cluster I	3	224-35(0.67), 225-11(0.33)
	1607	cluster I	1	205-1(1)
	691	cluster II	5	225-11(1)
	700	cluster II	8	264-15(0.88), 69-15(0.12)
	695	cluster III	5	354-27(1)
	708	cluster III	1	27-1(1)
	710	cluster IV	13	69-15(1)
	786	cluster IV	1	69-15(1)
	789	singleton	1	252-44(1)
	701	singleton	8	353-1(1)
	993	singleton	1	219-15(1)

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant.

[a]The relationship to the central genotype. Unassigned STs are clustered into groups that share four or more alleles.

Table 4.6. The distribution of *flaA* SVR alleles amongst *C. jejuni* genotypes isolated from lambs.

Clonal complex	Number of isolates	ST	Complex variant[a]	Frequency	*flaA SVR* allele/peptide (proportion)
45	1	137	SLV	1	85-24(1)
1332	1	696	SLV	1	15-5(1)
U	2	784	singleton	1	85-24(1)
		787	singleton	1	18[b](1)

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant.

[a]The relationship to the central genotype.

[b]Only the peptide allele could be obtained.

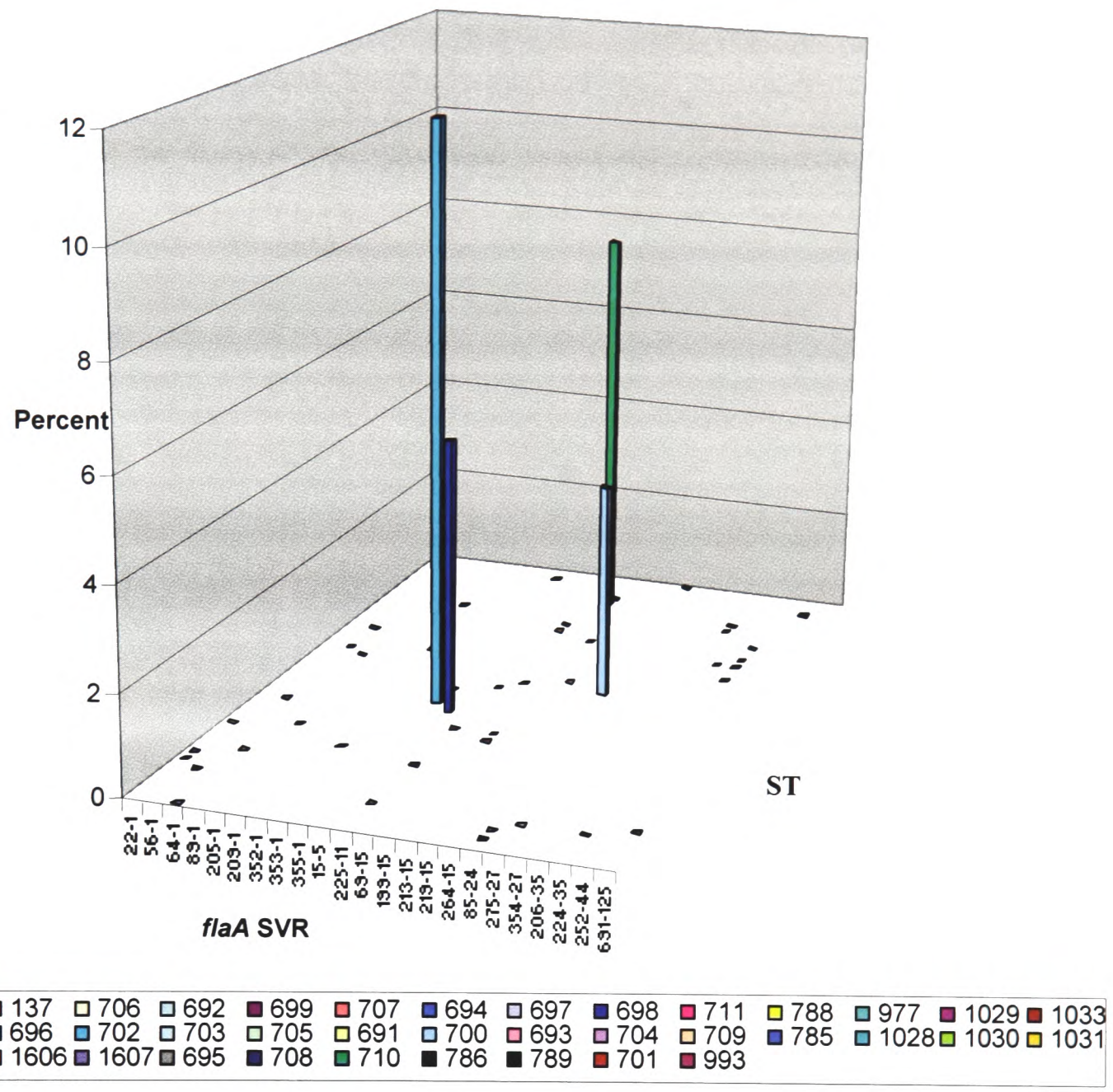


Figure 4.4.. The *flaA* SVR distribution amongst *C. jejuni* STs isolated from wild geese.

4.2.7 Comparison of *C. jejuni* isolates from geese with those from other sources.

Despite the low shedding rate of lambs grazing the pasture contaminated with geese faeces, two of the isolates were assigned to two clonal complexes, ST-45 and ST-1332, both of which were isolated from the geese. Two identical ST and *flaA* nucleotide/peptide combinations, ST-696 *flaA* SVR 15-5 and ST-137 *flaA* SVR 85-24, were identified amongst the lamb and geese isolates. A third genotype was identical at four of seven loci to one of the assigned isolates, and the fourth was completely unrelated.

The distribution of clonal complexes was compared with that identified amongst the farm and environmental isolates from the Chapter 3 (Figures 4.5 and 4.6). In addition the same set of human disease isolates was used for comparison since they were the most representative population sample available, and the temporally matched isolates available on the *Campylobacter* MLST database were unsuitable due to their bias of new and unusual STs. The geese isolates shared three clonal complexes, ST-21, ST-45 and ST-692 complexes with the farm and human isolates from Chapter 3. The remaining three clonal complexes identified amongst the geese isolates appeared to be host associated. Within the overlapping clonal complexes only ST-137 (ST-45 complex) was shared, between geese and human disease isolates.

Interrogation of the *Campylobacter* MLST database revealed one other clonal complex, ST-1034 complex to be isolated from three cases of human disease. Each of the clonal complexes potentially associated with geese were isolated from Mallard ducks captured in Sweden. Some also contained genotypes from cattle, environmental waters

and chicken isolated in Europe between 1999 and 2004. The ST-692 complex was also identified amongst environmental waters in Canada. Only four of 38 STs (10.5%) isolated from geese were identified amongst other sources on the *Campylobacter* MLST database. They were ST-137 (ST-45 complex) isolated from human disease, wild birds (species unknown) and sheep in Europe, Canada and Curaçao between 1982 and 2000. ST-193 (ST-21 complex) isolated from human disease in the UK in 1991; ST-977 (ST-1034 complex) isolated from human disease in the UK in 2003 and ST-696 (ST-1332 complex) isolated from cattle in the UK in 2001. Three of the clonal complexes isolated from geese, ST-702, ST-692 and ST-1034 complexes appeared to form a clade unrelated to those isolated from other sources (Figure 4.6).

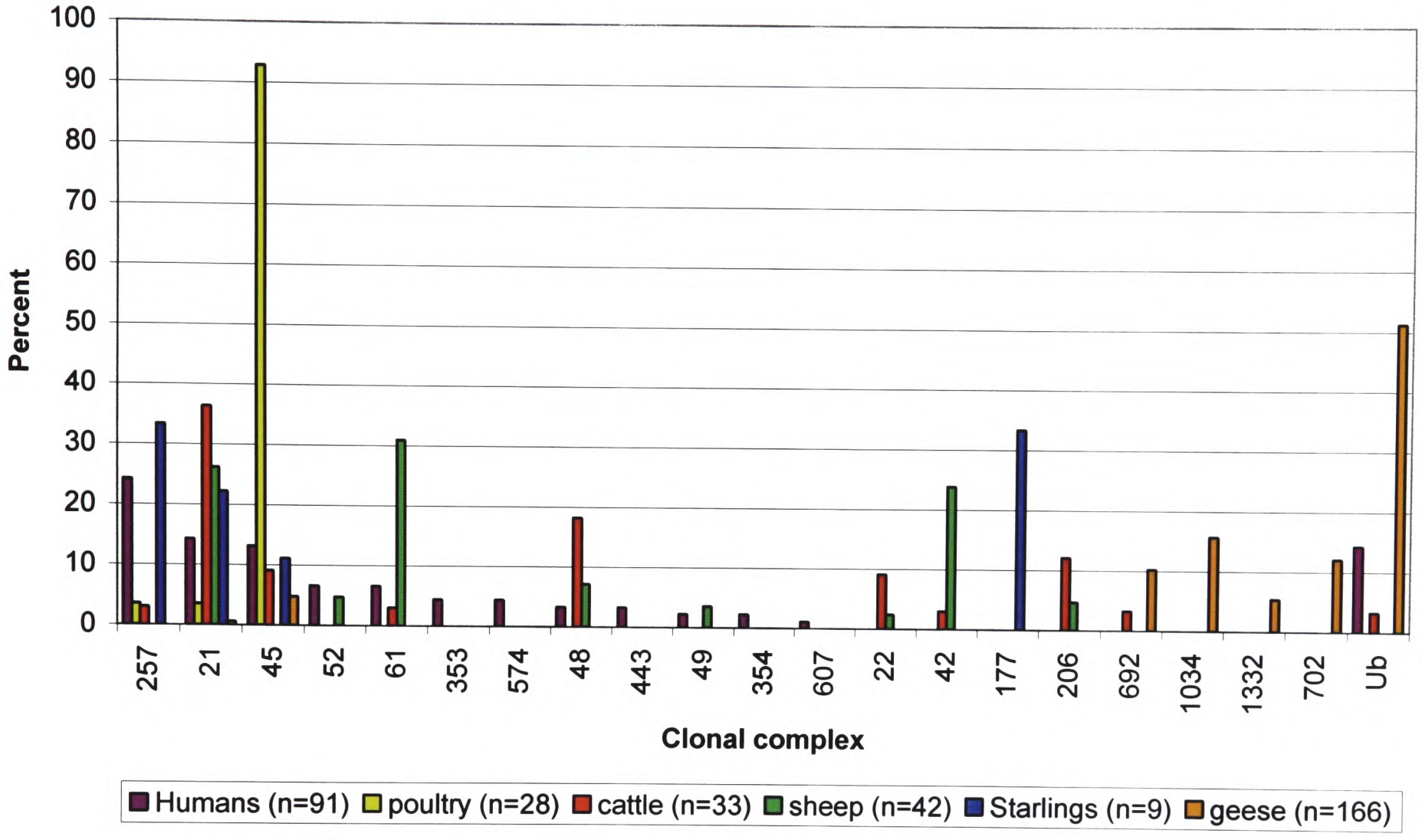


Figure 4.5. The distribution of *C. jejuni* clonal complexes amongst Oxfordshire geese and the farm, environmental and human disease isolates from Lancashire (Chapter 3).

The human disease isolates were every tenth isolate through the door of the Preston reference laboratory in 1999. U=STs that could not be assigned to a clonal complex.

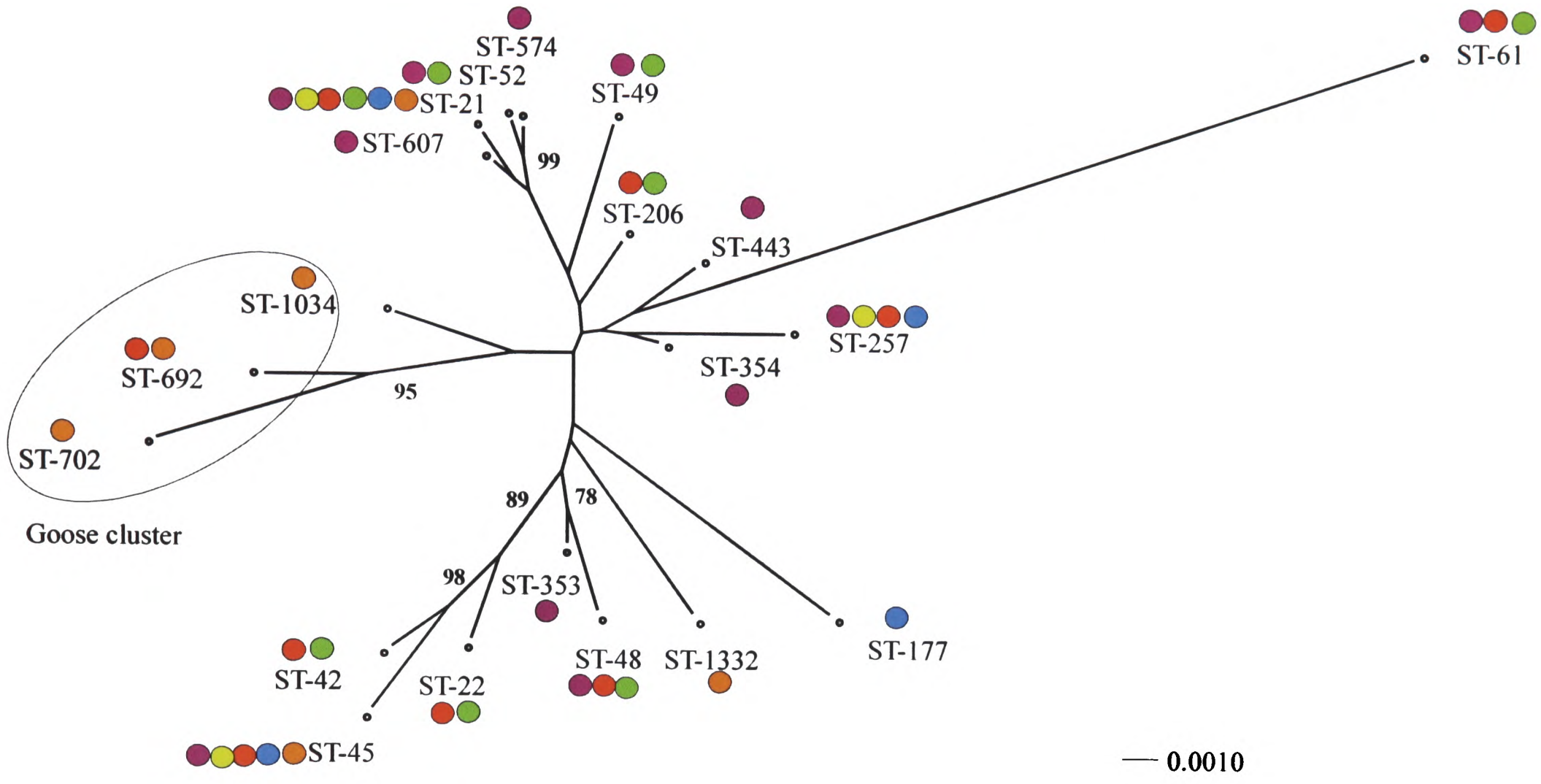


Figure 4.6. Neighbour joining tree showing the relationships of *C. jejuni* clonal complexes isolated from Lancashire farm animals and Oxfordshire geese (concatenated sequence). Boot strap values are given where they exceed 70%.

Purple= human isolates, yellow= poultry isolates, red = cattle isolates, green = sheep isolates, blue = starling isolates, orange = geese isolates.

4.3 Discussion.

The results demonstrated that *Campylobacter* could be successfully cultured from wild geese species and that the original MLST primers and method could be used on the isolates without modification. The nucleotide diversity present in the geese derived isolates was similar to that described in the farm animal isolates, and also human disease and retail food isolates (Dingle *et al.* 2001a), in terms of the number of alleles, nucleotide variability and (d_N/d_S) ratio at each locus. The number of positive isolates from lambs grazing the same pasture as the geese was very small, but since each was a different ST, the number of alleles and nucleotide variability was smaller, but the (d_N/d_S) ratios were similar to those obtained from the farm and geese isolates.

The overall shedding rate of *Campylobacter* by wild geese in this study was 50.4% (167 of 331 isolates) of which 50.2% (166 isolates) were *C. jejuni* and 0.3% (one isolate) was *C. coli*, which was not included in further analysis. The *C. jejuni* shedding rate varied between 32.9% on 26.2.03 and 74.4% on 3.2.03. Other studies have recorded carriage rates between 24.1% in Barnacle Geese in Sweden and 100% in domestic geese in Turkey (Aydin *et al.* 2001; Waldenstrom *et al.* 2005b). Differences are likely to reflect the varied host species, conditions, countries and sampling methods of the studies. The shedding rates in this study did not fit into the typical seasonal trend with a summer peak in shedding, which has been seen amongst human, poultry and farm animal sources in temperate countries in a number of studies (Wallace *et al.* 1997; Stanley *et al.* 1998c). Instead there was a peak in shedding rates in late September, in common with that seen in gulls (Broman *et al.* 2002). The diversity of genotypes varied between sampling

occasions and the trend appeared more typical of that seen in other animals, with greater diversity seen in late summer/early autumn (D=0.88-0.94) compared to the winter (D=0.82-0.84), although more samples would be required to confirm this. The increased diversity did not appear to be linked with particular genotypes, rather, the majority (57.9%, 22 of 38) of genotypes were sporadic and only isolated on one sampling occasion. The September peak in shedding and diversity of *Campylobacter* may be due to birds clustering together before migration, increasing stress levels and contact with other birds. Increasing rainfall could cause more agricultural run off, which together with falling UV light intensity and relatively mild temperatures may bring more *Campylobacter* organisms into the geese environment and aid their survival(Stanley *et al.* 1998a; Jones 2001b). The shedding rate and diversity may have been underestimated on occasions due to sampling error. Faecal samples that looked fresh were collected at the same time of day, but the survival of Campylobacters may have been affected by varying weather conditions and the age of the sample.

Only 4% (4 of 84) of faeces samples collected from lambs were positive for *C. jejuni*, a rate much lower than that seen in other studies, despite using enrichment culture. Stanley *et al*, 1998b isolated *Campylobacter* from 29.3% of fresh faeces and up to 100% from samples from the small intestines of lambs, although the lowest rate of 53% was recorded in October, the same month as the samples were collected in this study. The shedding rate in this study is likely to be an underestimate for the same reasons as the geese, since faeces were collected from the ground. Isolation of *Campylobacter* may have increased if samples had been taken from the small intestine where the bacteria may have been present, but not actually shed with the faeces (Stanley *et al.* 1998b). The low

shedding rate was seen amongst lambs despite them grazing the pasture highly contaminated with fresh goose faeces containing *C. jejuni*.

A total of 38 STs clustering into six clonal complexes and 19 unassigned isolates, were identified amongst the geese isolates, of which 34 STs were unique to this study. Four clonal complexes, ST-692, ST-702, ST-1034 and ST-1332 accounted for 43.3% (72 of 166) of the geese isolates, although only ST-1034 was isolated on all sampling occasions. The same clonal complexes were dominant amongst Swedish Mallard ducks and accounted for 65.9% of isolates (Waldenstrom *et al.* 2005a). Interrogation of the *Campylobacter* MLST database revealed that all except ST-702 complex also contained occasional isolates from cattle, water (Canada), poultry or Black Headed Gulls (Sweden). Thus, although the majority of STs isolated were unique, they may represent local clonal expansion of clonal complexes that are more widespread, and adapted to birds living in a particular aquatic environment rather than a specific host species. This may reflect the observation that geese in this study tended to stay local to Oxfordshire, despite being migratory species (A Gosler, personal communication). The relative longevity, worldwide spread and large number of STs within the ST-1034 complex suggests that this complex may be a particularly successful clone within this species with recent and rapid expansion within birds in Oxfordshire. A large number of STs (50% of STs, 51.2% of isolates) remained unassigned, in common with that of other studies of wild animal and environmental isolates (Petersen *et al.* 2001a; French *et al.* 2005). Many of the unassigned STs from the geese formed small genotypically related clusters and further sampling may help to resolve potential new clonal complexes and identify potential sources and host associations (French *et al.* 2005; Waldenstrom *et al.* 2005a).

Sequencing at the *flaA* SVR locus proved less discriminatory than MLST identifying 23 *flaA* SVR alleles compared to 38 STs, but was successful in providing an additional layer of differentiation between genotypes that clustered with MLST. As noted in a previous study, the antigen type appeared to be loosely associated with genotype although there was some mixing of combinations (Dingle *et al.* 2002). As might be expected there was no evidence to suggest that the evolutionary relationships of genotypes and antigen types were congruent. The antigen types were likely to have arisen by re-assortment of genes, or by recombination resulting in related *Campylobacter* genotypes having completely unrelated antigen types (Wassenaar *et al.* 1995). The fact that certain ST-*flaA* SVR combinations were identified on up to 18 times and over a period of one month suggests that the relationships between ST and antigen type were relatively stable, and may therefore be useful for short term epidemiological investigations.

Four STs were identified amongst the Oxfordshire lamb isolates, of which two were unique to this study. Although the numbers of Oxfordshire lamb isolates were very small, none of the cattle/sheep associated complexes were identified (Dingle *et al.* 2002). Results from this investigation suggest that lambs grazing the same pasture as the geese may have become colonised by 'the aquatic bird' genotypes since two identical ST and *flaA* SVR combinations were identified from both host sources. A third ST shared four of seven alleles with the one of the geese isolates, but not the same clonal complex and may represent a genotype not yet isolated from geese. Evidence from this study suggests the transmission of *Campylobacter* genotypes between geese and lambs may not be particularly common since the majority of lambs sampled from the field were not

shedding *Campylobacter* on this occasion, but further sampling and antigenic typing are required to confirm these observations. It may be that carriage of 'aquatic bird' adapted genotypes is transient making study more difficult. It would be interesting to discover whether in the long term, more lambs would became colonized by 'ruminant-type' genotypes, or whether sharing part of their habitat with geese would lead to a closer relationship, such as that discovered by French *et al* (2005), where isolates from cattle and rabbits were genetically closer than other host-type combinations. In general *Campylobacter* genotypes isolated from Oxfordshire geese demonstrated minimal overlap with those isolated from the Lancashire farm animals and their environment. Only two of the clonal complexes, ST-21 and ST-45 complexes and one ST, ST-137 were identified amongst the geese and the farm animal isolates. ST-137 was isolated from a Starling and may reflect closer physiological or environmental similarities between the wild bird species, than those encountered between wild bird species and farm animals.

There was evidence that the geese carried 'human-like' genotypes in low frequencies. ST-21 and ST-45 complexes, commonly isolated from human disease, accounted for 5.4% (9 of 166) of isolates. ST-706 (ST-45 complex) was unique to this study, but ST-137 (ST-45 complex) was seen 7 (0.5%) times amongst 1358 isolates from human disease on the *Campylobacter* MLST database, and the other, ST-193 (ST-21 complex) only once (0.07%). Thus it may be possible that the wild geese act as a source of infection for humans, albeit an infrequent one. Only ST-137 (7 of 166, 4.2%) was associated with isolates from chicken sources on the *Campylobacter* MLST database, suggesting that geese could also act as a source for contamination of chicken flocks, although it seems unlikely that they would mix directly with large flocks of commercially

reared chickens. It is possible that ST-137 is fairly successful and widespread within the environment, and geese, humans and chickens may acquire it from the same external source.

In conclusion, geese may be a significant reservoir of *C. jejuni* with an average of 50.2% samples being positive in this study. They carried human (5.4% of isolates) and chicken-like (4.2% of isolates) genotypes at low frequencies and cannot be ruled out as a potential source of infection, although their influence is likely to be minor. The isolates clustered into four stable clonal complexes thought to be adapted to an aquatic environment shared with Mallard ducks, and unassigned isolates in roughly equal proportions. More sampling could help to resolve the unassigned STs into clonal complexes and reveal further host associations, since they may be unusual STs occurring in low frequency. Lambs grazing on the same pasture as the geese became colonized by at least two 'aquatic bird' adapted *C. jejuni* genotypes suggesting that transmission was possible between the host species but that it did not occur at high frequency. Carriage of the shared genotypes may have only been transient if they were unable to adapt to living in ruminant hosts. More data would be required to confirm the findings. Future work should investigate other farmland bird species with greater potential to interact with farm animals, chickens and humans in order to survey the *Campylobacter* genotypes they carry, and to determine the risk they pose as a potential reservoir for infection. The data from this chapter provides a good foundation for further detailed comparison with such isolates.

Abstract

Chapter 5: *Campylobacter* populations in wild *Sturnus vulgaris* (Starlings) caught at the Oxford University farm in Wytham, Oxfordshire.

The aim of this chapter was to establish the population biology of *Campylobacter* in Starlings, expanding the work in Chapter 3. In addition the isolates could be compared with those isolated from free-range chickens reared in the same location at the same time to determine whether transmission of genotypes occurs between the two host sources. Approaching 1000 faecal samples were collected from 637 birds that were individually identifiable by numbered leg rings. Additional data relating to bird age, sex, weight and wing length were also recorded at time of sampling.

Campylobacters were recovered from 37% of samples following enrichment in Exeter broth. The majority of isolates were *C. jejuni* (81.6%), but *C. lari* (16.7%) and *C. coli* (1.7%) were also isolated. *C. jejuni* isolates were typed by MLST. There was a seasonal effect in shedding rate and genetic diversity with a peak in both occurring during the late spring/early summer months, in common with that seen in human disease. An age effect was also noted, with younger birds being more likely to shed *Campylobacter*.

A total of 192 birds were caught on more than one occasion. There was evidence that turnover of genotypes was rapid and carriage of multiple genotypes may occur, with no immunity inferred between *Campylobacter* species or between genotypes within the same species.

In contrast to that typically seen in poultry flocks, *C. jejuni* was isolated from some Starling nestlings aged approximately nine days, but siblings within the same nest were not readily colonised. Genotypes were unusual but did not form separate clusters from those isolated from other Starlings. There was a slight trend of smaller nests being more likely to contain chicks shedding *Campylobacter*.

There was little similarity between *Campylobacter* genotypes isolated from geese and Starlings. Clonal complexes ST-21 and ST-45 were isolated from both

sources but no STs were identical. Approximately 6% of isolates were STs associated with human disease and 3% of isolates were STs associated with retail chicken meat on the *Campylobacter* MLST database. ST-682 and ST-177 complexes were most commonly isolated from Starlings both in terms of frequency and time period over which they were isolated.

Chapter 5: *Campylobacter* populations in wild *Sturnus vulgaris* (Starlings) caught at the Oxford University farm in Wytham, Oxfordshire.

5.1 Introduction.

The work in this chapter aimed to determine whether wild Starlings, identified as a potential source of *Campylobacter* in Chapter 3, were a likely transmission vector for the free-range chickens on the farm site and ultimately humans at the top of the food chain. The population structure and genotypes of *Campylobacter* isolated from each of the host sources were compared in order to detect similarities amongst them. Starlings were commonly seen in large groups on the farm and were observed flying in and out of sheds housing cattle and pigs, and on occasions gained access to chick feed when sacks became split. Waldenström *et al* (2002) identified them as a species with relatively high carriage rates which is probably a reflection of their omnivorous ground feeding habits. They are also commonly seen in human environments being a garden bird and could potentially be a direct cause of infection for humans, for example through surface contamination of food when eating outdoors. Collaboration with the Edward Grey Institute for Ornithology based in the Zoology department enabled Starlings to be caught, monitored and identified with individual British Trust for Ornithology (BTO) leg rings. Large numbers of Starlings were required to provide sufficient *Campylobacter* isolates to enable statistical evaluation and detailed genetic analysis.

In order to investigate their role as transmission vector, the study aimed to determine the carriage rate of *Campylobacter* and the population biology of the organism amongst the Starlings. Isolates were typed by MLST to enable direct comparison of genotypes with those isolated from other host sources, and the original method was tested on this second extensive source of environmental isolates. The *Campylobacter* genotypes isolated from Starlings were compared with those isolated from the geese, farm animal and environmental isolates considered in Chapters 3 and 4 in order to highlight potential overlaps. In addition they were compared with data from human disease isolates stored on the *Campylobacter* MLST database http://pubmlst.org/campylobacter/. The chapter forms a basis for future work comparing *Campylobacter* populations and looking to identify potential transmission events between the Starlings and the free-range poultry reared in the immediate vicinity. The living conditions of wild birds are significantly different to those of domesticated poultry and may provide useful insights with respect to environmental influences.

5.2 Results.

5.2.1 Method amendments.

Thermophillic *Campylobacter* species were cultured from the wild Starlings although growth was generally poor, with small colonies that were difficult to remove from the agar plates, in comparison to other isolates from other sources. The samples from Starlings were enriched in Exeter broth before being plated onto mCCDA in order to aid recovery of cells that may be stressed. It was necessary to modify the MLST

primers for the *aspA* locus by replacing the forward *aspA*9 primer with the *aspA*8 primer for many, but not all, of the Starling isolates. The rest of the MLST method remained unchanged.

5.2.2 Shedding rate of *Campylobacter* species.

Throughout the 30 month study period a total of 964 samples were collected from 637 individual Starlings, of which 359 (37.2%) were positive for *Campylobacter* species. Using nucleotide sequence typing, 293 (81.6% of samples) were identified as *C. jejuni*, 6 (1.7% of samples) C. *coli* and 60 (16.7% of samples) *C. lari*. The isolation rate of *Campylobacter,* as well as species composition, varied from year to year during the study period, although the comparison is biased since sampling was limited during 2002 and 2005. The overall isolation rate for *Campylobacter* species ranged from 13.6% in 2005 to 58.3% in 2002 (Figure 5.1). The isolation rate of *C. jejuni* varied from 0.2% in 2005 to 58% in 2002 and the isolation rate of *C. lari* varied from zero to 13.4% of isolates in 2005. A more accurate analysis of year effect is achieved with the *C. jejuni* isolates in section 5.2.3.

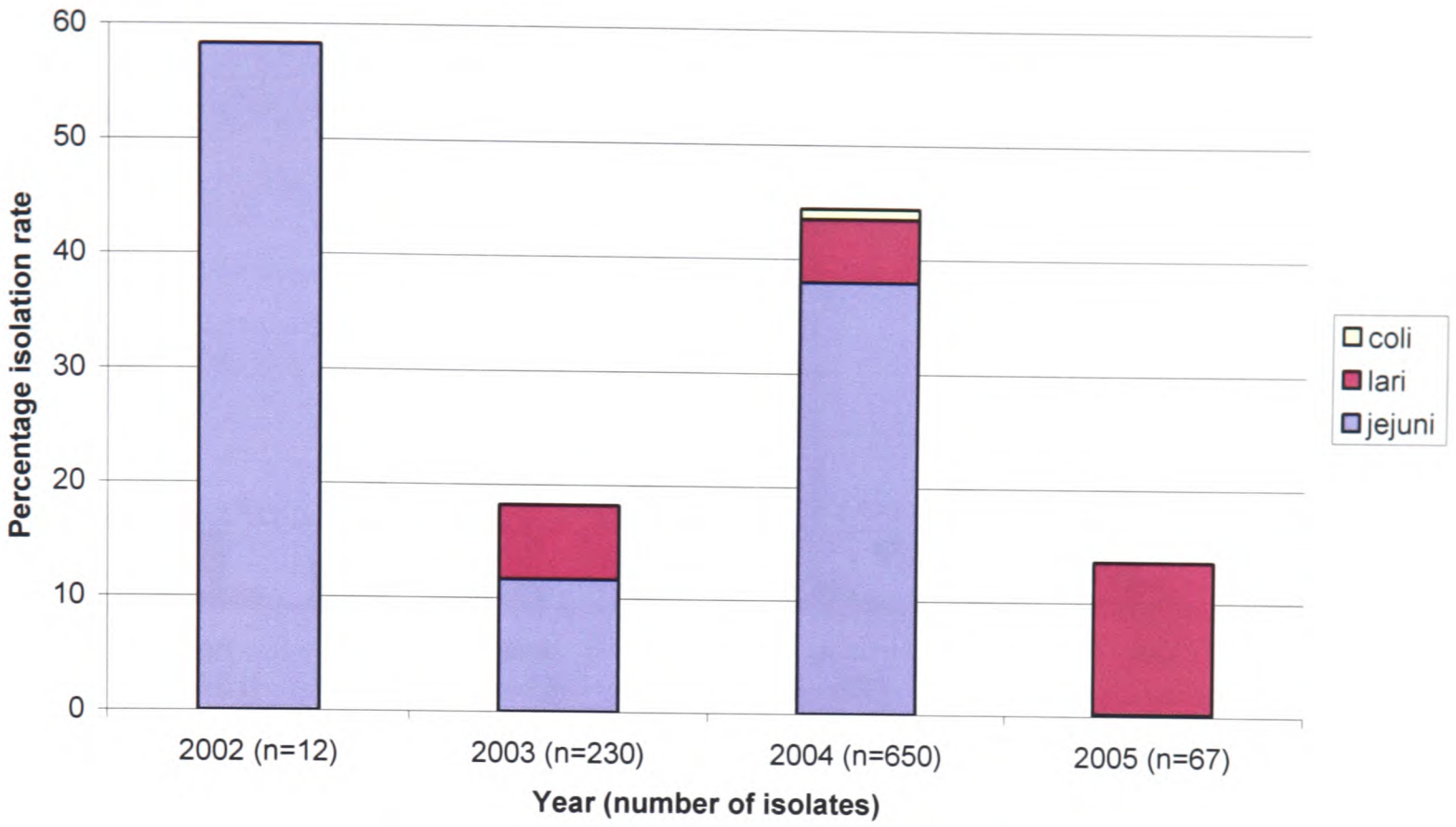


Figure 5.1. The percentage isolation rate of *Campylobacter* species collected from wild Starlings during the 30 month study period.

The isolation rate of the *Campylobacter* species also varied with month of the year (Figure 5.2). *C. jejuni* was most prominent during the summer months of June and July. Isolation of *C. lari* peaked during February and March, and *C. coli* was isolated in small numbers during January, June and November. Further analysis of *C. lari* and *C. coli* isolates was outside the scope of this project and only the predominant *C. jejuni* species will be considered from this point onwards.

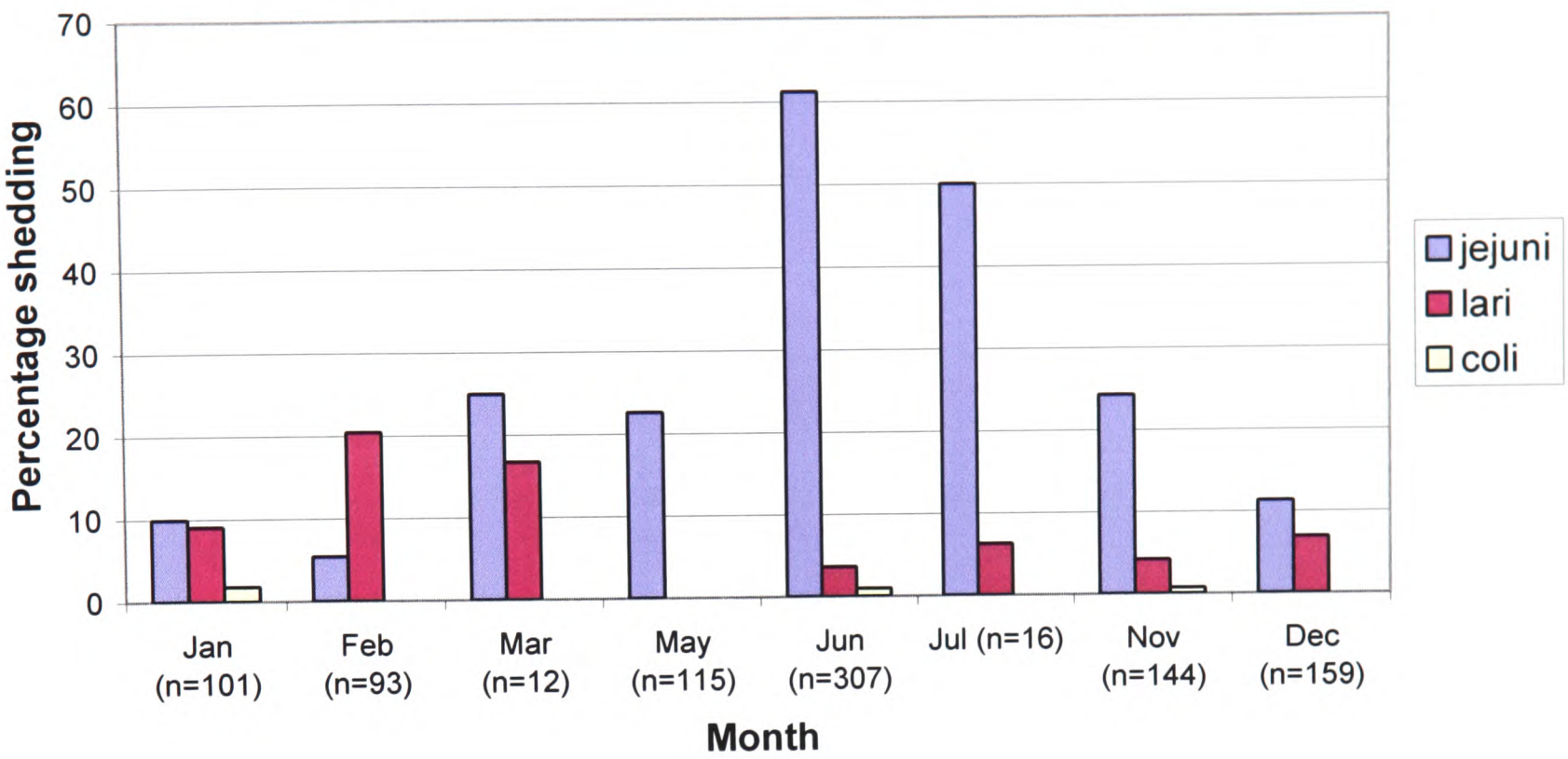


Figure 5.2. The isolation rate of *Campylobacter* species *vs* month of the year. Data from all years (2002-2005) of the study period are included. No data were available for April, August, September and October.

5.2.3 Seasonal effects on isolation of *C. jejuni* from Starlings.

Logistic regression analysis using sine and cosine models gave *p* values of <0.001, giving evidence that there was a significant seasonal effect in the isolation rate of *C. jeuni* from Starlings caught in both 2003 and 2004 (Figure 5.3). The highest isolation rates were recorded during the summer months of 2003 and 2004 with significantly lower levels isolated during the winter months. Samples collected during the single summer or winter months in 2002 and 2005 also fitted the trend. Logistic regression analysis gave *p* values of 0.485-0.716 for each year of sampling, giving evidence that the isolation rates

were not significantly different between the years, when the seasonal effect and month of sampling is taken into account.

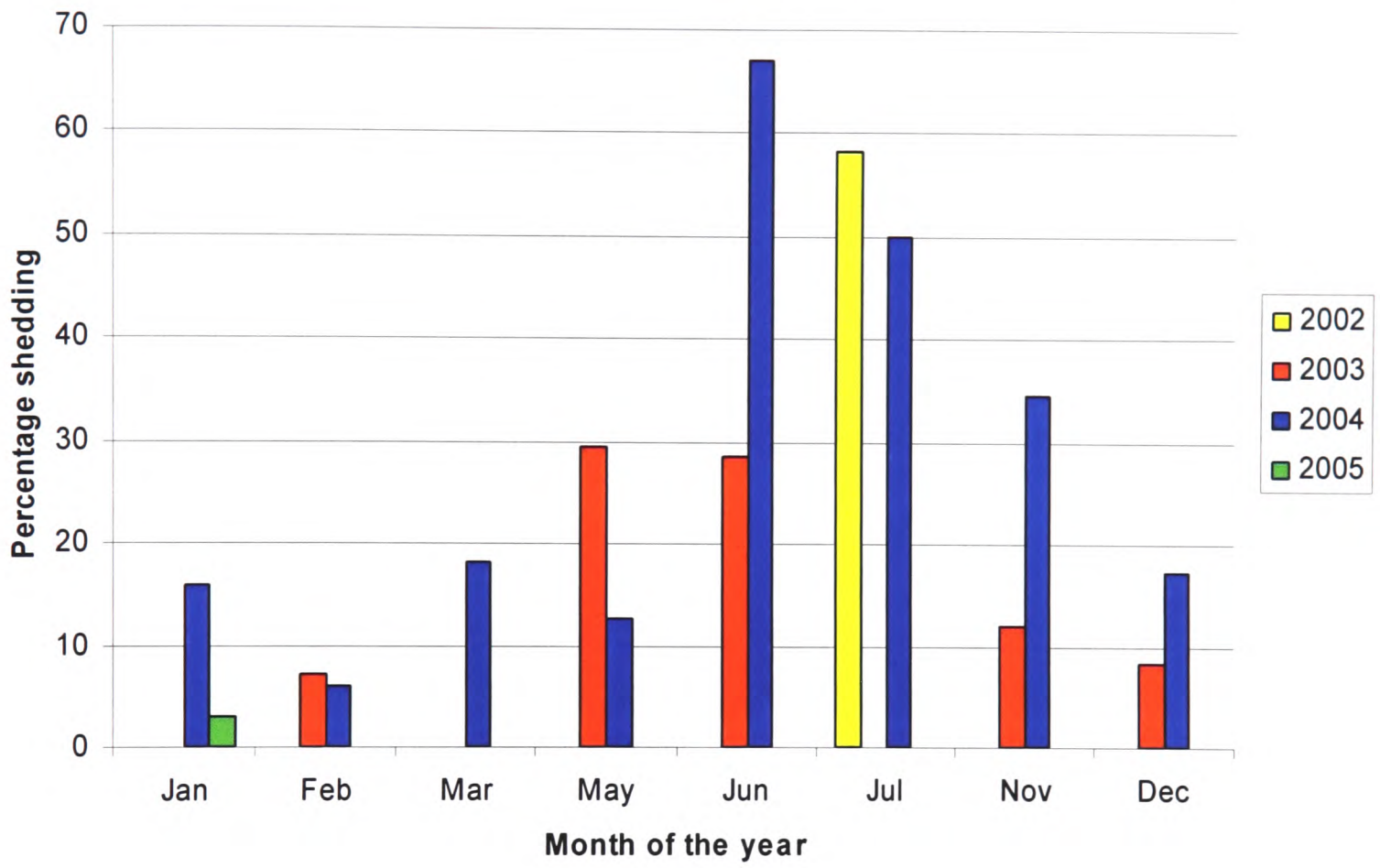


Figure 5.3. The *C. jejuni* shedding rate amongst Starlings sampled during 2002-2005 by month. No data were available for months April, August, September and October.

5.2.4 Clonal complex distribution.

The 359 *C. jejuni* isolates clustered into 11 clonal complexes, with 25 STs representing 48 (16.4%) isolates, remaining ungrouped (Figure 5.4.). The most common clonal complex was ST-682 complex accounting for 130 (44.4%) of isolates and 19 STS.

ST-177 complex was the second largest accounting for 71 (24.2%) of isolates and 16 STs. The remainder accounted for less than 5% of isolates and fewer than four STs each.

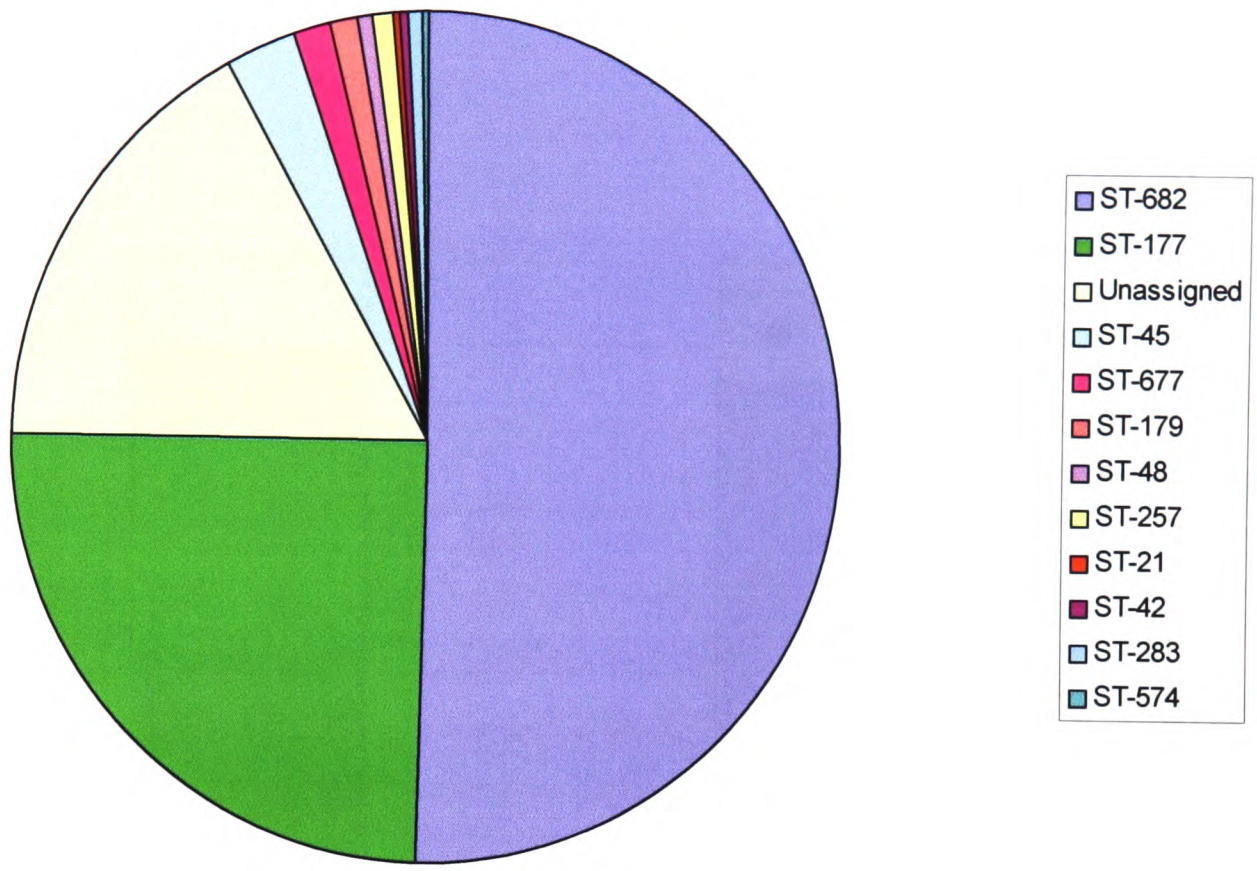


Figure 5.4 The *C. jejuni* clonal complexes detected amongst 291 *C. jejuni* isolates from wild Starlings.

5.2.5 Isolation of clonal complexes over time.

Of the 11 clonal complexes identified, six were isolated in 2003 and eight were isolated in 2004 (Figure 5.5). Only four complexes, ST-682, ST-177, ST-45 and ST-179 complexes, were isolated in both years. ST-682 and ST-177 complexes were also seen in July 2002. ST-682 complex was the dominant complex in both 2003 and 2004 accounting for 18.5% of isolates in 2003 and 53.2% of isolates in 2004. ST-177 complex was the next common and accounted for 14.8% of isolates in 2003 and 24.6% of isolates

in 2004. The remaining complexes accounted for less than 8% of isolates in 2003 and 3% of isolates in 2004. The changing proportions of clonal complexes between the years were not tested for statistical significance due to the low number of isolates obtained in 2003.

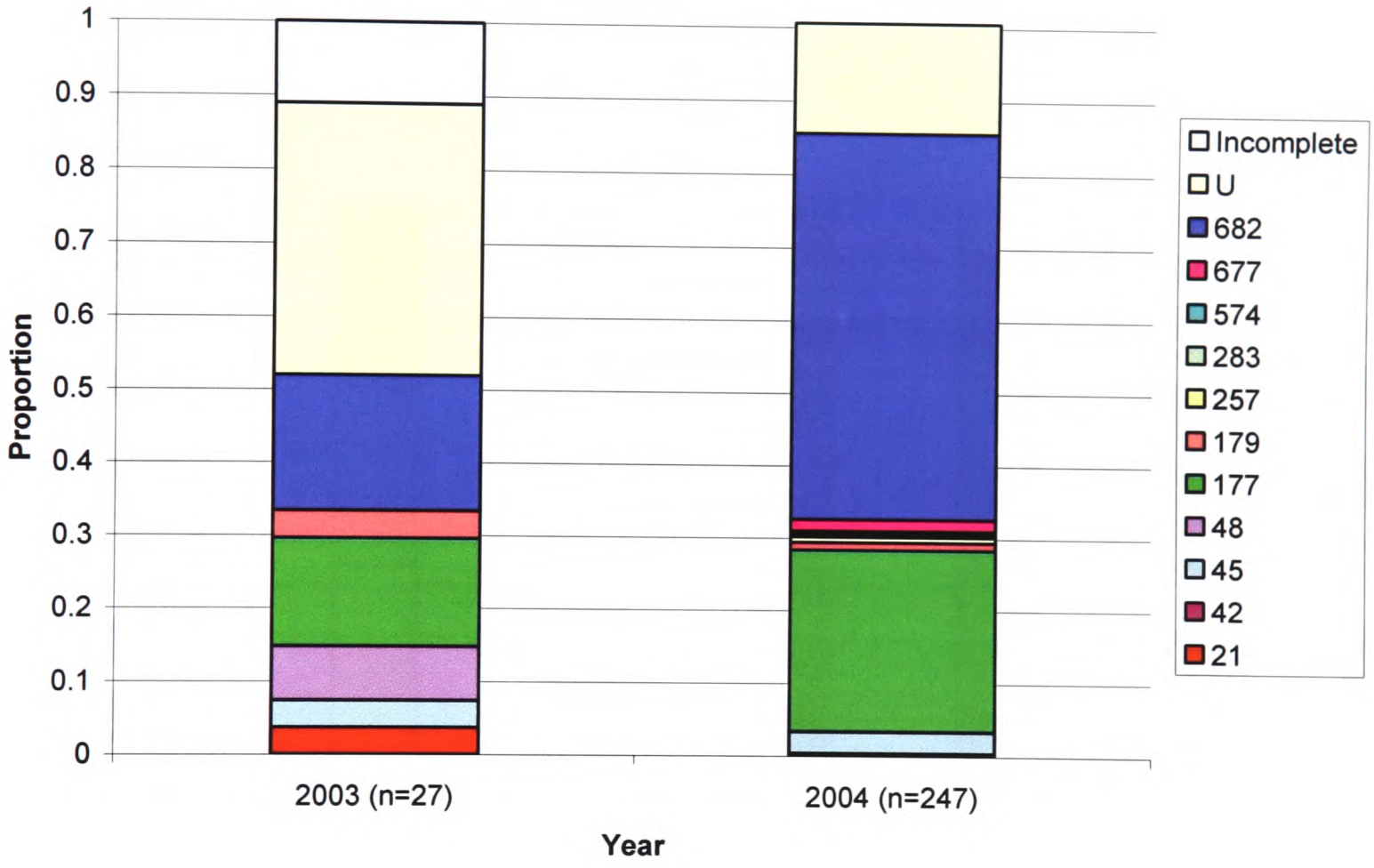


Figure 5.5 The *C. jejuni* clonal complexes isolated from Starlings during 2003 and 2004.

Between two and seven clonal complexes, plus unassigned isolates, were isolated during each month of 2004 (Figure 5.6). The lowest number was isolated during the months of March, November and December, and the highest during June. Only ST-682

complex, ST-177 complex, ST-45 complex and ST-179 complex were isolated from more than one month. ST-682 complex was isolated over five of the six months tested, with a peak in isolation rate in June. ST-177 complex was isolated over four of the six months and was predominant during the winter months. Chi squared analysis gave evidence that the distribution of six of the clonal complexes was not random over time (Table 5.1).

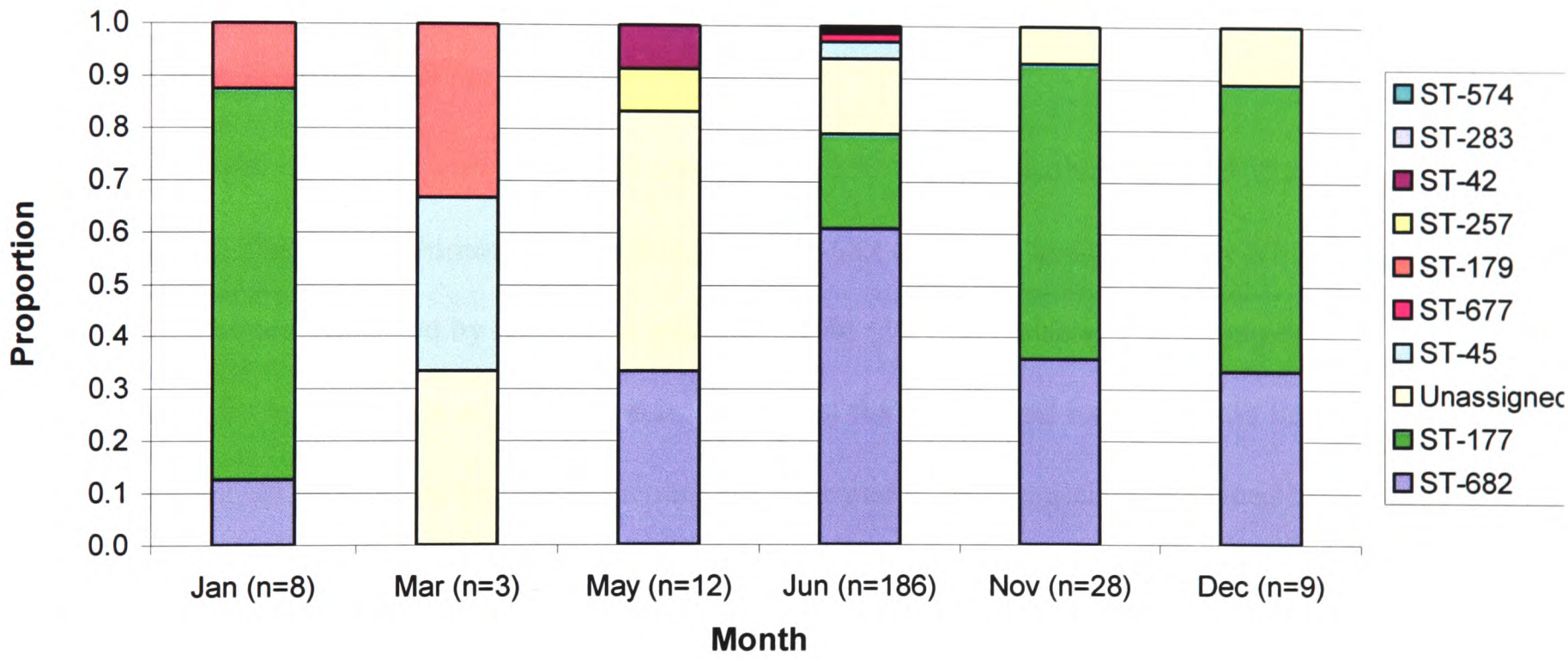


Figure 5.6 The *C. jejuni* clonal complexes isolated during 2004,by month. No data were available for February, April, July, August, September and October.

Table 5.1. Chi squared analysis to establish whether or not distribution of clonal complexes over time was random.

Clonal complex	$\chi 2$	*p* value	Significant
574	0.31	0.997	No
283	0.31	0.997	No
42	34.66	0.000	Yes
257	16.56	0.005	Yes
179	65.17	0.000	Yes
677	0.93	0.968	No
45	15.44	0.009	Yes
177	33.63	0.000	Yes
682	31.24	0.000	Yes

5.2.6 Distribution of STs.

A total of 75 STs were identified amongst the 359 *C. jejuni* isolates from Starlings (Table 5.1). The most common ST was ST-1020 (ST-682 complex) accounting for 63 (21.5%) isolates, followed by ST-177 accounting for 46 (15.7%) isolates. The remainder accounted for less than 5% of isolates each. Fifteen of the unassigned isolates could be grouped into small clusters but the remaining ten appeared to be completely unrelated to each other.

The most commonly isolated ST was not necessarily the one isolated over the longest period of time within the study period, for example ST-1020 (ST-682 complex) was isolated over 35 days, but ST-257 (ST-257 complex) was isolated on two occasions 369 days apart. Central genotypes were isolated from all of the clonal complexes except ST-21, ST-48, ST-179 and ST-283 clonal complexes. On average they were isolated over time period that was at least three times longer than the clonal complex variants (Table 5.2). The number of single locus variant STs isolated was greater than the number of double locus variant STs, which in turn was greater than the number of triple locus

variant STs. In terms of isolates, double locus variants were isolated almost twice as frequently as central genotypes that in turn were approximately twice as frequent as single and triple locus variants.

Table 5.2. The composition of clonal complexes identified amongst 293 *C. jejuni* isolates from Starlings in terms of STs, genetic variant and isolation period over the study.

C= central genotype, SLV=single locus variant, DLV=double locus variant, TLV= triple locus variant.

[a]The relationship to the central genotype. Unassigned STs are clustered into groups that share four or more alleles.

[b]The greatest number of days between sampling occasions on which the ST was isolated.

Clonal complex	No. of isolates	ST	Complex variant[a]	freq	Isolation period(d)[b]
21	1	1383	DLV	1	1
42	1	42	C	1	1
45	8	45	C	5	385
		998	SLV	1	1
		1025	DLV	1	1
		334	DLV	1	1
48	2	38	DLV	2	1
177	71	177	C	46	668
		144	SLV	3	11
		563	SLV	2	1
		1004	SLV	3	140
		1014	SLV	1	1
		1382	SLV	2	197
		1482	SLV	1	1
		1500	SLV	1	1
		1506	SLV	1	1
		1535	SLV	1	1
		1381	DLV	2	2
		1485	DLV	1	1
		1533	DLV	1	1
		685	TLV	1	1
		1388	TLV	2	8
		1394	TLV	3	14
179	3	220	SLV	3	84
257	2	257	C	2	369
283	1	267	SLV	1	1
677	4	677	C	2	10
		1024	SLV	1	1
		1534	TLV	1	1
682	143	682	C	9	695
		1385	SLV	1	1
		1386	SLV	1	1
		1390	SLV	1	1
		1392	SLV	1	1
		1542	SLV	2	2
		686	DLV	15	709
		1019	DLV	1	1
		1020	DLV	63	35
		1021	DLV	4	9
		1022	DLV	13	176
		1027	DLV	13	356
		1391	DLV	1	1
		681	TLV	1	1
		687	TLV	1	1
		818	TLV	11	504
		1387	TLV	1	1
		1503	TLV	2	2
		1505	TLV	1	1
		1507	TLV	1	1
574	1	574	C	1	1
Un-assigned	48	683	cluster I	14	867
		1023	cluster I	9	380
		1481	cluster I	1	1
		1502	cluster I	1	1
		1613	cluster I	1	1
		999	cluster II	1	1
		1026	cluster II	1	1
		1483	cluster II	1	1
		1539	cluster II	1	1
		1536	cluster III	1	1
		1384	cluster III	1	1
		1609	cluster IV	1	1
		1612	cluster IV	1	1
		1537	cluster V	1	1
		1000	cluster V	1	1
		436	singleton	2	1
		684	singleton	1	1
		997	singleton	2	1
		1002	singleton	1	1
		1389	singleton	1	1
		1484	singleton	1	1
		1486	singleton	1	1
		1501	singleton	1	1
		1504	singleton	1	1
		1508	singleton	1	1

Table 5.3 The summary statistics for the genetic variants within the clonal complexes isolated amongst the 293 *C. jejuni* isolates from Starlings.

Complex variant[a]	Average isolation period (days)	Longest period of isolation (days)	Number of STs	Frequency of isolates
C	304	695	7	66
SLV	25	196	18	27
DLV	93	709	14	119
TLV	49	504	11	25
U	51	867	25	48

C= central genotype, SLV=single locus variant, DLV=double locus variant, TLV= triple locus variant.

[a]The relationship to the central genotype. U=STs that could not be assigned to a clonal complex.

5.2.7 Isolation of *C. jejuni* STs over time.

Although the number of isolates collected in 2003 was small, of the 75 STs identified, five were isolated during 2003 and 2004. These included ST-682 (ST-682 complex), ST-1027 (ST-682 complex), ST-818 (ST-682 complex), ST-177 (ST-177 complex), ST-1382 (ST-177 complex), ST-45 (ST-45 complex) and ST-179 (ST-179 complex). ST-682 was also isolated during 2002.

The composition of the ST-682, ST-177, ST-45 and ST-179 clonal complexes isolated over more than one month in 2004 varied with respect to ST (Table 5.3.). A total of 34 STs were identified of which only seven were isolated during more than one month.

Table 5.4 The *C. jejuini* clonal complexes isolated in more than one month during 2004. Figures given are the proportion of isolates. No data were available for February, April, July, August, September and October.

Clonal complex	ST	Month					
		Jan (n=8)	March (n=3)	May (n=12)	June (n=186)	Nov (n=28)	Dec (n=9)
682	1020			0.17	0.32		
	686				0.08		0.11
	1022				0.005	0.04	0.22
	1027				0.06		
	818				0.005		
	1021				0.002		
	682			0.17			
	1503				0.001		
	1542				0.001		
	1391	0.13					
	1385				0.005		
	1386				0.005		
	1019				0.005		
	1387				0.005		
	1390				0.005		
	1392				0.005		
	1505				0.005		
	1506				0.005		
177	177	0.25			0.10	0.5	0.44
	1381	0.50					
	1500				0.005		
	1388				0.001		
	563				0.001		
	144				0.02		
	1004				0.005	0.07	
	1394				0.001		
	1382				0.005		
	1485				0.005		
	1506				0.005		
	1533				0.005		
	1535						0.11
45	45		0.33		0.09		
	1026				0.005		
179	220	0.13	0.33				

5.2.8 The diversity of genotypes.

The diversity of genotypes isolated during 2003 and 2004 was calculated using the modified Simpson's Index of Diversity, a *D* value of 1.0 indicating that all genotypes within the population are different and a *D* value of 0.0 indicating that all genotypes with the population are the same (Hunter 1990) (Figure 5.7). The level of diversity was 0.96 in 2003 and 0.90 in 2004. The diversity of genotypes was calculated by month for 2004. Values greater than 0.64 were recorded throughout the year, but the highest values were recorded in May (0.95) and June (0.88), and the lowest in November (0.64) and January (0.75). Unfortunately monthly diversity amongst isolates for the other years could not be calculated since there were insufficient numbers of samples.

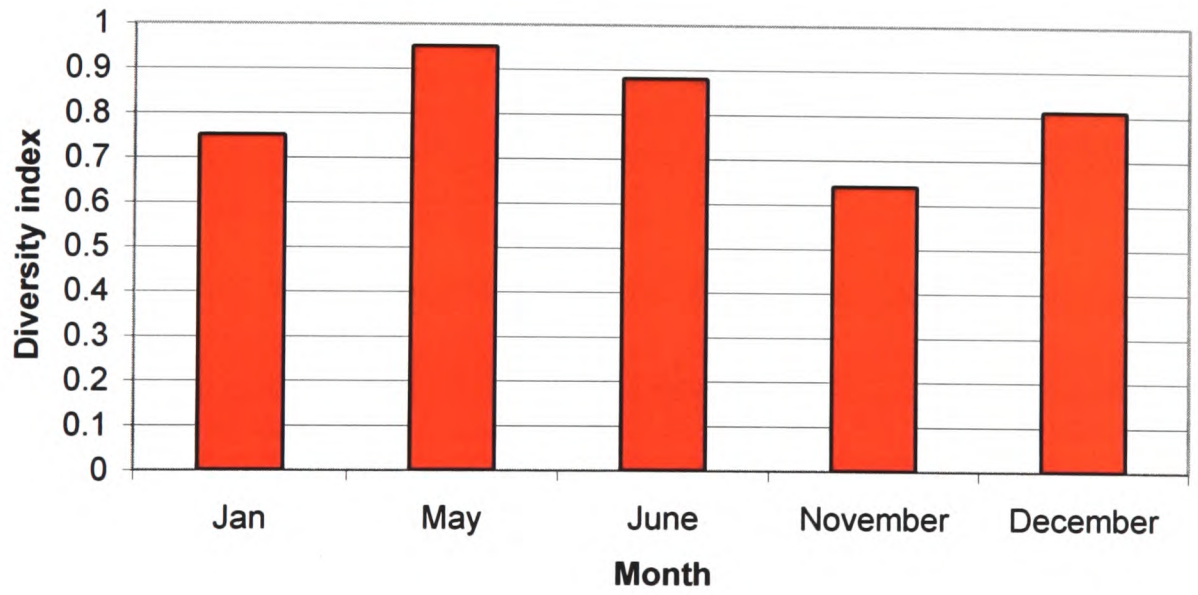


Figure 5.7. The seasonal peak in diversity of *C. jejuni* STs during 2004. No data was available for February, March, April, July, August, September and October.

5.2.9 Grouping of unassigned STs.

Fifteen of the 25 unassigned STs grouped into clusters of between two and five STs, based on sharing four or more identical alleles. The central genotype could not be identified and the number of variants was not sufficient to designate a clonal complex. Two large clusters of STs, and another containing more distantly related genotypes, can be seen on the Neighbour Joining tree suggesting that increased sampling may help to resolve them into clonal complexes (Figure 5.8).

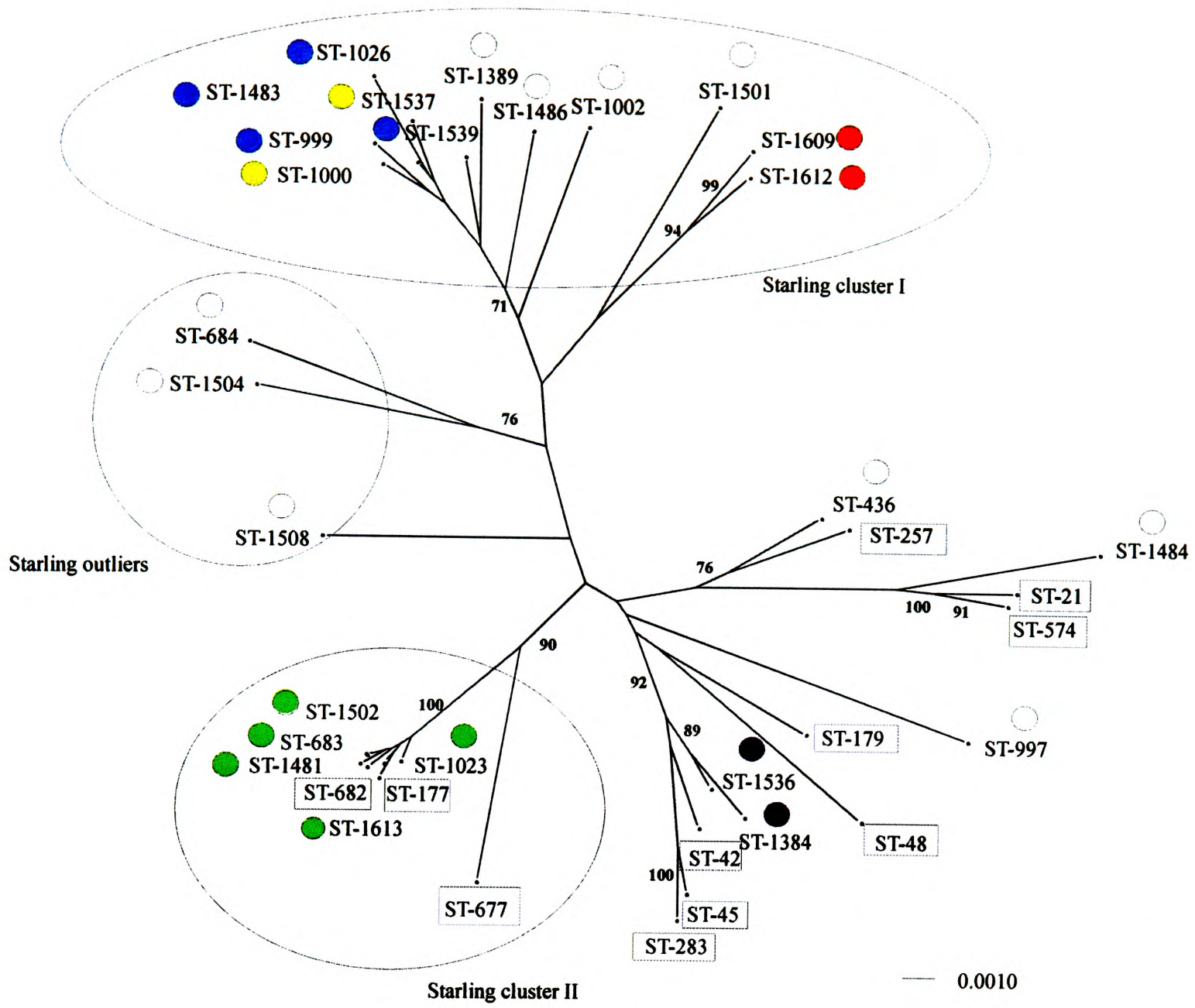


Figure 5.8. A Neighbour Joining tree showing the relationship of unassigned STs with the central genotype (shown in boxes) identified amongst *C. jejuni* isolates from Starlings (concatenated sequence). Bootstrap values are given where they exceed 70%. STs sharing four or more alleles are marked with the same colour, singleton STs are colourless.

5.2.10 Allelic diversity.

The number of alleles ranged from 14 at the *aspA* locus to 25 at the *tkt* locus (Table 5.4.). The number of variable sites varied from 16 (3.4%) at the *aspA* locus to 49 (9.8%) at the *pgm* locus. The d_N/d_S were all considerably below one indicating the absence of positive selection at each of the loci.

Table 5.5. The allelic diversity amongst *C. jejuni* isolates from Starlings.

Locus	Fragment size (bp)	No.of alleles	No. of variable sites	% variable sites	d_N/d_S ratio
aspA	477	14	16	3.4	0.011
glnA	477	20	30	6.3	0.014
gltA	402	24	31	7.7	0.018
glyA	507	21	43	8.5	0.029
pgm	498	21	49	9.8	0.031
tkt	459	25	42	9.2	0.026
uncA	489	17	28	5.7	0.014
All loci	3309	75	240	7.3	0.015

5.2.11 Antigenic diversity.

A total of 37 *flaA* SVR peptides, and 54 *flaA* SVR allele-peptide combinations were detected amongst the 291 *C. jejuni* isolates (Table 5.5.). There was evidence that *flaA* SVR was not randomly distributed amongst clonal complexes, but instead formed clustered associations (Figures 5.9 and 5.10). Clonal complexes were often associated with more than one *flaA* SVR type, the largest, ST-682 and ST-177 complexes having 19 and 16 different *flaA* SVR peptides each. In addition the same *flaA* SVR was sometimes shared between more than one complex, for example *flaA* SVR type 234-3 was associated with ST-177 and ST-677 complexes. Similarly there was evidence of non random but

mixed associations between ST and *flaA* SVR type. Sometimes STs from the same clonal complex had some *flaA* SVR types in common, but this was not always the case.

Table 5.6 The distribution of *flaA* SVR alleles amongst *C. jejuni* genotypes isolated from Starlings.

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant; [a]The relationship to the central genotype. Unassigned STs are clustered into groups that share four or more alleles. [b]Only the peptide allele could be obtained.

Clonal complex	ST	Complex variant[a]	freq	*flaA* SVR allele-peptide (proportion)
21	1383	DLV	1	37-1(1)
42	42	C	1	274-42(1)
45	45	C	5	21-2(0.4), 22-1(0.4), 2-27(0.2)
	998	SLV	1	21-2(1)
	1025	DLV	1	275-27(1)
	334	DLV	1	239-9(1)
48	38	DLV	2	41-4(1)
177	177	C	46	124-68(0.2),403-67(0.2),86-18(0.2),454-24(0.1),85-24(0.1) 322-19(0.1),234-3(0.02),400-43(0.02),404-128(0.02),460-142(0.02)
	144	SLV	3	86-18(0.67), 124-68(0.33)
	563	SLV	2	181-94(0.5), 457-141(0.5)
	1004	SLV	3	447-136(1)
	1014	SLV	1	322-19(1)
	1382	SLV	2	349-67(0.5), 400-43(0.5)
	1482	SLV	1	322-19(1)
	1500	SLV	1	77-56(1)
	1506	SLV	1	400-43(1)
	1535	SLV	1	86-18(1)
	1381	DLV	2	432-45(1)
	1485	DLV	1	2-27(1)
	1533	DLV	1	400-43(1)
	685	TLV	1	-24(1)b
	1388	TLV	2	80-48(0.5), 400-43(0.5)
	1394	TLV	2	461-46(1)
179	220	SLV	3	82-5(0.67), 68-5(0.33)
257	257	C	2	16-12(1)
283	267	SLV	1	239-9(1)
677	677	C	2	234-3(0.5), 400-43(0.5)
	1024	SLV	2	234-3(0.5), 400-43(0.5)
	1534	TLV	1	234-3(1)
682	682	C	8	62-29(0.88), -46(0.12)b
	1385	SLV	1	62-29(1)
	1386	SLV	1	400-43(1)
	1390	SLV	1	456-140(1)
	1392	SLV	1	81-46(1)
	1542	SLV	2	62-29(1)
	686	DLV	15	85-24(0.67), 400-43(0.13), -24(0.07)b, 86-18(0.07), 456-140(0.07)
	1019	DLV	1	No data(1)
	1020	DLV	61	400-43(0.8),81-46(0.05),124-68(0.03),80-48(0.02),85-24(0.02), 86-18(0.02),181-94(0.02),405-45(0.02), 448-137(0.02),449-18(0.02)
	1021	DLV	4	81-46(0.75), 85-24(0.25)
	1022	DLV	13	450-45(0.92), 451-138(0.08)
	1027	DLV	13	456-140(0.5), 86-18(0.08), 181-94(0.08), 349-67(0.08),NT(0.23)
	1391	DLV	1	68-5(1)
	681	TLV	1	-67(1)b
	687	TLV	1	-45(1)b
	818	TLV	11	04-128(0.55),85-24(0.1),86-18(0.1),181-94(0.1),186-95(0.1),447-136(0.1)
	1387	TLV	1	456-140(1)

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Table 5.6. *continued.*

Clonal complex	ST	Complex variant[a]	freq	*flaA* SVR allele-peptide (frequency)
682-continued	1503	TLV	2	124-68(0.5), 450-45(0.5)
	1505	TLV	1	404-128(1)
	1507	TLV	1	349-67(1)
574	574	C	1	8-1(1)
Unassigned	683	cluster I	14	405-45(0.3),341-18(0.2),-45(0.1)b,NT(0.1),62-29(0.1) 85-24(0.1),323-67(0.1),400-43(0.1),459-18(0.1)
	1023	cluster I	9	80-48(0.78), 81-46(0.22)
	1481	cluster I	1	80-48(1)
	1502	cluster I	1	341-18(1)
	1613	cluster I	1	186-95(1)
	999	cluster II	1	455-139(1)
	1026	cluster II	1	514-118(1)
	1483	cluster II	1	434-134(1)
	1539	cluster II	1	508-18(1)
	1536	cluster III	1	81-46(1)
	1384	cluster III	1	433-3(1)
	1609	cluster IV	1	No data(1)
	1612	cluster IV	1	372-18(1)
	1537	cluster V	1	504-155(1)
	1000	cluster V	1	399-118(1)
	436	singleton	2	413-131(1)
	684	singleton	1	-24(1)b
	997	singleton	2	306-93(1)
	1002	singleton	1	No data(1)
	1389	singleton	1	462-143(1)
	1484	singleton	1	348-15(1)
	1486	singleton	1	452-37(1)
	1501	singleton	1	No data(1)
	1504	singleton	1	400-43(1)
	1508	singleton	1	234-3(1)

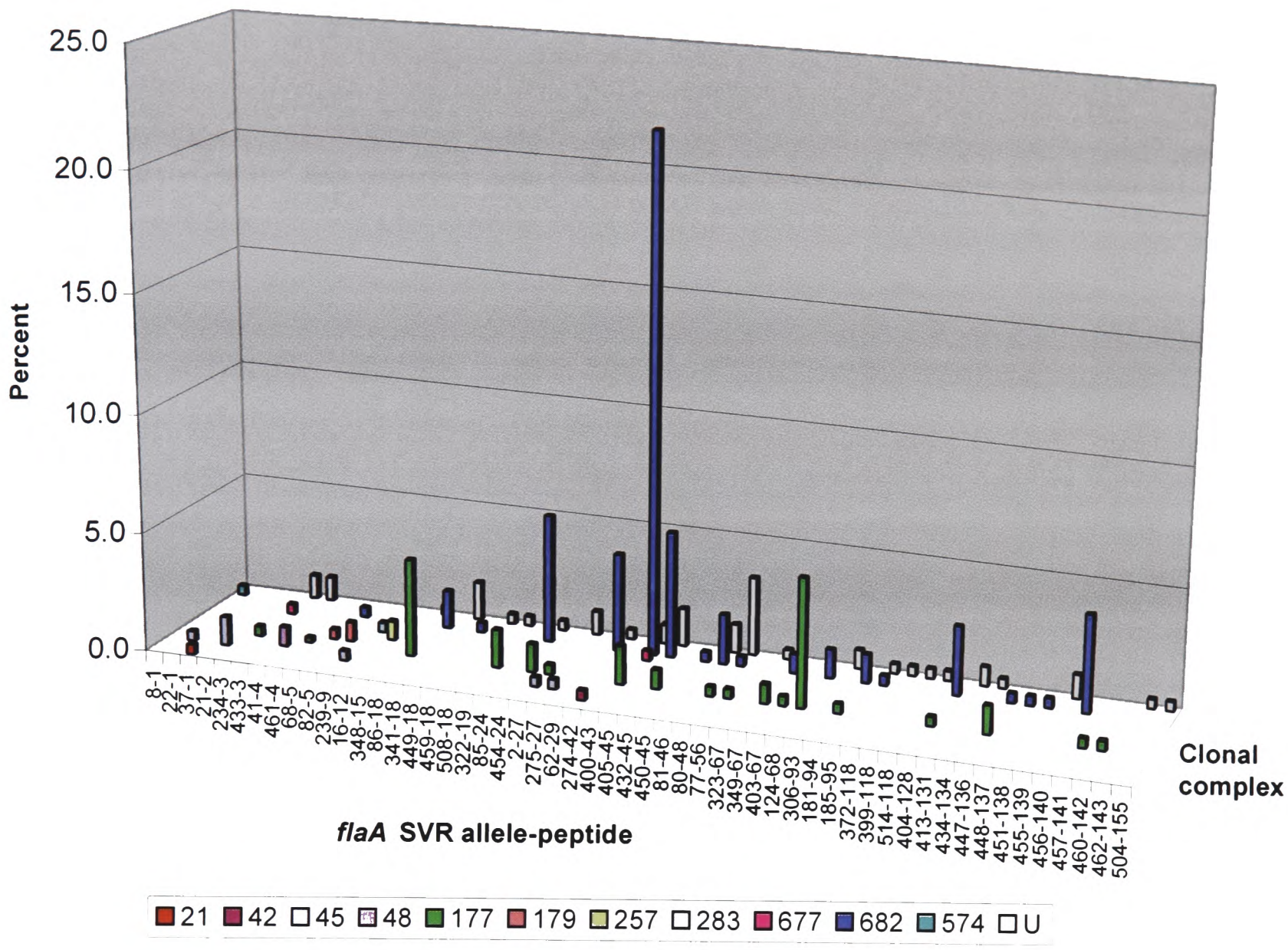

Figure 5.9. The structure of *flaA* SVR diversity amongst *C. jejuni* clonal complexes isolated from Starlings.

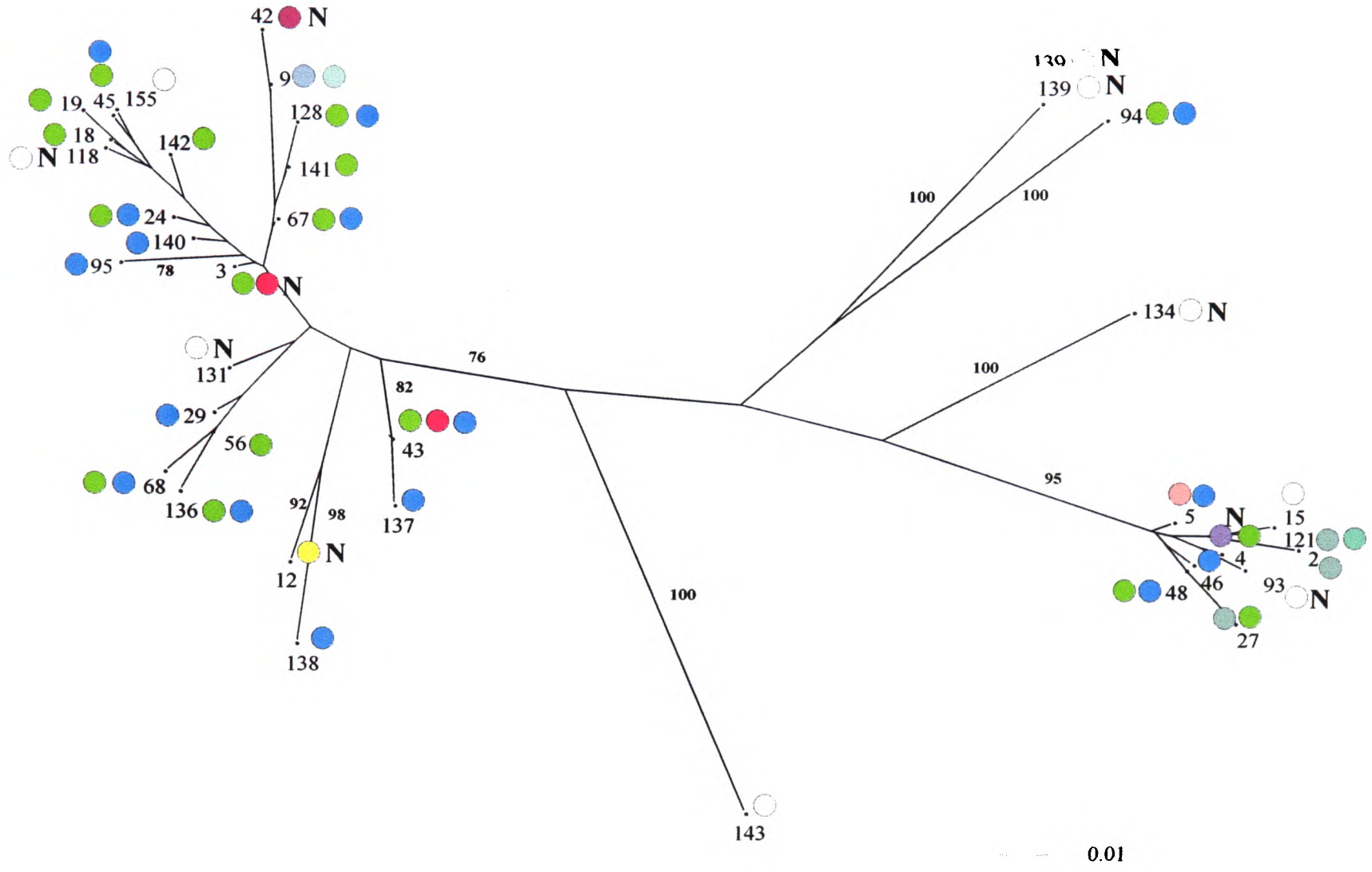

Figure 5.10. A Neighbour Joining tree showing the relationship between *flaA* SVR peptides and *C. jejuni* clonal complexes (concatenated sequence).

ST-42 complex aubergine; ST-45 complex pale blue; ST-48 complex purple; ST-177 complex bright green, ST-179 complex salmon pink; ST-257 complex yellow; ST-283 complex mint green; ST-574 complex aqua; ST-677 complex bright pink; ST-682 complex blue; Unassigned isolates white; *flaSVR* peptides isolated from Nestlings are represented by an N.

Variation of ST-*flaA* SVR combinations over a year.

ST-177 was the ST most frequently isolated from Starlings during 2004. Most of the ST-177 isolates were associated with different *flaA* SVR types (Figure 5.11). Only three flaA SVR types, 124-68, 403-67 and 86-18 were identified in more than one month. The remaining seven antigenic types were seen for the course of one month only. Other STs were not isolated in sufficient numbers to investigate variation over time in detail.

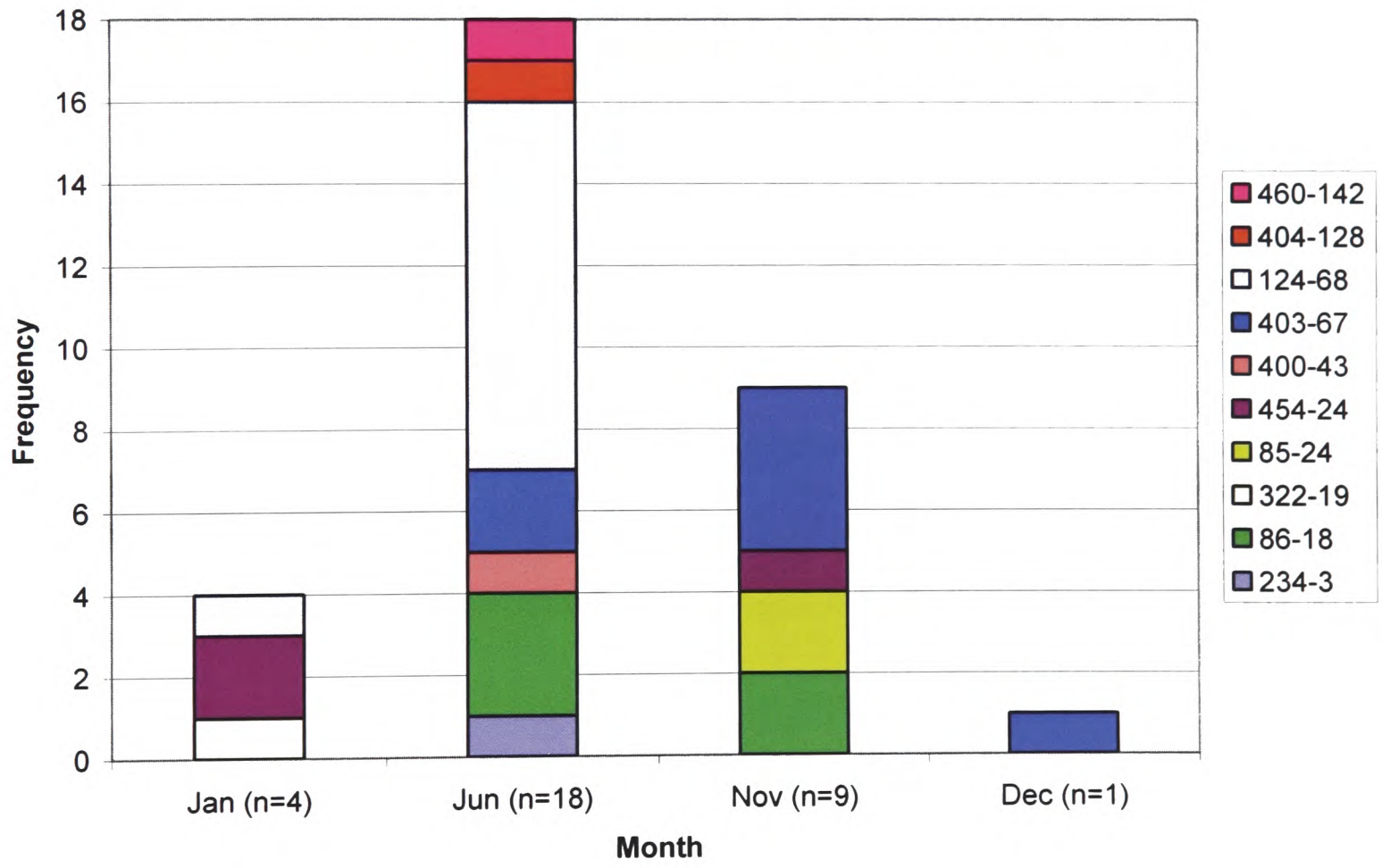

Figure 5.11 The *flaA* SVR types associated with ST-177 isolates from 2004. Data was not available for February, March, April, May, July, August, September and October.

5.2.12 Carriage of *Campylobacter* amongst re-captured Starlings.

A total of 192 Starlings were caught on more than one occasion, with one bird (ring number CL95058) being caught nine times. From these birds 199 *Campylobacter* isolates were obtained, of which 176 were *C. jejuni* and 23 *C. lari.* A total of 35 (18.2%) Starlings were shedding *Campylobacter* species on each sampling occasion (time period between sampling was between one and 588 days), 77 (40.1%) Starlings were negative for *Campylobacter* species on each occasion (time period between one and 364 days) and 80 (41.7%) Starlings switched between positive and negative status (time period between one and 392 days). *C. lari* was isolated from 16 (8.3%) of the re-captured Starlings. Eight of these switched between shedding and not shedding *C. lari* (time period six to 519 days), three were shedding *C. lari* on every occasion (time period one to 20 days), and five switched between *C. lari* and *C. jejuni* (time period one to 57 days).

The majority of *C. jejuni* isolates from Starlings positive on more than one occasion were different genotypes. The shortest time period between different genotypes was a day, seen in three birds. Up to four different genotypes were isolated from an individual Starling over a time period of nine days. There were six instances where the same genotype was isolated from an individual Starling more than once, two of which were sampled on consecutive days, three separated by two days and one separated by five days. Antigenic typing using the *flaA* locus revealed that four the six birds carried the same ST-*flaA* SVR strain on two consecutive occasions (Table 5.6). The remaining two birds, CW04696 and CW04766, carried the same ST on consecutive occasions separated by two and five days, but the *flaA* SVR had changed. The *flaA* SVR was unlikely to have changed by genetic drift since the two types in each of the birds differed by 16 and 31 of

321 nucleotides, with polymorphic sites scattered throughout the region. Two days was the longest time period over which an identical ST-*flaA* SVR clone was isolated from the same bird.

Table 5.7. Starlings with the same *C. jejuni* ST isolated on more than one occasion, with associated *flaA* SVR types.

Bird Id.	Date/ ST(clonal complex);flaA SVR-peptide			No. of days between identical isolates
	Isolate 1	Isolate 2	Isolate 3	
CT86854	2.6.04	4.6.04	-	2
	1020(682);400-43	1020(682);400-43	-	
CW04696	22.6.04	24.6.04	-	Not applicable
	1020(682);86-18	1020(682);400-43	-	
CW04712	24.6.04	25.6.04	-	1
	1020(682);400-43	1020(682);400-43	-	
CW04714	24.6.04	25.6.04	29.6.04	1
	1020(682);400-43	1020(682);400-43	1027(682)	
CW04766	17.11.04	19.11.04	24.11.04	Not applicable
	177	177;403-67	177;85-24	
CW04772	24.11.04	26.11.04	-	
	1022(682);450-45	1022(682);450-45	-	2

-; no further Campylobacters were isolated.

5.2.13 *Campylobacter* shedding amongst nestlings.

A total of 20 samples in 2003 and 81 samples in 2004 were collected from nestling Starlings when they were approximately nine days of age. Of these, 12 (11.9%) were positive for *C. jejuni*. No other *Campylobacter* species were isolated. Ten STs were identified and they were grouped into ST-42, ST-48 and ST-257 clonal complexes

with eight STs remaining unassigned. Only one of the ten STs from nestlings, ST-257, was isolated from the older Starlings. Pairwise F_{ST} comparing *C. jejuni* genotypes isolated from nestling and adult birds gave a value of 0.255 and p value <0.001 suggesting that the *Campylobacter* populations were significantly different. Splits tree analysis gave evidence of networking and recombination between the STs, although none of them had more than three alleles in common with each other (Figure 5.12). There were 140 of 3309 polymorphic sites identified amongst the concatenated sequences. The Neighbour Joining tree (concatenated sequence) gave evidence that the nestling STs are scattered amongst the clusters of STs isolated from adults (Figure 5.13)

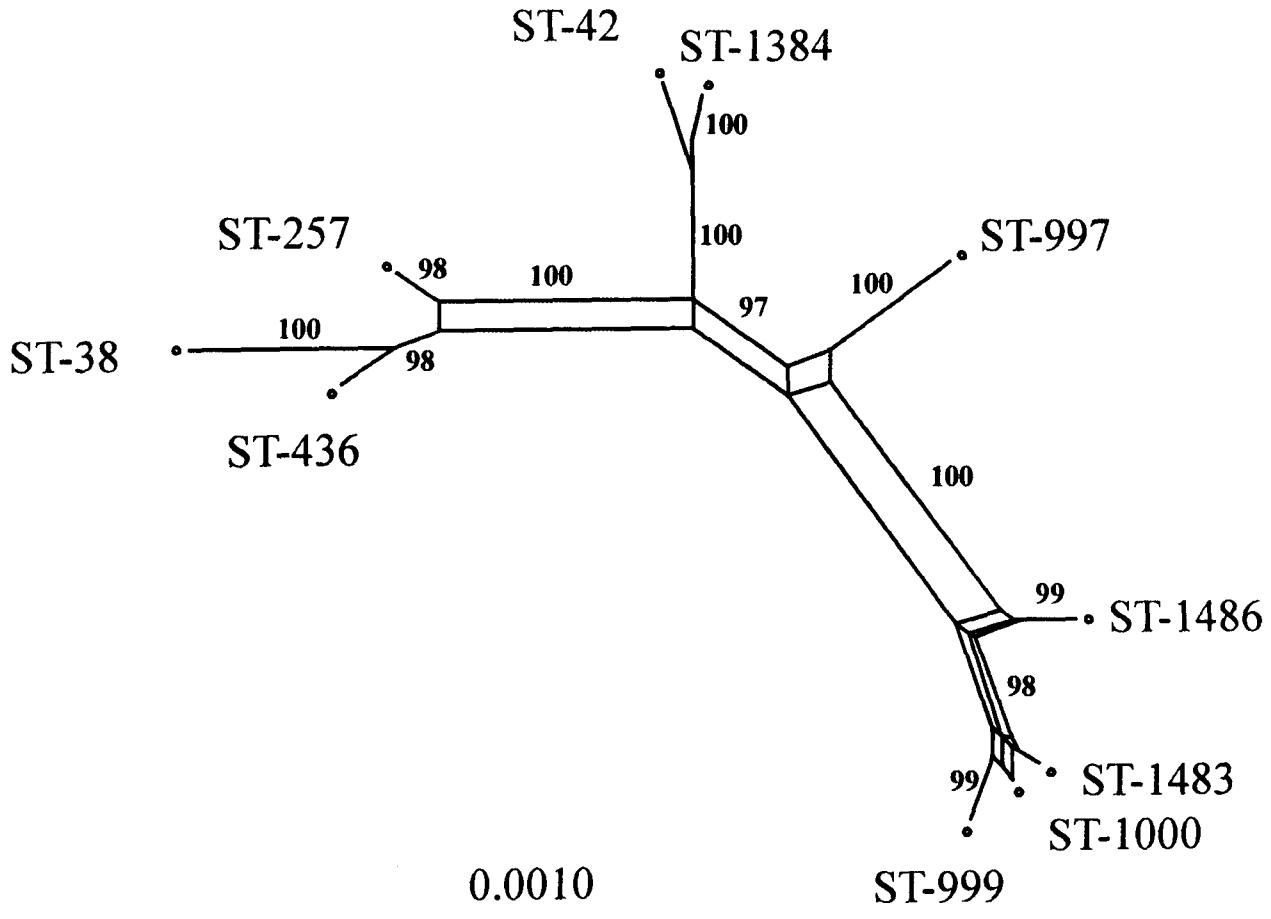

Figure 5.12. Splits graph demonstrating recombination between *C. jejuni* STs isolated from Starling nestlings (concatenated sequence). Bootstrap values are given where they exceed 70%.

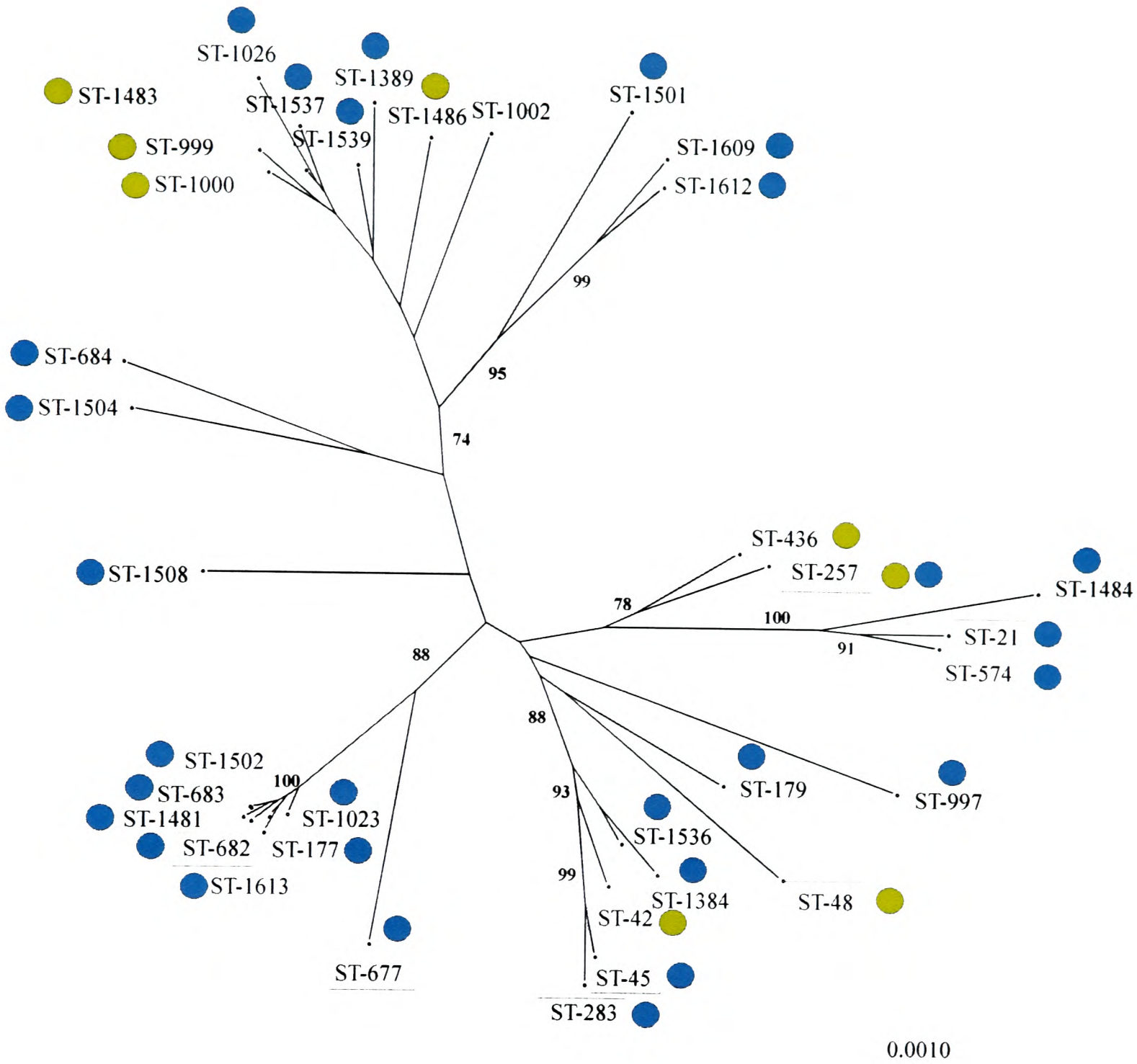

Figure 5.13. A Neighbour Joining tree showing the relationship of *C. jejuni* STs isolated from Starling nestlings (yellow) and adult birds (blue) (concatenated sequence). Clonal complexes are represented by the central genotype (shown in boxes), the remainder are unassigned. Boostrap values are given where they exceed 70%.

The 24 nests contained between two and six chicks, and averaged 4.0 chicks per nest (Figure 5.14). Nine of the 24 (37.5%) nests contained at least one chick shedding *C. jejuni*. Seven of the nine (78%) 'positive' nests contained only one chick shedding *C. jejuni,* one nest (nest 3, 2003) contained two of four chicks shedding *C. jejuni* and one nest (nest 32, 2004) contained at least three of four chicks shedding *C. jejuni* – no sample could be obtained from the fourth chick in the nest. The *C. jejuni* isolates from the positive siblings in nest three in 2003 had identical ST-*flaA* SVR alleles, and of the isolates from the three positive siblings in nest 32, 2004, two had identical ST-*flaA* SVR alleles and the third had a different and unrelated ST. Two nestlings from 2003 were sampled at day 15. One, from the nest 2 that contained no chick shedding *Campylobacter* at nine days was still negative at 15 days. The other, from nest 6 with three nestlings that were shedding *Campylobacter* was not sampled at day nine, but was negative at day 15. The *flaA* SVR types isolated from nestlings were unusual, with eight of ten being isolated from nestlings only, but they were distributed amongst the other *flaA* SVR types isolated from adult birds and did not form a related cluster on their own. (Figure 5.10.)

The distribution of *C. jejuni* positive nests and chicks was random with respect to location and year. An odds ratio was calculated to determine if nest size was predictive of *C. jejuni* colonization giving a value of 0.58, a 95% confidence interval of 0.29-1.13 and Z value of –1.61. There was evidence to suggest a slight negative correlation, with smaller nest sizes being more likely to contain chicks shedding *C. jejuni* (Table 5.7).

		Chick					
	Nest	1	2	3	4	5	6
				2003			
Farm	44						
	6	1486(U) 452-37	997(U) 306-93	No sample	997(U) 306-93		
	32						
	27						
	2						
	20		38(48) 41-4				
				2004			
Farm	14				257(257) 16-12		
	23						
	6						
	20				1384(U) 433-3		
	32	436(U) 413-131	436(U) 413-131				
	44						
	47						
	35		42(42) 274-42				
Sawmill	6	No sample	No sample				No sample
	3				1483(U) 434-134		
	11						
	19				999(U) 455-139		
	24						
	38						
	4	1000(U) 399-188					
	22						
	23						

Figure 5.14. Distribution of *C. jejuni* STs (clonal complex) and *flaA* SVR type amongst Starling chick siblings at nine days of age.

U=STs that could not be assigned to a clonal complex. Blank cells = negative for *Campylobacter.*

Table 5.8. The number of chicks shedding *Campylobacter vs* nest size; data used to calculate the odds ratio to determine whether or not nest size is predictive of *Campylobacter* status.

Nest size (no. of chicks)	*Campylobacter;* no.(proportion) of chicks Negative	Positive	Total no. of chicks reared within nest size
2	3(0.75)	1(0.25)	4
3	8(0.67)	4(0.33)	12
4	40(0.91)	4(0.09)	44
5	28(0.93)	2(0.07)	30
6	5(0.83)	1(0.17)	6
Total	84(0.88)	12(0.12)	96

5.2.14 Correlation of *Campylobacter* shedding with the Starling variables of age, sex, weight and wing length.

The correlation between *Campylobacter* shedding and host variables was tested using *z* tests (standard normal deviate) that test if the means of the age, weight, and wing length of birds with *Campylobacter* positive samples were the same or different to the means of those relating to birds with *Campylobacter* negative samples. Recaptured birds with multiple isolates were discarded from the analysis. The average age of Starlings with positive isolates was 3.15, and with negative isolates 3.59. The *t* test (-20.07) gave a *p* value of <0.0001, demonstrating that there was a significant age effect, with younger birds being more likely to shed *Campylobacter*. With 95% confidence, the reduction in mean age was between 0.27 and 0.61 for those Starlings with positive isolates compared to those with negative isolates. The average weight of Starlings that were shedding *Campylobacter* was 75.2g and the average weight of Starlings that were not shedding *Campylobacter* was 79g. The *t* test (-8.33) for the differences in means in bird weight

gave a *p* value of <0.0001 showing there was also a significant weight effect, with lighter birds being more likely to shed *Campylobacter*. With 95% confidence there was a reduction of between 2.92 and 4.68g in the mean weight of birds shedding *Campylobacter* compared to those where not. The average wing length of Starlings with *Campylobacter* was 126.7mm, and 129.9mm without. The *t* test (-9.94) gave a *p* value of <0.0001, showing there was a significant wing length effect, with birds having a shorter wing length being more likely to shed *Campylobacter*. With 95% confidence the reduction in wing length in *Campylobacter* positive birds was between 2.7 and 3.6mm, compared to *Campylobacter* negative birds. Since the age, wing length and weight variables were likely to interlinked, for example older birds being bigger and heavier, logistical regression analysis was performed to determine which was the most influential variable. Age proved to be the most significant factor with *p* value of <0.001 (odds ratio 0.50), although there was still some effect by weight (odds ratio 0.96, *p* 0.108) and wing length (odds ratio 0.94, *p* 0.048). More specifically, juvenile birds (age code 3) were significantly associated with *Campylobacter* colonization (odds ratio 1.69), with older birds becoming progressively less associated (Figure 5.15).

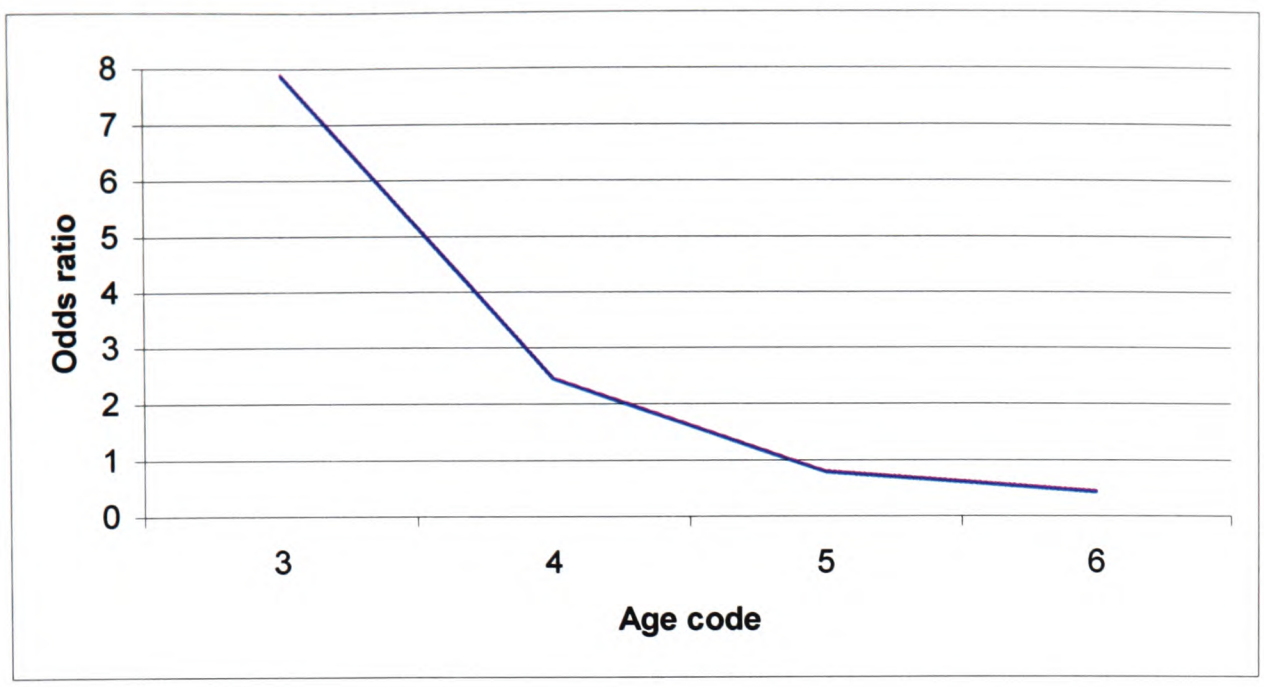

Figure 5.15. Odds ratios give evidence that isolation *Campylobacter* is significantly associated with juveniles (age code 3).

The effect of sex was tested using contingency tables and the χ^2 test of significance (Table 5.7). The χ^2 value comparing sex was 0.29, with p value greater than 0.5, giving no evidence of a sex effect on *Campylobacter* status.

Table 5.9. The contingency tables comparing sex and *Campylobacter* status.

Observed

	Campylobacter		
	Positive	**Negative**	**Total**
Male	48	209	257
Female	53	205	258
Total	101	414	515

Expected

	Campylobacter		
	Positive	**Negative**	**Total**
Male	50.4	206.6	257
Female	50.5	207.4	258
Total	101	414	515

5.2.15 Comparison of *C. jejuni* isolates from Starlings with those from other sources.

The distribution of clonal complexes was compared with those identified amongst the Oxfordshire geese, farm and environmental isolates from Lancashire from Chapters 3 and 4 (Figure 5.16). The original population sample set of human disease isolates was used for comparison since temporally matched isolates were unavailable. Five of the 11 clonal complexes, isolated from Starlings, namely ST-257, ST-21, ST-45, ST-48 and ST-42 complexes, overlapped with those isolated from the other sources. ST-21 complex was isolated from all of the sources, ST-257 complex was isolated from all sources except cattle, ST-45 complex was isolated from all sources except sheep, ST-48 and ST-42 complexes were isolated from Starlings, cattle and sheep. Within the overlapping clonal complexes four STs were identified amongst the Starling isolates and other sources. They included ST-257 (ST-257 complex) isolated from Starlings in Lancashire, poultry and human disease, ST-45 (ST-45 complex) isolated from cattle, poultry and human disease, ST-38 (ST-48 complex) isolated from cattle and sheep, and ST-42 (ST-42 complex) isolated from cattle and sheep. Six clonal complexes, ST-177, ST-179, ST-283, ST-677, ST-682 and ST-574 complexes were isolated from Starlings only, with ST-682 and ST-177 complexes showing predominance.

A total of 60 of the 75 STs isolated from Starlings were unique to this study. Ten had been previously isolated from cases of human disease and were recorded on the *Campylobacter* MLST database. Four, ST-42 (ST-42 complex), ST-45 (ST-45 complex), ST-38 (ST-48 complex) and ST-257 (ST-257 complex) have been isolated world wide over a number of decades. More specifically, ST-42 was isolated in Europe, the USA

and Australia in 1961-2002, and included isolates from cases of MF and GBS. ST-45 was isolated in Europe, North and South America and Australia in 1982-2003, ST-38 was isolated in the UK and USA in 1983-2001 and ST-257 was isolated in the UK and Australia in 1990-2001. ST-267 (ST-283 complex), ST-436 (unassigned) and ST-1384 (unassigned) have been isolated from across Europe from 1981-2004. ST-563 (ST-177 complex) and ST-677 (ST-677 complex) have been isolated from the UK only from 1981-2001. Three STs, ST-177 (ST-177 complex), ST-144 (ST-177 complex) and ST-220 (ST-179 complex) had been previously isolated from sand in the UK during 1994. The neighbour joining tree using concatenated sequence (Figure 5.17) indicates that complexes isolated from Starlings only, form a separate cluster, far removed from the cluster of geese associated genotypes. A minority of the genotypes from Starlings were isolated from other host sources also.

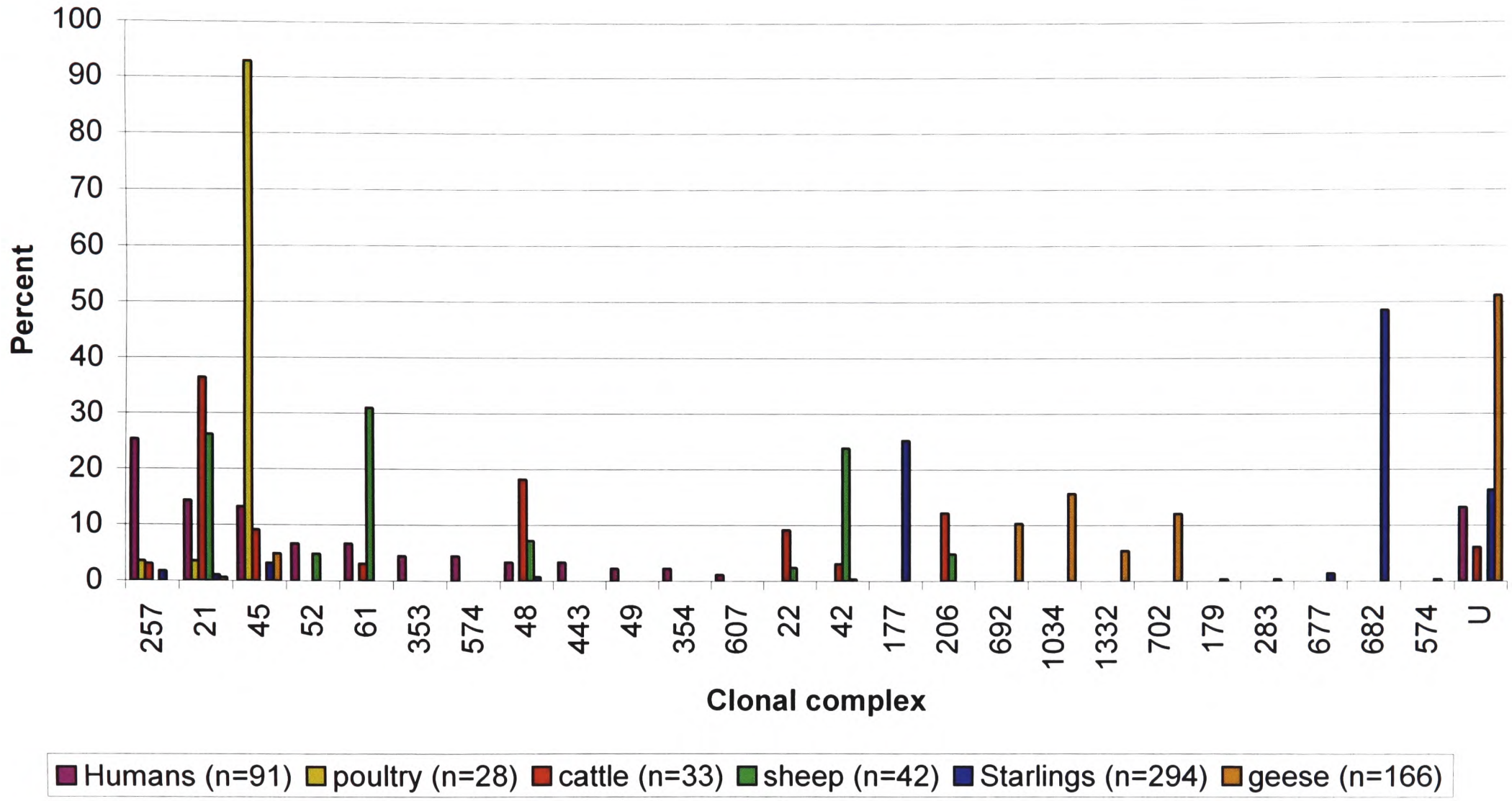

Figure 5.16. Distribution of *C. jejuni* clonal complexes amongst isolates from Oxfordshire Starlings and the Oxfordshire geese and Lancashire farm and environment isolates from Chapters 3 and 4.

The human disease isolates were every tenth isolate through the door of the Preston reference laboratory in 1999. U=STs that could not be assigned to a clonal complex.

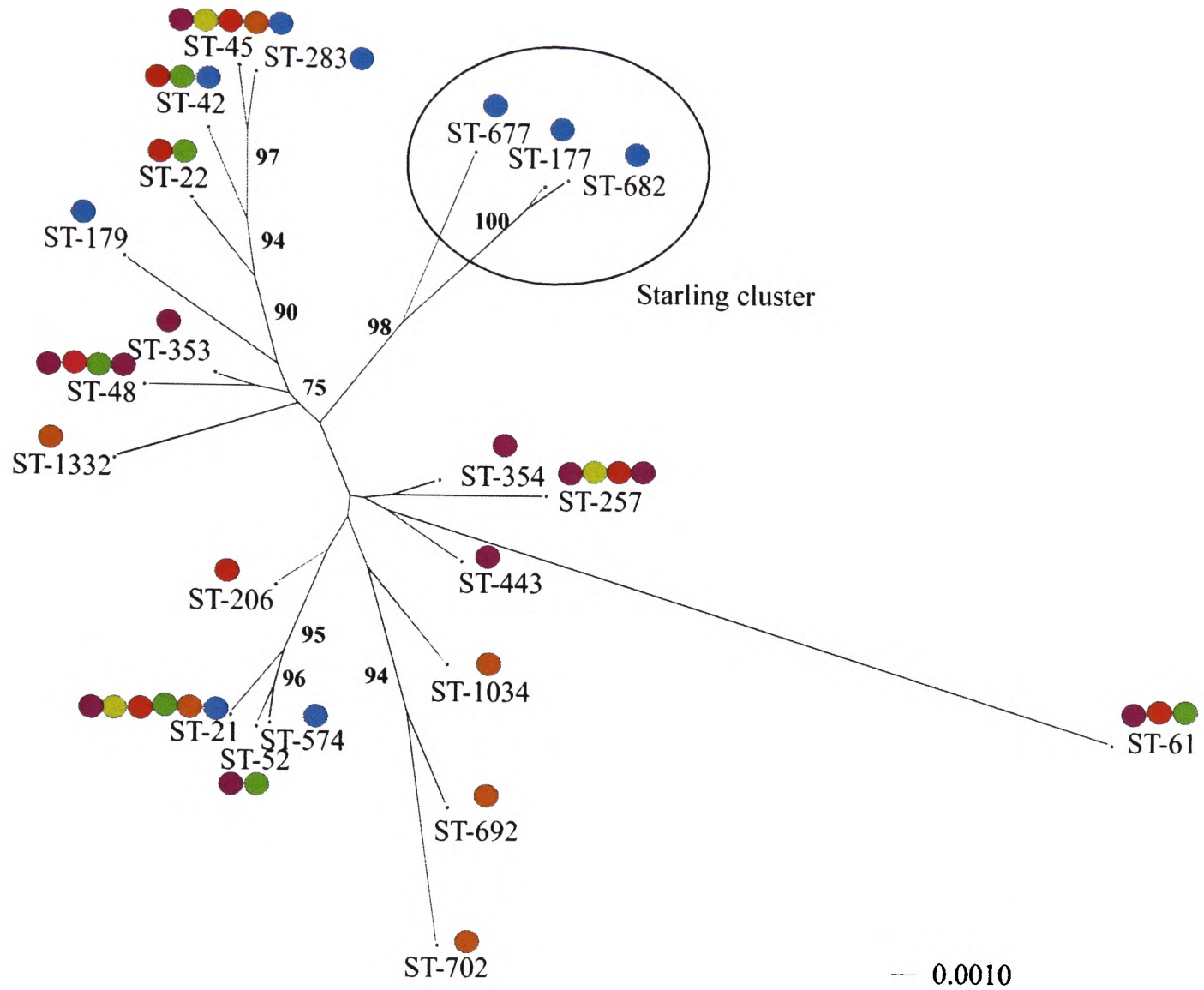

Figure 5.17. A Neighbour Joining tree showing the distribution of clonal complexes amongst *C. jejuni* isolates from Starlings and geese, farm animals and human disease (concatenated sequence).

Purple= human isolates, yellow= poultry isolates, red = cattle isolates, green = sheep isolates, blue = starling isolates, orange = geese isolates.

5.2 Discussion.

Three thermophillic *Campylobacter* species, *C. jejuni*, *C. coli* and *C. lari* were isolated from the Starlings using the Exeter enrichment method of culture. Although the media were not specifically developed for growth of *C. lari,* the growth of all *Campylobacter* species was relatively poor compared to isolates from other samples, and suggests that the media and/or culture conditions could be optimised further in the future. The carriage rates of 30.4% *C. jejuni* and 6.2% *C. lari* amongst the Starlings captured in this study, were similar to rates found by Kapperud and Rosef (1983) who isolated *Campylobacter* from 28.4% of mixed species of wild birds, Leuchtefeld *et al.* (1980) who isolated *C. jejuni* from 35% of ducks, and Brown *et al.* (2004) who isolated *C. lari* from 7% of wild birds. Whilst the proportions of *C. jejuni* and *C. lari* appeared to be consistent from year to year, there was evidence to suggest that carriage of *C. jejuni* was highest in June and July whilst *C. lari* was more common in the winter months. Other studies have found carriage rates amongst wild birds to vary between 2.6% and 89.9% depending on the time of year, the bird species and their associated environment and feeding habits (Luechtefeld *et al.* 1980; Chuma *et al.* 2000; Broman *et al.* 2002; Waldenstrom *et al.* 2002; Devane *et al.* 2005). Highest levels have been found amongst Gulls and Crows scavenging on rubbish dumps, and lowest levels have been found amongst insectivores and granivores (Kapperud and Rosef 1983; Waldenstrom *et al.* 2002). The low and erratic carriage of *C. coli* (0.6%) amongst Starlings in this study may be accounted for with evidence from other studies that *C. coli* is predominant amongst aquatic and marine birds (Waldenstrom *et al.* 2002). In addition, a *C. coli* clone potentially adapted to survival in water has recently been identified (Kemp *et al.* 2005).

A minor modification to the primers for the *aspA* locus, and a change in the DNA extraction method from boiled cell suspensions to Isoquick preparations, were required for the MLST method, but it otherwise was unchanged allowing direct comparison with genotypes isolated from other sources. The *C. jejuni* genotypes isolated were diverse, with 75 different STs identified amongst 293 isolates. They clustered into 11 clonal complexes of which two, ST-682 complex and ST-177 complex, were predominant amongst the Starlings both in frequency and number of genetically related variants. They also showed temporal stability and were the only clonal complexes to be identified over three years, 2002-2004. There was some evidence that the ST-682 complex was more common than ST-177 complex in the summer and the ST-177 complex more common than the ST-682 complex in the winter.

ST-1020 (ST-682 complex) was the most frequently isolated ST (21.5% of isolates), but was only identified during 2004 and is possibly a reflection of sampling bias during this year. Many of the birds caught during June 2004 were feeding on an open sack of chick feed that was in the near vicinity and became contaminated with bird faeces. This may have helped to increase the local transmission of ST-1020 during June, although the situation is probably not usual with regards to the birds feeding behaviour. A further four STs within the ST-682 complex, ST-686, ST-818, ST-1022 and ST-1027 were isolated in roughly equal proportions of 3.75% to 5.12%. ST-1022 was isolated during 2004, but the others were isolated during 2002 and 2003. Thus whilst ST-682 complex was prevalent amongst the Starlings, the actual STs varied from year to year, perhaps through local clonal expansion. In contrast, ST-177 (ST-177 complex), the second most frequently isolated ST (15.7% of isolates) was the central genotype, as well

as the most common genotype of the ST-177 clonal complex, with the remaining STs accounting for less than 4% of isolates. ST-177 itself may be particularly successful at surviving within the environment since it was the ST isolated most consistently throughout 2004 and has also been isolated from wild birds in Sweden (Waldenstrom *et al.* 2005a).

The differences in ST distribution within the clonal complexes suggested that ST-682 complex and ST-177 complex have been established within Starlings longer than the others. The ST-682 complex appeared to have undergone greater clonal expansion, at least amongst Starlings in Oxfordshire, since it had a large proportion of double and triple locus variants, and the central genotype was isolated in almost equal proportions to three double locus variants and one triple locus variant. In contrast ST-177 complex had a larger proportion of single locus variants but fewer double and triple locus variants. In addition the central genotype of the ST-177 clonal complex was significantly more common that the other variants within the complex, suggesting that there has been less evolution and genetic drift. Alternatively, ST-177 may just be a more successful genotype than ST-682.

The clonal complex model gave a give a good indication of the underlying population biology of *Campylobacter* in the Starling host and fits with the partially clonal structure identified by other studies using MLST and serotyping (Waldenstrom *et al.* 2005a). The central genotypes were isolated over a prolonged period of time, with the exception of ST-42 (ST-42 complex) which may be a rare genotype within Starlings. The number of single locus variants was greater than the number of double locus variants

which in turn was greater than the number of triple locus variants which fits with the model.

The majority of STs (61 of 75, 81.3%) from various clonal complexes were isolated from Starlings only. None of the STs had been isolated from the large numbers of wild geese sampled at the same site suggesting that mixing of the genotypes between the wild bird species was negligible, perhaps due to the different ecological niches they fill, or that one host species was immune to the others *Campylobacter* genotypes. It seems likely that the genotypes within the ST-682 and ST-177 clonal complexes may be specifically adapted to survival in Starlings and their environment, and interrogation of the *Campylobacter* MLST database also revealed a predominance of isolates from Starlings and environmental samples from sand, with occasional isolates from other wild bird species. Other studies have found evidence of avian-associated genotypes amongst poultry and wild bird species such as Thrushes and Mallards, and results from this study are consistent with those of Waldenström *et al* (2005a) who found *Campylobacter* genotypes to be differentiated by species of wild bird (Stanley and Jones 1998, Colles *et al.* 2003).

A relatively high proportion (25 of 75 STs accounting for 16.4% of isolates) remained unassigned. Other studies also found a high proportion of unassigned isolates from environmental and wild animal samples (Waldenstrom *et al.* 2005a). This is likely to reflect the predominance of genotypes isolated from human disease and meat on the *Campylobacter* MLST database upon which the majority of the clonal complexes have been established. There is evidence that *C. jejuni* genotypes amongst environmental and wild animal isolates are often different to those from human disease and thus they are

likely to form different clonal complexes (Petersen *et al.* 2001a; Broman *et al.* 2002; French *et al.* 2005; Meerburg *et al.* 2006). Evidence from this study suggests that more than half of the unassigned isolates formed related clusters and it is possible that more sampling may help to resolve new clonal complexes and identify the central genotype. Alternatively, since BURST, a programme used to identify clonal complexes does not directly take into account the number of polymorphisms and therefore the distance of the relationship between genotypes, a more refined model may be required to establish clonal complexes. It is possible that the Starlings acquired the unusual *C. jejuni* genotypes, particularly the singleton unassigned genotypes, at low levels and from diverse environmental sources.

Sequence typing of the *flaA* SVR was less discriminatory than MLST giving 54 alleles compared to 75 STs, but proved useful in adding another layer of differentiation to genotypes clustered with MLST. As seen in the previous chapters the antigen type appeared to be loosely associated with genotype but relationships were not exclusive. Certain *Campylobacter* strains identified by identical ST and *flaA* SVR allele appeared to be stable over a period of at least 11 months. Some STs, e.g. ST-177 were isolated in high frequency but antigen typing suggests that they probably originated from numerous different sources rather than being a 'super clone' that is homogenous within the Starling population.

There was a marked seasonality both in shedding and diversity of *C. jejuni* isolates from this study, with highest levels of both occurring during the late spring/early summer months. This is consistent with the peak in human disease in temperate countries, but in contrast to findings by Waldenström *et al* (2002) who found no seasonal

effect with Gulls, and only limited effects with Starlings, ducks, geese and Blackbirds. In a separate study Broman *et al* (2002) did find a pronounced seasonal variation in carriage rates amongst Gulls, with highest numbers being isolated in autumn months, and Sopwith *et al* (2003) found human disease incidence associated with bird pecked milk to be highly seasonal with greatest numbers occurring in May and June. The differences in the findings could perhaps be explained by the different bird species and sampling methods, for example Broman *et al* (2004) sampled birds on migration and it is possible that lingering and mixing with large numbers of birds at stop over points promoted colonization by *Campylobacter*.

The spring peak seen in this study and in that by Sopwith *et al* (2003) could be linked with increased feeding activity associated with raising offspring, together with warmer temperatures allowing better survival of the bacteria. This is supported by Stanley and Jones (1998) who noticed an increase in bird activity amongst sheds on a farm during June. Starlings are typically ground feeding birds and a greater variety of food, perhaps with differing levels of *Campylobacter* contamination, would be available during the warmer months when the soil is not frozen. This may also explain the increased diversity of *Campylobacter* genotypes during the summer months, although no particular genotype was associated with increased carriage, consistent with results gained by Sopwith *et al* (2003). The spring peak in carriage could also be partly explained by the increase in young birds at this time, since there was evidence that *Campylobacter* carriage was significantly more common amongst the juvenile age group. The most likely explanation is that of immature host immunity, but the findings contrast with those of Waldenström *et al* (2002) who detected no age effect in passerine birds, and the

opposite effect in shorebirds where adults tended to have the higher prevalence of *Campylobacter*. Again this may reflect the different bird species and their differing ecology as well as sampling methods and sample size.

There was some association of bird weight and to a lesser extent wing length with *Campylobacter* carriage, although both factors are largely interlinked with age. The smaller birds were more likely to be colonized by *Campylobacter*, which may be linked with host immunity or general health status, though there is no evidence that *Campylobacter* colonization has any effect on the health of wild birds or vice versa. This in contrast to findings by Waldenström *et al* (2002) who found that migratory birds with increasing body mass were more likely to colonized. Some human studies report differences in the susceptibility of different sexes to *Campylobacter* but there appeared to be no effect of sex on *Campylobacter* carriage amongst the Starlings (Louis *et al.* 2005). Similarly Waldenström *et al* (2002) found no effect of sex amongst several different species of wild bird.

A total of 192 birds were captured on more than one occasion. Equal proportions (40 and 42%) of birds were either negative for *Campylobacter* species on each occasion, or switched between positive and negative, a similar finding to that by Waldenström *et al* (2002). Fewer (17.7%) were positive on each sampling occasion, but the time period between sampling was up to 392 days. Some Starlings were colonized by both *C. jejuni* and *C. lari* indicating that colonization by one species did not infer immunity to the other. The majority of Starlings were colonized by different *C. jejuni* genotypes on each occasion implying that colonization by one genotype did not infer immunity to another. Three birds had different genotypes on consecutive days suggesting that they carried

mixed *Campylobacter* populations or that turnover of genotypes was extremely rapid. Six Starlings, re-captured over a period of two to five days, were colonized by the same ST on each occasion. Of these, four birds were colonized with identical ST-*flaA* SVR strains on each occasion, but two birds had different ST-*flaA* SVR strains on each occasion. The change in *flaA* SVR allele was unlikely to have arisen from genetic drift but could possibly have arisen by exchange of the *flaA* SVR allele amongst STs within a mixed population. The most likely explanation is that mixed cultures were sequenced since it proved extremely difficult to separate then on occasions. Unfortunately the propensity of an individual to be positive, the precise timescale of carriage, or the rate of loss of particular genotypes could not be determined from the data, but the model of multiple carriage, and/or rapid replacement of genotypes was upheld and the model of long term carriage of single genotypes rejected. Broman *et al* (2002) similarly found a rapid turnover of colonizing strains in Gulls, and Waldenström *et al* (2002) found urease positive thermophilic *Campylobacter* (UPTC) strains to be frequently acquired and lost by Redshank. More data are required to determine whether birds become immune to individual genotypes, but none of the birds sampled more than two days apart in this study were colonized by exactly the same genotype.

The presence of Starling nestbox colonies on the farm allowed the unique study of *Campylobacter* populations in nestling wild birds. The nestlings from 24 nests were sampled in April 2003 and April 2004 when they were approximately nine days of age. Ten of the 81 nestlings were positive for *C. jejuni* with no apparent ill effects. The finding contrasts with numerous studies of young poultry chicks that rarely become colonized before 21 days of age in the 'natural' setting. Reasons for this are unknown,

but early protection of poultry chicks is likely to be afforded by maternal antibodies or competing gut flora (Sahin *et al.* 2003). Studies have shown that poultry chicks may be experimentally infected as young as one day, although precise effects are unknown (Hald *et al.* 2001). The majority of siblings of *Campylobacter* positive nestlings did not appear to be colonized, despite being in close contact with each other in the nest. A nest containing a chick shedding *Campylobacter* was not any more likely to contain a second chick shedding *Campylobacter.* In contrast, high numbers of poultry within a flock become colonized with *Campylobacter* very rapidly after it has first been detected. The difference could perhaps be explained by their different living conditions. The Starling nests are kept clean from faeces by the parents who remove the faecal sacs produced by the chicks, and thus a potential source of contamination is avoided. In addition they are fed a more varied and natural diet in comparison with farmed poultry chicks, which may help to promote competitive gut flora and protect the Starling nestlings that are not yet colonized. In contrast to farmed poultry, it seems likely that *Campylobacter* infection is introduced to Starling chicks on an individual basis through contaminated feed provided by their parents. Two nests contained two chicks colonized by indistinguishable *Campylobacter* genotypes and it cannot be certain whether they infected each other, or were exposed to the same external source. In contrast, farmed poultry chicks are not kept scrupulously clean and their coprophagic behaviour could promote the rapid spread of *Campylobacter* throughout the flock. The feed is manufactured although it is very unlikely to be a source of *Campylobacter,* the diet unvaried, which may influence the gut flora and natural immune defense mechanisms of the poultry chicks (Evans and Sayers 2000; Newell and Fearnley 2003).

The *Campylobacter* genotypes isolated from the nestlings were diverse, suggesting they had originated from a number of different sources. The two Starling associated clonal complexes, ST-682 and ST-177 complexes, were not isolated and F_{ST} results suggested that nestling genotypes were significantly different to those isolated from adult birds, although they are not so unusual that they form separate clusters of genotypes. The result was probably largely influenced by the fact that three 'human-like' STs were isolated. This may be a reflection of feed availability, season, or a stochastic effect resulting from a relatively small sample of isolates from the nestlings. It is possible that nestlings raised on the farm would be fed with local pickings, and that the 'human-like' genotypes originate from faecal contamination by farm animals.

Comparison of the *C. jejuni* genotypes isolated from Starlings in this chapter with those isolated from geese, farm and environmental isolates in Chapters 3 and 4 revealed that five clonal complexes overlapped. Genotypes isolated from Oxfordshire Starlings clustered with those isolated from Lancashire Starlings and poultry in ST-257 complex, ST-21 complex and ST-45 complex. Genotypes from the ST-177 complex were isolated from both Oxfordshire and Lancashire Starlings but the ST-682 complex, predominant amongst Oxfordshire Starlings was not. This may be due to the significantly different sample sizes and sampling techniques or due to local clonal expansion of the ST-682 complex in Oxfordshire. Starling isolates also clustered with genotypes from Lancashire ruminants in ST-257 complex, ST-21 complex, ST-45 complex and ST-42 complex. Four STs, ST-38 (ST-48 complex), ST-42 (ST-42 complex), ST-45 (ST-45 complex) and ST-257 (ST-257 complex), were found amongst Starlings and the other hosts. ST-257 (ST-257 complex) and ST-45 (ST-45 complex) were isolated from Oxfordshire Starlings,

and Lancashire Poultry. ST-257 (ST-257 complex) and ST-45 (ST-45 complex) were also isolated from the human disease isolates collected in 1999. ST-38 (ST-48 complex) and ST-42 (ST-42 complex) were isolated from Oxfordshire Starlings and Lancashire ruminant animals. The ST-177 complex, although isolated from both Oxfordshire and Lancashire Starlings, consisted of different genotypes. ST-521 (ST-177 complex) isolated from Starlings in Lancashire had only one mutation in the *pgm* allele when compared to ST-177 (ST-177 complex) which was prevalent amongst the Oxfordshire starlings.

A total of 17 STs (5.8% of isolates) belonging to seven different clonal complexes, with one ST unassigned, had been isolated from cases of human disease on the *Campylobacter* MLST database. Other studies have also found evidence that wild birds harbour low levels of genotypes indistinguishable from those causing human disease (Rosef *et al.* 1985; Colles *et al.* 2003; Sopwith *et al.* 2003; Broman *et al.* 2004). It is thought that bird species showing greatest interaction with the human and urban environments are the ones most likely to be shedding 'human-like' genotypes. It is highly likely that birds could acquire such genotypes from humans, for example by frequenting rubbish dumps or by pecking animal waste used as fertilizer on fields, but the extent to which they are transported back to the human environment is unknown (Petersen *et al.* 2001a; French *et al.* 2005). Although it is possible that *C. jejuni* genotypes originating in wild birds could cause human disease, they are carried in low numbers and evidence from the *Campylobacter* MLST database and other studies suggests that they are not a major source of human disease (Petersen *et al.* 2001a; Waldenstrom *et al.* 2005a). A total of four of the 75 STs isolated from the Starlings were

found to be associated with isolates from live chickens or chicken meat on the *Campylobacter* MLST database. As with humans, it is unclear whether Starlings become colonized with genotypes from the chickens or their environment, or vice versa. The levels were very low (9 of 293, 3.1% of isolates) suggesting that although they could not be discounted as a potential source of infection, they are unlikely to act as a major reservoir.

In conclusion, the *C. jejuni* population amongst Starlings captured in Oxfordshire over a three year period was diverse, but dominated by two clonal complexes, ST-682 and ST-177 complexes. None of the STs were isolated from the wild geese on the farm, and it is possible that they are adapted to survival in the Starling species and their environment. Low levels of STs indistinguishable to those causing human disease (5.8% of isolates) and from chicken sources (3.1% of isolates) were identified, although they are unlikely to be a major source for human disease or contamination of poultry flocks. Carriage and genotypic diversity appeared to be seasonal amongst the Starlings, with a peak occurring in late spring/early summer. This may be partly associated with increased numbers of juvenile birds who were found to be more frequently colonized by *C. jejuni* than other age groups. Colonization of young Starlings appeared very different to that of young farmed poultry. Nestlings were shedding *Campylobacter* at nine days of age, whilst farmed poultry are rarely shedding *Campylobacter* before 21 days of age. In addition the majority of siblings of positive nestlings were not shedding *Campylobacter*, whilst infection spreads very rapidly amongst farmed poultry. This is most likely to be a reflection of the different life styles and ecology of the birds.

Future work should include sampling of Starlings in other locations to determine whether or not the ST-682 complex is local to Oxford, and whether other Starling associated clonal complexes exist in other areas. The STs isolated from more than on animal host should be investigated in more detail to determine whether they are the same strains in different host sources, and in both regions of the UK. Antigenic loci such as *flaA* and MOMP (*porA)* could be sequenced to give a high level of differentiation between the genotypes. Comparison of the genotypes isolated from Oxfordshire Starlings with those isolated from farm animals in the same location and sampled in a similar way would give a more accurate comparison. If the differences between the *Campylobacter* populations in Starlings and free-range poultry are upheld, explanations could be sought by investigating differences in their contrasting environments as well as studying other factors such as behavioural patterns, health and condition.

Abstract

Chapter 6: Longitudinal study of *Campylobacter* populations in two free-range broiler chicken flocks.

Free-range broiler chickens were tested for *Campylobacter* at the same time as the wild bird studies. The first flock tested was the first reared in the farms entire history. Samples were collected on a weekly basis to determine when birds initially began shedding *Campylobacter* and how quickly it spread through the flock. A large number of individual birds were sampled to capture the potential genetic diversity. The sampling regime was repeated later in the year with a second flock, Flock 43.

Chicks first began shedding *Campylobacter* aged 35 days in Flock 1 and aged 28 days in Flock 43; both coinciding with moving from brooding to rearing arcs and being allowed out onto the range. 100% of the birds tested in Flock 1 were shedding *Campylobacter*, but numbers were generally slightly lower in Flock 43, probably as a result of seasonal effects. Approximately 100 birds were retained from Flock 1 only, and removed to a barn whereby the shedding rate dropped to 60% of birds tested.

A succession of genotypes was isolated from both flocks, the proportions of which fluctuated from week to week. Genetic diversity increased with age of bird. Only one ST was isolated from both flocks and differences may be a seasonal or temporal effect. In common with other studies of free-range chickens a high proportion of isolates were *C. coli* that tended to be late colonisers.

ST-21 and ST-45 clonal complexes were identified amongst free-range chickens and the wild geese and Starlings discussed in Chapters 4 and 5, but ST-45 was the only ST in common, isolated from chickens and Starlings. The ST was relatively uncommon in both host sources.

Three new clonal complexes, the *C. jejuni* ST-661 and ST-574 complexes and the *C. coli* ST-828 complex, were identified amongst the free-range chicken isolates. Allele *asp*87 was discovered to be an usual hybrid between *C. jejuni* and *C. coli* with an obvious cross-over point in the nucleotide sequence.

Chapter 6: Longitudinal study of *Campylobacter* populations in two free-range broiler chicken flocks.

6.1 Introduction.

Previous studies have shown *Campylobacter* genotypes isolated from chicken sources to overlap with those isolated from human disease. However the levels of discrimination and repeatability associated with genotyping have been insufficient to study the population genetics of the organism in detail, or identify the proportion of genotypes that are transmissible between the sources. It is essential to identify which genotypes colonize chicken flocks and determine how they relate to those from human disease, in order that preventative measures may be developed. Free-range chickens are becoming an increasingly popular choice for consumers but it is impossible to raise them under strict bio-security measures that have proved the most effective course in preventing colonization by *Campylobacter* of housed birds. Free-range broiler chicken flocks were reared on the Oxford University farm at Wytham for the first time in its history, as part of a commercially viable enterprise and research project called Poultry in Natural Environments (PINE) which was funded by the UK Department for the Environment, Food and Rural Affairs (DEFRA). Work was done in collaboration with the Food Animal Initiative (FAI), based at Wytham.

The aim of this study was to determine the genetic diversity and population structure of *Campylobacter* colonizing free-range chicken flocks and to establish how it may vary within individual flocks over time. Two flocks were sampled on a weekly basis from when they arrived

at the farm as day-old chicks until they left for slaughter, typically at 56 days of age, although some birds were retained from the first flock and reared until 112 days of age. Genotypes isolated from free-range chickens in this chapter were compared with those surveyed from two wild bird species occupying the same farm site in Chapters 4 and 5, to determine whether or not it is appropriate to consider wild birds a source of *Campylobacter* for chicken flocks.

6.2 Results.

6.2.1. Method amendments.

A total of 549 *Campylobacter* isolates were successfully isolated from flocks 1 and 43 using direct culture on mCCDA plates. The MLST method was not altered, although a chequer boarding technique was used to choose primers that worked optimally with the isolates. Primers chosen were a combination of those designed for the original scheme in Oxford, and others that were developed by Dingle *et al* (2001a) and Miller *et al* (2005). It was necessary to re-extract many of the boiled cell preparations with the 'Isoquick' DNA extraction kit to improve the quality of the PCR reactions and sequencing data. The most likely reason for the failure of the boiled cell preparations was haem from blood agar plates inhibiting PCR reactions. Care was taken to ensure that the isolates were pure culture, but if mixed electropherogram traces were obtained during nucleotide sequencing, the original isolate was taken from storage, re-cultured and the DNA re-extracted.

6.2.2 Shedding rate.

Campylobacter was first detected in flock 1 on day 35, when 100% of chickens tested were positive (Figure 6.1). The shedding rate continued at 100% of tested birds until partial

depletion at day 56. When sampling was resumed at day 77, the shedding rate had fallen to 60% of birds tested, and remained at this level or lower until the last sampling occasion on day 112. The lowest shedding rate was 40% on day 105. *Campylobacter* was first detected in flock 43 on day 28, a week earlier than flock one. Flock 43 generally had lower shedding rates than flock 1, with 90% birds testing positive on the day *Campylobacter* was first detected. The highest rate, 100% of birds shedding, was detected the following week on day 35, but fell to 90% again on day 42. The lowest subsequent shedding rate was 80% on day 49, but by depletion on day 56, 95% of birds tested were shedding *Campylobacter*.

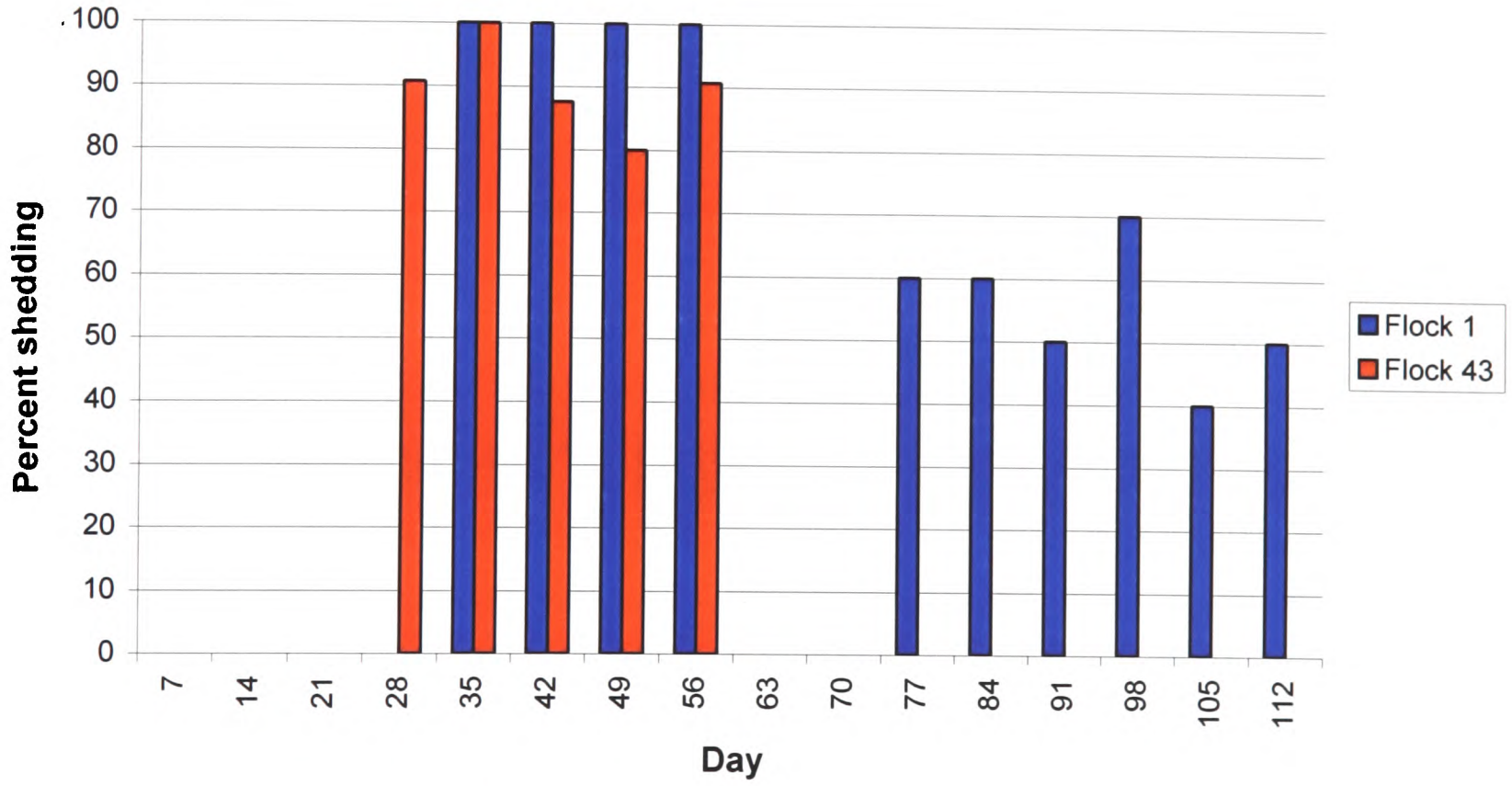

Figure 6.1. Shedding rate of *Campylobacter* amongst free-range broiler chickens from flocks 1 and 43.

6.2.3 Clonal complex distribution.

The STs isolated from both flocks clustered into eight clonal complexes (Figure 6.2). Five of the clonal complexes had been previously recognised but three, ST-661 complex, ST-574 complex and the *C. coli* ST-828 complex were defined for the first time using the data from this study, together with that held on the *Campylobacter* MLST database. Isolates from flock 1 clustered into five clonal complexes, of which ST-21 complex and ST-828 were predominant, accounting for 97% of isolates between them. Isolates from flock 43 clustered into four clonal complexes plus one unassigned genotype. The complexes were more evenly distributed with ST-661 and ST-574 complex accounting for approximately 30% of isolates each. Only two clonal complexes, ST-21 complex and ST-661 complex were identified amongst both flocks. The proportions of both varied largely between the two flocks.

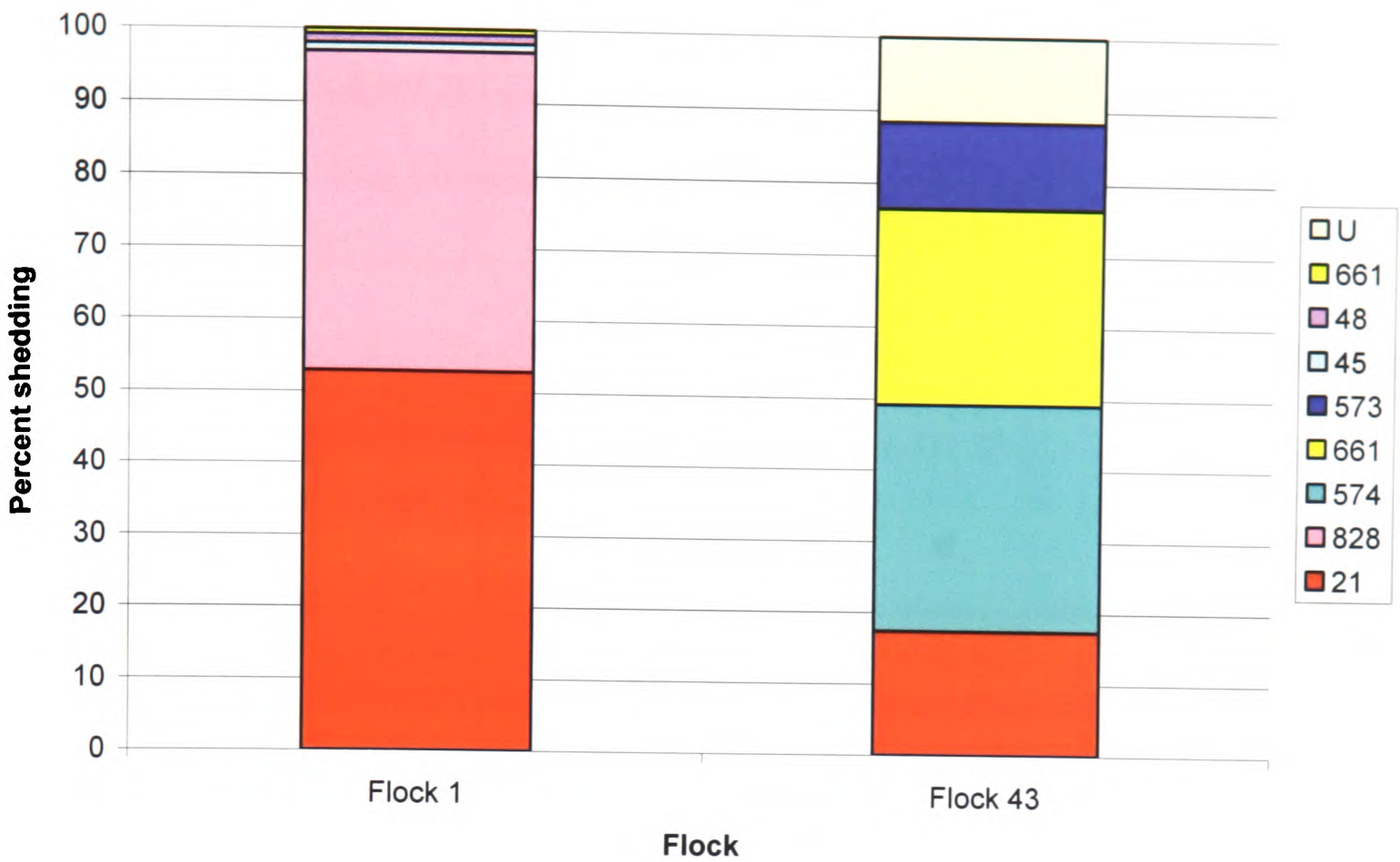

Figure 6.2. The *Campylobacter* clonal complexes isolated from flocks 1 and 43.

6.2.4 Distribution of STs.

A total of 11 STs were isolated from both flocks, seven from flock 1 and five from flock 43 (Table 6.1). Only one, ST-814 (ST-661 complex) was isolated from both flocks. The dominant STs in flock 1 were ST-19 (ST-21 complex) accounting for 129 of 258 (50%) of isolates, and ST-854 (ST-828 *C. coli* complex) accounting for 111 of 258 (43%) of isolates. They were also the STs that were isolated over the longest period of time, with ST-19 being isolated on seven sampling occasions and ST-854 being isolated on five sampling occasions. Other STs accounting for the remaining 7% of isolates in flock 1 appeared to be more sporadic and were identified on between one and three sampling occasions. The dominant STs in flock 43

were ST-574 (ST-574 complex) accounting for 93 of 291 (32%) of isolates and ST-814 (ST-661 complex) accounting for 80 of 291 (27.5%). ST-262 (ST-21 complex) and ST-814 (ST-661 complex) were isolated over the longest period of time, being identified on four sampling occasions. Few STs were related to each other with the exception of ST-854, ST-902 and ST-855, all part of the *C. coli* ST-828 complex. Central genotypes were identified for three of the seven clonal complexes but they were not predominant in terms of frequency or longevity.

Table 6.1. The composition of clonal complexes identified amongst 549 *Campylobacter* isolates from flocks 1 and 43 in terms of STs, genetic variant and isolation period over the study.

	Clonal complex	No. of solates (%)	ST	Complex variant[a]	Freq	Isolation period(d)[b]
Flock 1	21	129 (50)	19	SLV	129	77
	45	3 (1)	45	C	3	7
	48	1 (0.4)	813	DLV	1	1
	661	2 (0.8)	814	SLV	2	1
	828[C]	123 (47.8)	854	SLV	111	49
			902	SLV	5	18
			855	DLV	7	18
Flock 43	21	51 (17.5)	262	SLV	51	28
	573	35 (12)	573	C	35	21
	574	93 (32)	574	C	93	14
	661	80 (27.5)	814	SLV	80	28
	Unassigned	32 (11)	1090[C]		32	1

C= central genotype, SLV=single locus variant, DLV=double locus variant, TLV= triple locus variant.

[a]The relationship to the central genotype. [b]The greatest number of days between sampling occasions on which the ST was isolated. [C]= *C. coli.*

6.2.5 Succession of genotypes.

A succession of genotypes was apparent in both flocks. In flock 1, ST-21 complex was dominant at days 35 and 42, after which it was largely replaced by ST-828 *C. coli* complex, although ST-21 complex was still present in small numbers (Figure 6.3a). When sampling was resumed on the retained birds, ST-21 complex showed a small resurgence on days 77, 84 and 112 but was absent for the three consecutive weeks between days 84 and 112. ST-48, ST-45 and ST-661 complexes were more sporadic in appearance and were only present as a small proportion of the isolates. The clonal complexes were generally represented by a single ST, with the exception of ST-828 *C. coli* complex and ST-48 complex where STs occurred in a succession (Figure 6.3b). ST-854 (ST-828 complex) was dominant on days 49-91 but was replaced by two related genotypes ST-855 (ST-828 complex) and ST-902 (ST-828 complex) on days 98 to 112. ST-814 (ST-48 complex) was isolated on day 56, but a related genotype ST-38 (ST-48 complex) was isolated on day 98.

In flock 43 ST-661 complex was dominant on days 28 and 35 but the proportion was greatly reduced on days 42 and 56 (Figure 6.4a). ST-574 complex became dominant on days 42 and 49 but the proportion was reduced on day 56. ST-573 complex present in small numbers on days 35 and 49 became co-dominant with an unassigned ST on day 56. ST-21 complex was present on days 28, 35, 42 and 56 but never gained dominance. The STs remained consistent within the clonal complexes isolated from flock 43 (Figure 6.4b). Genotypes from flock 1 were generally less diverse than those from flock 43. The largest number of genotypes present in a week in flock 1 was three clonal complexes and four STs and in flock 43 four clonal complexes and five STs.

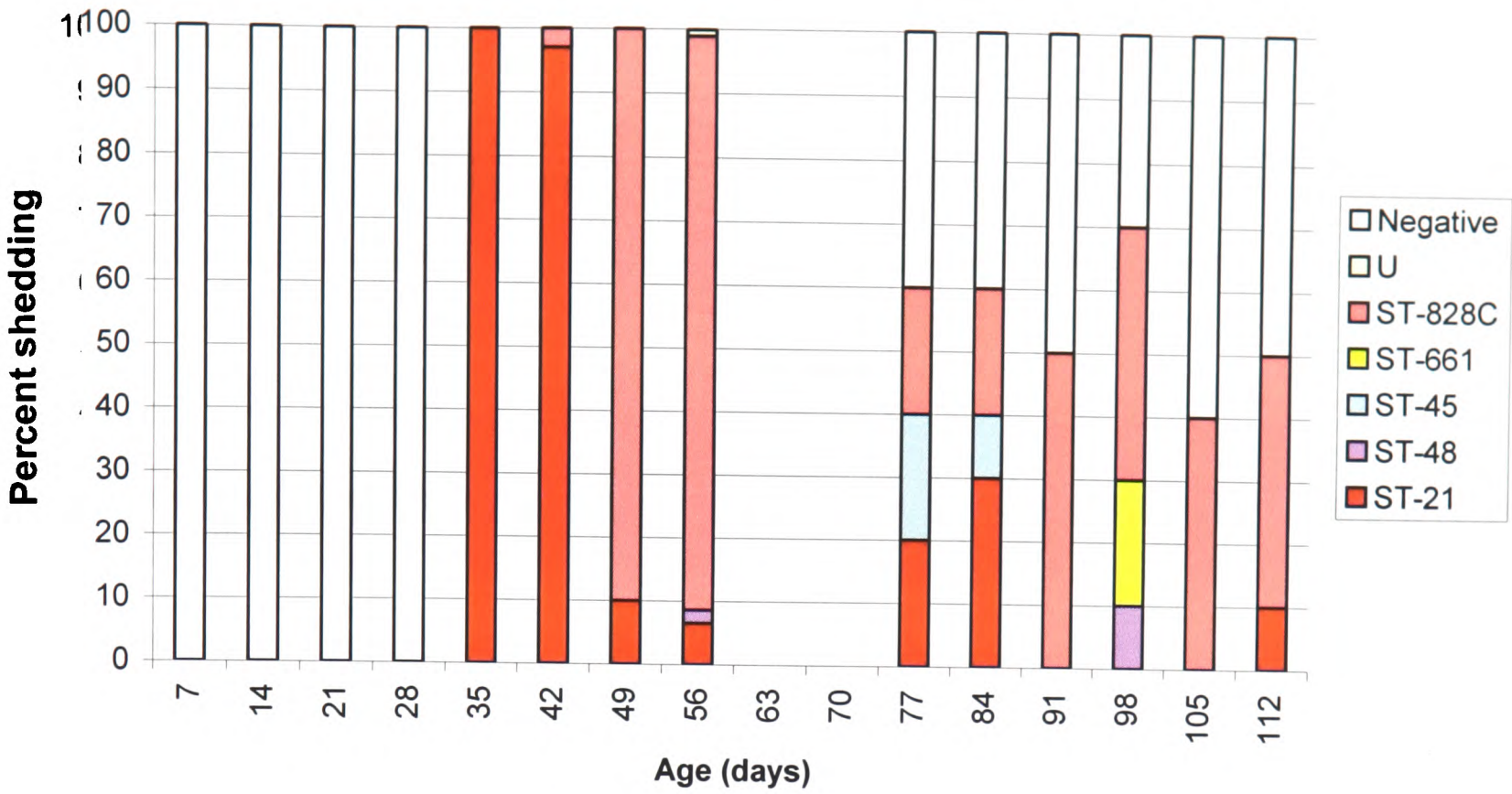

Figure 6.3a Shedding rate and clonal complexes amongst *Campylobacter* isolates from flock 1. U=isolates that could not be assigned into a clonal complex. C= *C. coli.*

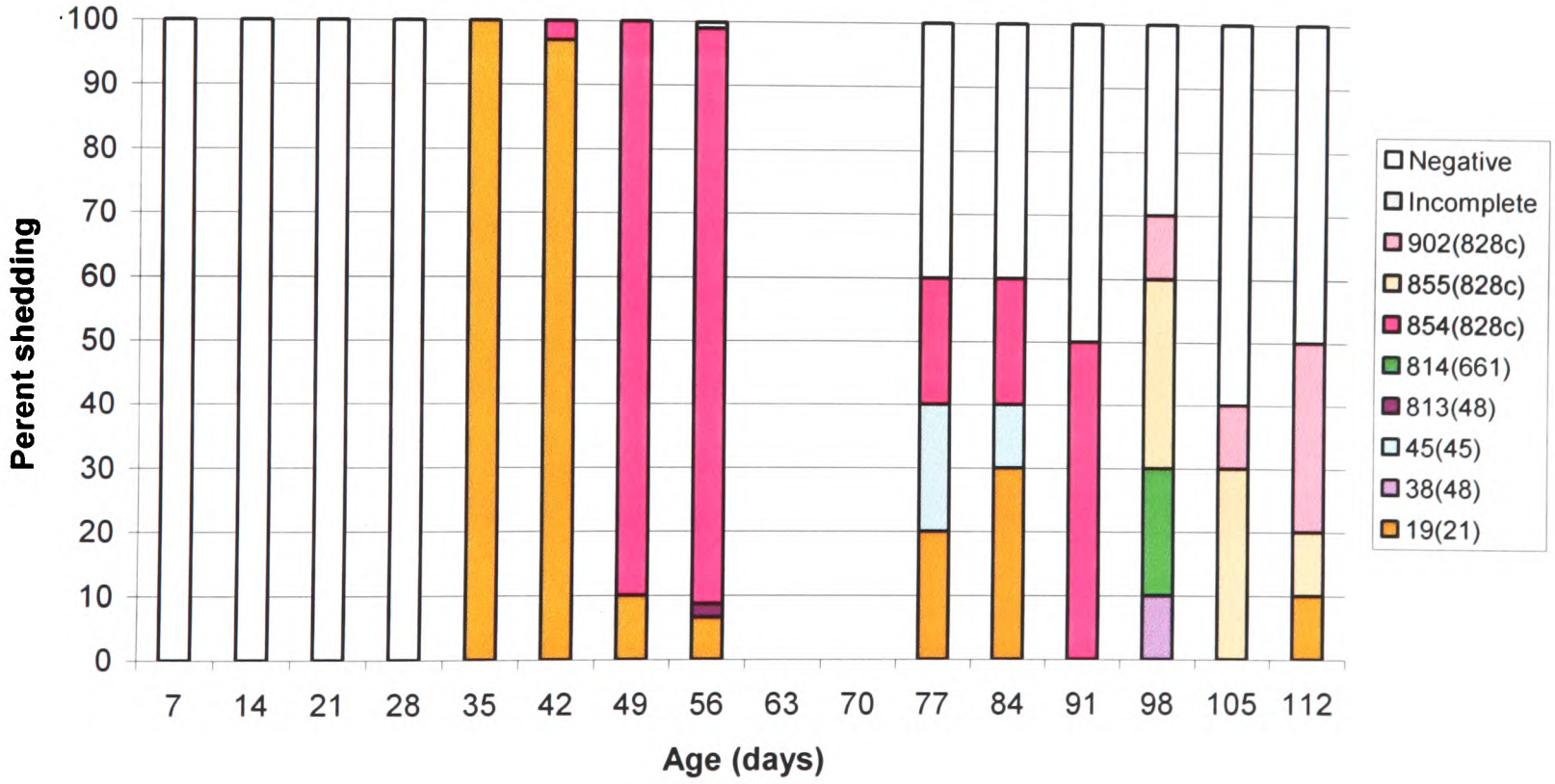

Figure 6.3b. Shedding rate and STs of *Campylobacter* amongst isolates from flock 1.

C=C. coli.

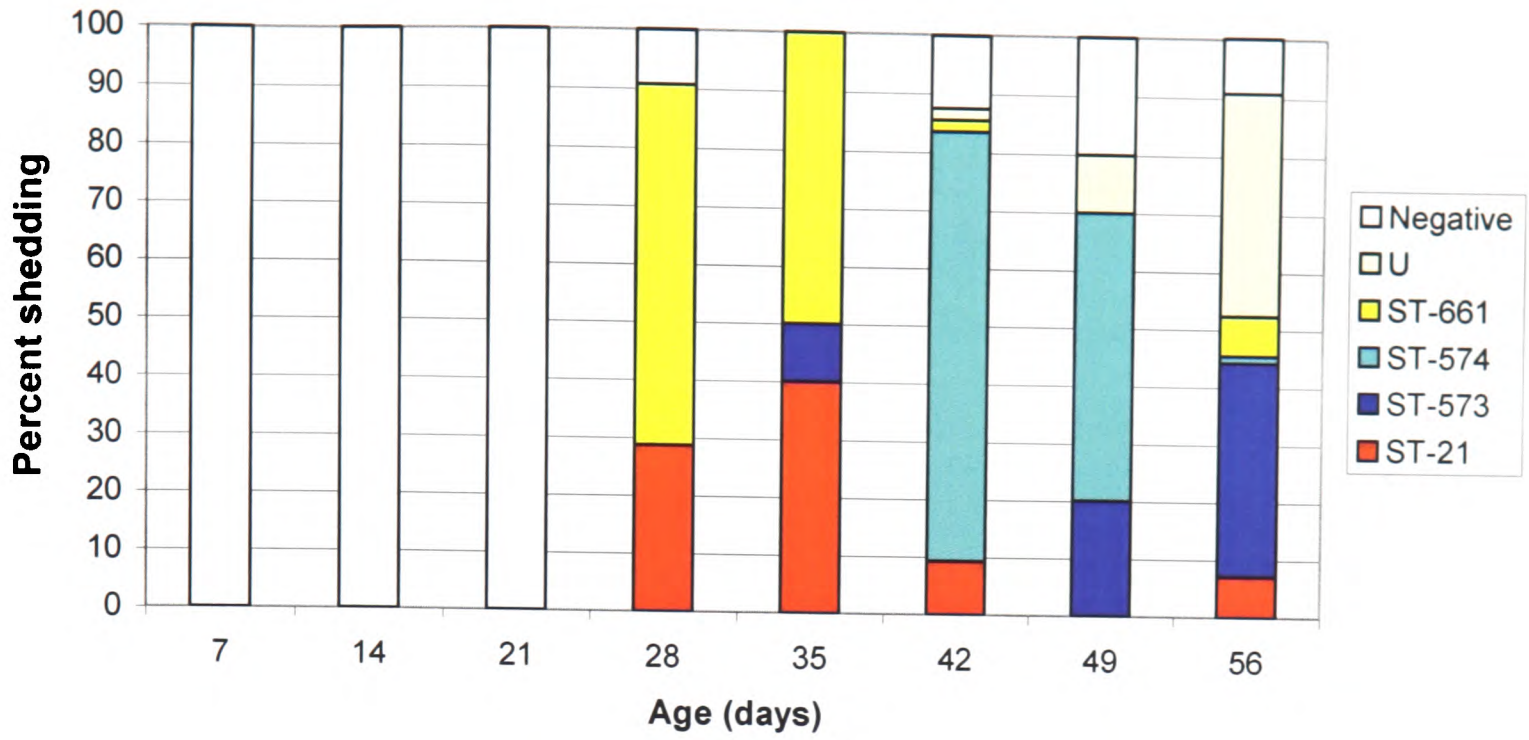

Figure 6.4a. Shedding rate and clonal complexes of *Campylobacter* amongst isolates from Flock 43. U=isolates that could not be assigned into a clonal complex. C= *C. coli.*

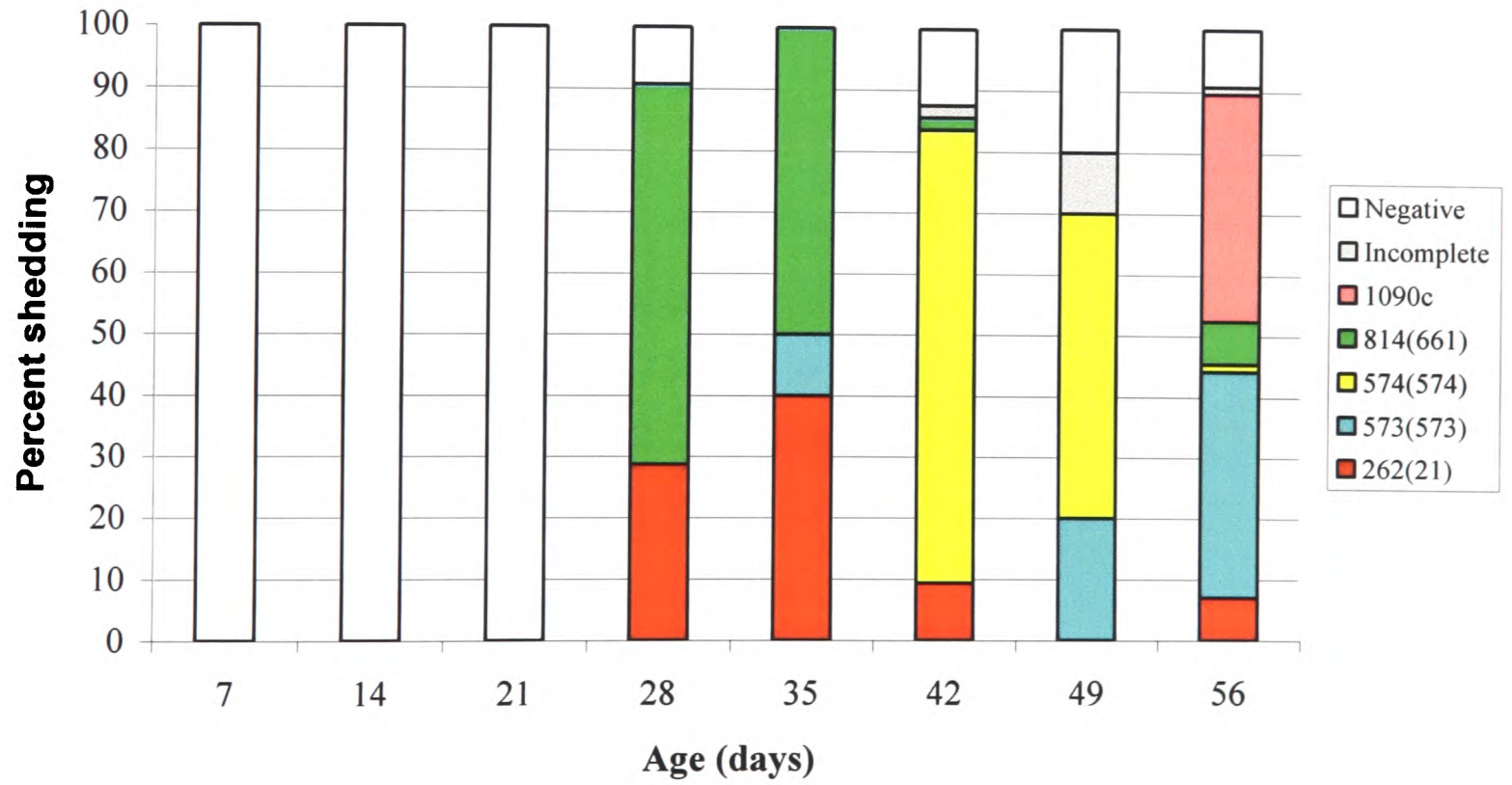

Figure 6.4b. Shedding rate and STs of *Campylobacter* amongst isolates from Flock 43. C= *C. coli.*

6.2.6 Allelic diversity.

The origin of all of the alleles were identified as *C. jejuni* or *C. coli*, with the exception of the *aspA* 87 allele which appeared to a hybrid of both species; a cross over event is evident between nucleotides 355 and 368 (Figures 6.5a and 6.5b). The number of alleles amongst the *Campylobacter* isolates from flocks 1 and 43 ranged from five at the *aspA* locus to eight at the *glyA* locus. The number of alleles amongst the *C. jejuni* isolates ranged from three at the *aspA* locus to six at the *glnA* and *glyA* loci, and the number of alleles amongst the *C. coli* isolates ranged from one at the *gltA* locus and three at the *tkt* locus. The number of variable sites ranged from 49 (12.2%) at the *gltA* locus to 97 (19.1%) at the *glyA* locus when alleles from both *Campylobacter* species were included. The number of variable sites ranged from five (1%) at the *uncA* locus to 30 (5.9%) at the *glyA* locus amongst *C. jejuni* isolates. The number of variable sites ranged from 1 (0.2%) at the *glnA*, *glyA* and *pgm* loci to 37 (7.8%) at the *aspA* locus (including the divergent *aspA* locus) amongst *C. coli* isolates. The d_N/d_S were all considerably below one indicating the absence of positive selection.

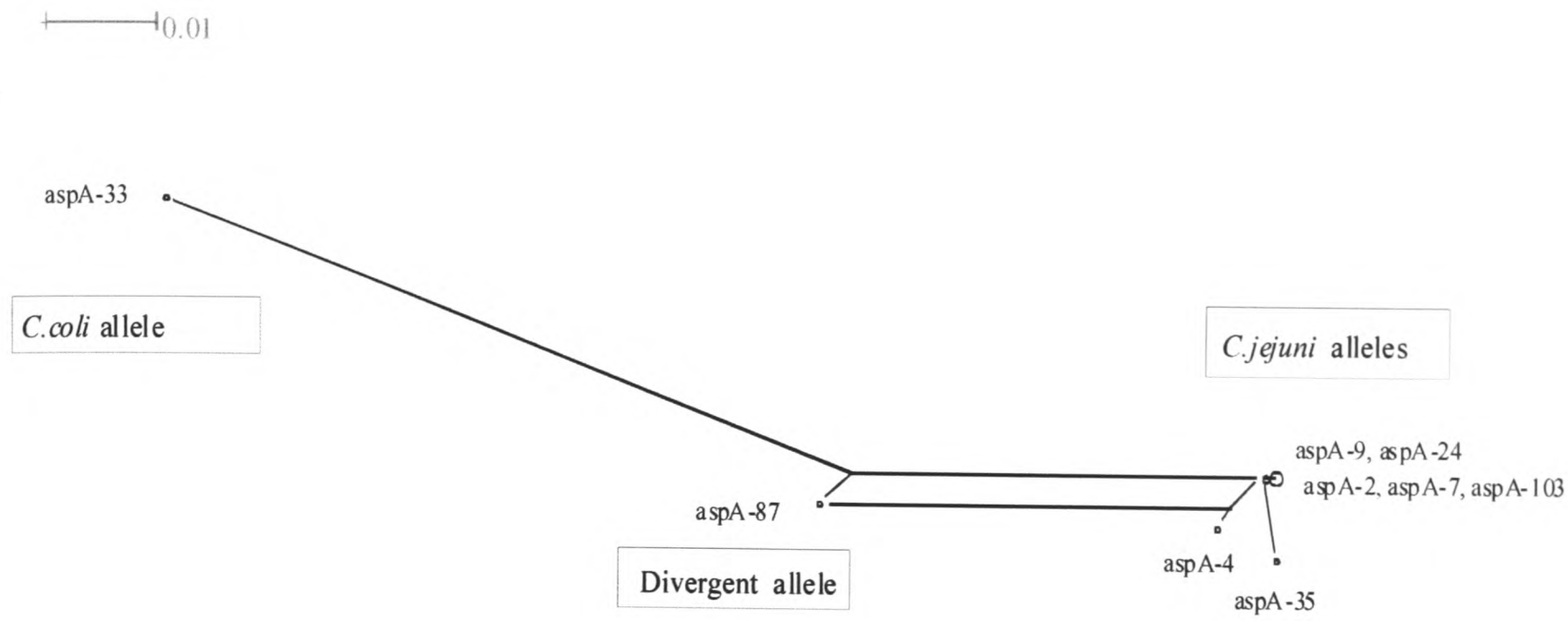

Figure 6.5a. A splits graph demonstrating the relationship of the divergent *aspA* 87 allele with *aspA* alleles from *C. jejuni* and *C. coli* (concatenated sequence).

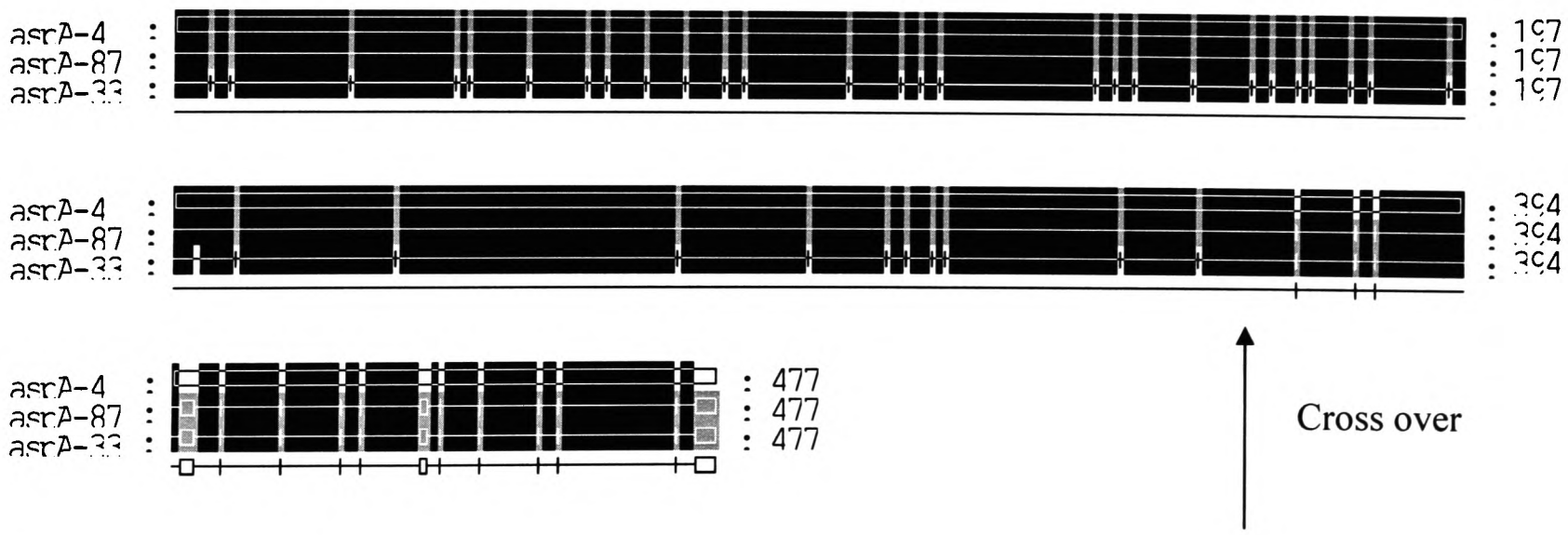

Figure 6.5b. Alignment of the *C. jejuni aspA* 4 allele, the *C. coli aspA* 33 allele and the hybrid *aspA* 87 allele. A cross over event has occurred between nucleotide residues 355 and 368.

Table 6.2. Diversity of allele sequences amongst *Campylobacter* isolates from flocks 1 and 43.

Locus	Fragment size (bp)	No. of alelles			No. of variable sites			% variable sites			d_N/d_S ratio		
		jej	*coli*	*total*	*jej*	*coli*	*total*	*jej*	*coli*	*total*	*jej*	*coli*	*total*
aspA[a]	477	3	2	5	8	37[a]	60	1.7	7.8[a]	12.6	0.011	0.082[a]	0.066
glnA	477	6	2	8	14	1	61	2.9	0.2	12.0	0.015	0.002	0.061
gltA	402	5	1	6	8	na	49	2.0	na	12.8	0.010	na	0.047
glyA	507	6	2	8	30	1	97	5.9	0.2	19.1	0.030	0.002	0.103
pgm	498	5	2	7	25	1	81	5.0	0.2	16.3	0.027	0.002	0.090
tkt	459	4	3	7	12	2	70	2.6	0.4	15.3	0.014	0.003	0.095
unc	489	4	2	6	5	3	74	1.0	0.6	15.1	0.005	0.006	0.088
all loci	3309	7	4	11	102	45	492	3.1	1.4	14.9	0.013	0.007	0.080

[a]The divergent *aspA* 87 allele is included. Na= not applicable.

6.2.7 Antigenic diversity.

The *flaA* SVR type was not consistent with clonal complex or *Campylobacter* species (Table 6.3). For example ST-19 and ST-262 both belonging to the ST-21 clonal complex had different *flaA* SVR nucleotide alleles, although the peptide remained the same. Similarly, two STs grouping into the *C. coli* ST-828 complex, ST-855 and ST-902 isolated from flock 1 had identical *flaA* SVR types, but a third, ST-854 also isolated from flock 1 had a different nucleotide and amino acid profile. The *flaA* SVR type was largely correlated with ST, but not exclusively. Seven STs from both flocks were always associated with a particular *flaA* SVR nucleotide and peptide combination, including ST-814 with *flaA* SVR type 191-33 which was the only ST-*flaA* SVR type to be isolated from both flocks. A particular *flaA* SVR type was sometimes associated with more than one ST and clonal complex, for example ST-573 and ST-574, both central genotypes of complexes were both associated with *flaA* SVR type 8-1 in this study.

Table 6.3. The *flaA* SVR and ST combinations of *Campylobacter* isolates from flocks 1 and 43.

C=*C. coli*. U = STs that could not be assigned to a clonal complex.

Clonal complex	ST	*flaA* SVR allele-peptide (frequency)									
		8-1	36-1	37-1	66-1	41-4	15-5	111-8	319-15	86-18	191-33
21	19		138						1		
	262	7		44							
45	45		1				2				
48	38							1			
	813					2					
573	573	34								1	
574	574	93									
661	814										82
828[C]	854[C]		4						101		
	855[C]				6						
	902[C]				5						
U	1090[C]				32						

6.2.8 Comparison with *Campylobacter* genotypes isolated from wild birds in the same location.

A total of 18 clonal complexes were identified amongst the isolates from the chickens sampled in this chapter and the geese and Starling isolates collected in Chapters 4 and 5 (Figure 6.6). Of these, two clonal complexes, ST-21 complex and ST-45 complex, overlapped all three sources and ST-48 complex overlapped the chicken and Starling sources. ST-45 (ST-45 complex) was the only genotype isolated from more than one source and was identified amongst chicken and Starling isolates. The genotype was associated with the same *flaA* SVR allele in both sources giving further evidence that the same *Campylobacter* strain may have transferred between them. Other STs within the overlapping clonal complexes had between two and 21 nucleotide differences along the concatenated sequence of seven loci.

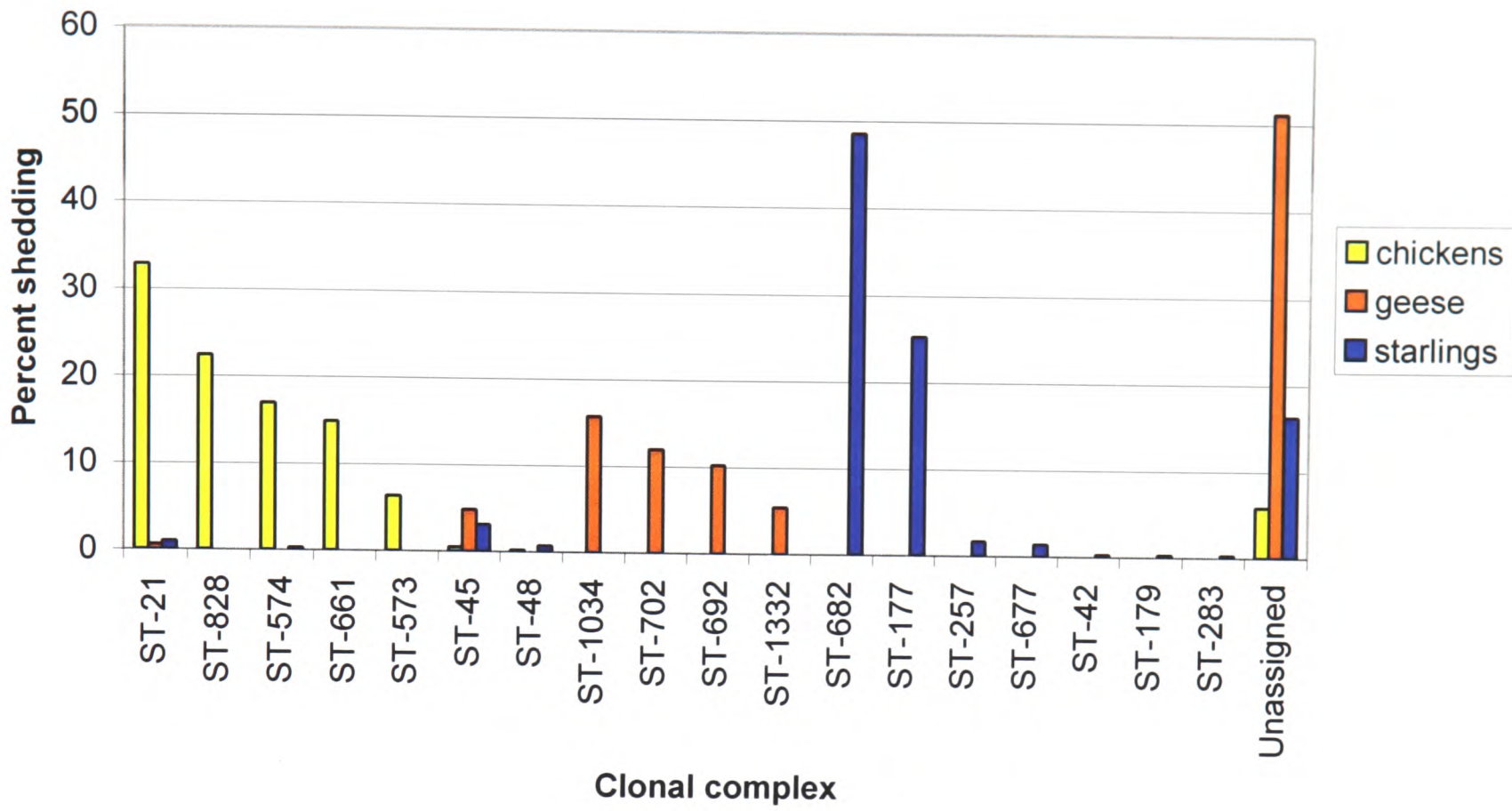

Figure 6.6. The clonal complexes isolated from free-range broiler chickens, geese and Starlings sampled on the Oxford University Farm, Wytham.

6.3 Discussion.

Chicks within both flocks began shedding *Campylobacter* within a single sampling point of each other at days 28 and 35. Flock 1 was the first batch of chickens commercially reared on the farm site in its history, and was the one that became positive slightly later. However the timing in both coincided with chicks being let out onto the range for the first time, a factor noted previously, and it has been suggested that some flocks at least, became colonized by *Campylobacter* genotypes from the soil (Rivoal *et al.* 2005). It was not possible to test this theory in this study, but comparison with environmental and sand isolates on the *Campylobacter*

MLST database did not reveal any genetic similarities those isolated from the chickens. Contamination from the soil, at least with the first flock, was likely to be limited as the field had been fallow for a long period prior to rearing the chickens. Another potential confounding factor was the change of feed in these chickens at ten days, as is common practice. *Campylobacter* has rarely been isolated from feed prior to flocks becoming positive, but different feeds have been found to influence gut behaviour and therefore may influence colonization (Newell and Fearnley 2003). It has been noted that a change to solid dry food from a moist food gave rise to different intestinal structures and that hindgut viscosity reduced the longer birds were kept on a particular diet (Petersen *et al.* 1999; Bouwknegt *et al.* 2004). Colonization at three to four weeks of age has been observed in many studies with both conventionally housed, as well as free-range chickens (Berndtson *et al.* 1996b; Shreeve *et al.* 2000, Corry and Atabay 2000; Newell and Fearnley 2003; Bouwknegt *et al.* 2004; Boyd *et al.* 2005; Rivoal *et al.* 2005; Bull *et al.* 2006). The microflora of young chicks is unlikely to be mature but it is possible they may influence early coloniszation by *Campylobacter* by producing inhibitory substances (Humphrey *et al.* 1993; Grant *et al.* 2005). In addition maternal antibodies are thought to play at least a partial role in protection of young chicks, but levels fall substantially at 14 days of age (Sahin *et al.* 2003).

More than 90% of birds tested in both flocks were shedding *Campylobacter* on the day it was first detected. The finding, in common with those from other studies, implies that the spread between individual chickens was very rapid, and that most individuals within the flock became colonized (Berndtson *et al.* 1996b; Evans and Sayers 2000; Corry and Atabay 2001; Hald *et al.* 2001). It appeared that shedding levels of individuals within flock 43 were generally lower than in flock one over the 56 day rearing period. There was not an obvious explanation for the finding, and it may have been a stochastic or seasonal effect. However, numerous factors such

as differing colonization potential of *Campylobacter* genotypes, age of bird when first infected, health status and environmental factors, particularly temperature and sunlight may all influence shedding rates (Corry and Atabay 2001; Bouwknegt *et al.* 2004; Patrick *et al.* 2004). The shedding rates amongst individuals in flock 1 were significantly lower once sampling resumed between days 77-112, an age not often investigated since most conventional flocks, and even organic poultry are slaughtered before this time. This is consistent with lower shedding rates found in birds of eight weeks and older and suggests that host immune factors could be responsible (Newell and Fearnley 2003). There was no evidence to link *Campylobacter* genotype with the reduced shedding rate within the extended sampling period of flock 1, since both new and old genotypes were isolated from the retained birds. The lower shedding rate may reflect the fact that the retained birds were moved into a barn and thus had a reduced exposure to the environment. In addition the birds were kept at lower density and provided with water bells rather than nipple drinkers, which may have reduced the stress of competition and unnatural behaviours.

A total of 11 STs grouping into seven clonal complexes were identified amongst the 549 *Campylobacter* isolates from both flocks. The complexes most commonly isolated amongst the flocks, ST-21 complex and ST-828 complex were also the most common and diverse on the *Campylobacter* MLST database. Only one genotype, ST-814 (ST-661 complex) was isolated from both flocks. The *flaA* SVR allele was also identical suggesting a common source of infection for both. The proportional make-up of clonal complexes varied between the flocks with two STs being dominant in flock 1, and five being more evenly spread in flock 43. This may reflect seasonal differences between the flocks, or the fact flock one was the first on the farm and there had been chance for contamination to build by flock 43. Although the central

genotypes were isolated from three of the seven clonal complexes they were not necessarily the most common or long lived and there was little evidence of structuring of the *Campylobacter* population as seen amongst other host sources in previous chapters. Instead the chicken flock could be considered as a unit containing limited genotypes. More flocks should be sampled to determine the population biology of *Campylobacter* within chickens as a host source.

Both flocks had mixed genotypes from both *C. jejuni* and *C. coli,* with up to five STs being isolated per sampling occasion. The findings were consistent with multiple exposure to different sources of *Campylobacter* for both flocks and *flaA* SVR typing proved to be a useful tool in highlighting this. Other studies have identified co-colonization of flocks by the two subspecies, although detailed population analysis using sequence data has been limited (Thomas 1997; Camarda *et al.* 2000; Corry and Atabay 2001; Petersen *et al.* 2001b; Shreeve *et al.* 2002, Newell and Fearnley 2003; Schouls *et al.* 2003; Bull *et al.* 2006). It appeared that diversity became greater as the flocks became older, presumably since there would have been more opportunities for acquisition of new genotypes. There is some discussion as to whether *Campylobacter* genotypes can co-exist within a flock, or whether they are displaced by new genotypes (Petersen *et al.* 2001b; Hook *et al.* 2005). Evidence from this study suggests that both situations could occur in the short time period of 56 days, and that the *Campylobacter* populations were flexible and dynamic. Some genotypes eg ST-855 (ST-828 complex) and ST-902 (ST-828 complex) co-existed over a number of weeks with their proportions fluctuating significantly on occasions, but others, eg ST-45 (ST-45 complex) appeared to be displaced very quickly. A greater number of samples per flock would be required to confirm the findings since sampling sensitivity is likely to be low.

The differences in longevity of colonization and genotype composition could be influenced by intermittent external sources of infection, or different colonization potentials and competitive advantages of the *Campylobacter* genotypes over one another (Pearson *et al.* 1996; Hanel *et al.* 2004; Rivoal *et al.* 2005; Bull *et al.* 2006). Perhaps the most likely explanation in this case is a stochastic effect described by Grant *et al* studying transfer of labelled *Campylobacter* mutants within a limited number of birds under experimental conditions (Grant *et al.* 2005). They were unable to predict which mutants could be isolated from which birds, a week after they each received a large inoculum of a specific type. Some birds lost their mutants and others became co-colonized, whilst over time a limited number of mutants came to predominate. When the experiments were repeated the same mutants did not act in the same way. They hypothesized that *Campylobacter* populations were formed randomly as a result of bottlenecks in colonization or transmission between the birds. The situation may be further influenced by coprophagic behaviour of the chickens, which could allow continual reseeding of gut by particular genotypes.

The *C. coli* ST-854 (ST-828 complex), present on five sampling occasions disappeared rather abruptly after day 91 when it had been isolated from 50% of the birds tested. Two related STs from the same clonal complex were isolated the following week. ST-902 (ST-828 complex) differed at only two nucleotides, one at the *glnA* locus and one at the *glyA* locus. ST-855 (ST-828 complex) had the same differences at the *glnA* and *glyA* loci, and an additional nucleotide change at the *tkt* locus. Genetic drift can not be ruled out in this instance, although it may be unlikely. Since ST-855 (ST-828 complex) and ST-902 (ST-828 complex) only differed from each other by a single nucleotide and were detected at the same time, they may have originated from a similar source. The fact that ST-855 (ST-828 complex) and ST-902 (ST-828 complex)

isolates had identical *flaA* SVR alleles and ST-854 (ST-828 complex) had a completely unrelated antigen type lends support for the latter hypothesis. The ST-828 complex which all of the *C. coli* isolates from flock 1 clustered into, appears to be very large and diverse when taking into account all of the STs on the *Campylobacter* MLST database and it may be that the three related genotypes occurred by chance (Miller *et al.* 2006).

ST-19 (ST-21 complex) was isolated on day 35 when flock 1 first became positive, and on the last sampling occasion on day 112, a span of 11 weeks. The reappearance of ST-19 (ST-21 complex) after three weeks absence could perhaps be explained by sampling sensitivity, and its failure to be isolated if it was present at low levels, or by an intermittent external source. Typing of the *flaA* SVR demonstrated that the isolates were identical throughout the study and that no genetic drift, even under immune pressure from the host, had occurred at all in this instance. This was inconsistent with results from another study where greater diversity was found amongst isolates using phage and serotyping over MLST (Bull *et al.* 2006). The re-emergence of ST-19 (ST-21 complex) amongst poultry previously colonized by the genotype implies that it has entered some sort of commensal relationship with the host, some birds have cleared the infection whilst others have become chronic shedders or that immunity was not conferred when it was encountered at a young age (Sahin *et al.* 2003; Boyd *et al.* 2005).

Some studies have found *C. coli* genotypes to replace *C. jejuni* genotypes as flocks near the end of their lifespan, although Bull *et al* found an early colonizing *C. coli* genotype to be displaced by a *C. jejuni* genotype in one particular flock (El-Shibiny *et al.* 2005; Bull *et al.* 2006). The results from this study are consistent with the finding that initial colonizers were *C. jejuni* genotypes. *C. coli* genotypes were not detected until day 42 in flock one and day 56 in flock 43. It is not known why *C. coli* tend to be later colonizers, but perhaps it is a reflection of

changes in the gut of the chicken host as it matures, or differing exposures through chicken behaviour or flock management. It is possible that this situation is more commonly seen in free-range and organic chickens since they have a longer life span and are potentially exposed to a greater number of environmental sources (El-Shibiny *et al.* 2005).

A total of 18 clonal complexes were identified amongst the chicken isolates from this chapter and the geese and Staring isolates from Chapters 4 and 5. Only two clonal complexes, ST-21 complex and ST-45 complex were found in all the sources and only one ST, ST-45 was isolated from chickens and Starlings. No STs isolated from the chickens were isolated from the geese, although some of the STs were closely related having as few as two nucleotide differences in the concatenated sequence. Thus the wild birds can not be ruled out as a potential source of contamination for the chickens although they probably play a minor role. The population structure of *Campylobacter* has not yet been fully determined amongst free-range chickens, but other differences were observed in the colonization potential of *Campylobacter* amongst the chickens and wild birds. For instance the Starling chicks were shedding *Campylobacter* at nine days of age whilst the poultry chicks in this study, in common with other studies, did not shed *Campylobacter* until at least 28 days of age. The carriage rate of *Campylobacter* was very much lower amongst wild birds and, in contrast to chickens, *C. coli* was rarely isolated from wild birds. The differences in *Campylobacter* colonization are likely to reflect the contrasting environments in which the birds live. Early shedding amongst wild birds may result from ingestion of contaminated feed, whilst this is rarely a problem in domestic poultry, at least until a flock becomes colonized. Once poultry chicks become colonized the crowded conditions and un-natural diet may help to promote the spread of *Campylobacter* whilst wild birds are more widely dispersed through their environment.

All of the clonal complexes identified amongst the isolates from the two flocks were associated with isolates from human disease on the *Campylobacter* MLST database. Three of the 11 STs, ST-19 (ST-21 complex), ST-262 (ST-21 complex) and ST-45 (ST-45 complex) accounting for 183 of 549 (33.3%) isolates were indistinguishable from isolates from human disease. Four of the complexes, ST-573 complex, ST-574 complex, ST-661 complex and ST-45 complex showed bias for isolates from chicken samples on the *Campylobacter* MLST database (Dingle *et al.* 2002). ST-257 complex, another complex previously associated with chicken, was absent in this study and perhaps reflects the differences between *Campylobacter* genotypes colonizing live chickens and chicken meat. Five STs, ST-813, ST-855, ST-902, ST-814 and ST-1090, accounting for 127 of 549 (23.1%) isolates have only been detected in chicken sources to date, despite other STs within the same clonal complex being isolated from cases of human disease. STs isolated from the two flocks have also been reported elsewhere; ST-814 (ST-661 complex) was identified by Connerton *et al.* (2004) amongst barn kept chickens; ST-45 (ST-45 complex) was almost exclusively isolated from turkeys and chicks by Colles *et al.* (2003) and was also isolated by Bull *et al.* (2006) from broilers, broiler breeders and puddle samples; Bull *et al.* (2006) also isolated ST-855 (ST-828 complex) from transport crates, ST-262 (ST-21 complex) from puddles, ST-573 (ST-573 complex) from broilers and drinkers and ST-574 (ST-574 complex) from broiler breeders. It is possible that each of the STs listed are common and stable colonizers of chickens and their environments in the UK, since each of the reports are from different regions and span several years (Bull *et al.* 2006).

The *C. coli* ST-854 was identified exclusively amongst pigs by Miller *et al.* (2006) in the US. The *glnA* 38 allele in particular appeared to be a marker of pig specificity, suggesting an early source of infection for flock 1 could have been the pigs on the farm which were located in

close proximity to the brooding sheds. Similarly ST-855 has the *tkt* 35 allele which was also noted to be a marker of pig specificity. Limited samples were collected from pigs on the University farm at a later date, giving three STs that grouped in the ST-828 *C. coli* clonal complex but differed at three of the seven loci, and between three and eight nucleotides (Dingle *et al.* 2005). Thus pigs can not be completely disregarded as a potential source, although it seems unlikely. Other studies have found *C.coli* genotypes from pigs to group separately from chicken and human isolates (Rivoal *et al.* 2005; Siemer *et al.* 2005).

Three STs, ST-813, ST-902 and ST-1090 were unique to this study and could reflect localised sources of contamination, or recombinants that are unsuccessful evolutionary 'dead ends'. Although the sampling in this study was very localised, the genotypes appear to be stable over a number of decades and have been identified nationally and sometimes internationally amongst human disease and chicken samples. The results suggest that free-range chicken flocks may be a significant source of human infection, and that genotypes present on the farm are capable of surviving the processing stages to contaminate the final food product. It appears there are also 'chicken adapted' genotypes, such as ST-661 complex, but it is unclear whether these are incapable of causing disease in humans, or more likely whether they are just not encountered, perhaps due to failure to survive through to the end stage of food production.

In conclusion, high numbers of individual chickens within both flocks were shedding *Campylobacter* between days 28/35 and 56 days. Levels had dropped substantially when rejected birds from flock 1 were sampled for another six weeks, which was perhaps a reflection of maturing host immunity, reduced stress levels, or reduced interaction with the environment. There was a succession of genotypes, both *C. jejuni* and *C. coli*, with up to five genotypes being present on one sampling occasion. Genotypes were diverse, but a number had been isolated

from chicken sources in other studies or shown chicken bias on the *Campylobacter* MLST database. Wild birds could not be ruled out as a source of *Campylobacter* for the chicken flocks although their role is likely to be small. Free-range chickens are likely to be a significant source of infection for humans with a quarter of genotypes and nearly a third of isolates being indistinguishable to those isolated from human disease. More data are needed to establish the true population dynamics of *Campylobacter* within free-range chickens by sampling a larger number of flocks. In addition flocks should be sampled over a longer time period in order to discover whether there are seasonal effects such as those seen amongst wild birds in the previous chapters. Further work is required to explore other potential sources of *Campylobacter* for the chicken flocks.

Abstract

Chapter 7: *Campylobacter* populations amongst free-range broiler flocks sampled at depletion.

The aim of this study was to determine the population biology of *Campylobacter* amongst free-range broiler chickens and to determine whether genotypes are obtained from wild birds and ultimately passed on to the human food chain. In addition, seasonal effects, differences between two different farm sites, and differences between flocks reared on fallow land and flocks reared on land contaminated by previous flocks could be tested. The flocks reared at two Oxfordshire farm sites on a rolling production cycle were sampled at depletion (56 days), the time point identified in Chapter 6 to have the greatest diversity of *Campylobacter* genotypes.

At least 70% of birds, and more typically 90-100% of birds tested were shedding *Campylobacter*, with a small but significant peak occurring in late Spring/early Summer. There was a succession of genotypes over the year that appeared to occur at random. There were some differences in colonisation potential, for example ST-828 complex was commonly isolated throughout the year but ST-45 complex was isolated from one flock only. There was a peak in genetic diversity in late Spring/early Summer, with some flocks being colonised by up to seven genotypes. *C. coli* was isolated from 84% of flocks and accounted for 41.7% of isolates.

There was no significant effect of farm site, arc or free-range plot on the *Campylobacter* populations isolated from free-range flocks. A small proportion of isolates clustering in ST-21 and ST-45 complexes were identified amongst free-range chickens and the wild birds sampled at the same time and location. Of these only three identical strains identified by ST and *flaA* SVR type were isolated from the chickens and Starlings. Together, the results suggest that the local environment may not be as important a source of contamination of free-range poultry as previously thought.

Genotypes most commonly isolated from the free-range broiler flocks were ST-828 complex (30.2% isolates), ST-661 complex (14.1%), ST-21(13.1%) complex and ST-573(12.1%) complex. Three hybrid STs with sequence originating from both *C. jejuni* and *C. coli*; ST-1487, ST-1090 and ST-1615 were identified, together with a hybrid allele, *tkt* 164.

Chapter 7: *Campylobacter* populations amongst free-range broiler flocks sampled at depletion.

7.1 Introduction.

Contamination arising from environmental sources such as wild birds, is often suggested as a cause for the spring peak in *Campylobacter* colonization in domestic chickens in temperate countries (Riordan *et al.* 1993; Broman *et al.* 2002; Karenlampi *et al.* 2003; Sopwith *et al.* 2006). The peak in colonization often precedes or coincides with a peak in the number of cases of human Campylobacteriosis and it may be that they are related in some way. The aims of the work described in this chapter were to continue to establish the population structure of *Campylobacter* within free-range chicken flocks in sufficient depth to allow detailed comparison with that identified in wild birds. Flocks were sampled in a contemporary time period and at the same location as the wild birds, both migratory species, studied in Chapters 4 and 5 in order that seasonal effects may be directly compared.

Free-range poultry flocks were reared on two Oxfordshire sites, Wytham and Northmoor, on a rolling production schedule. Neither site had previously been used for chicken rearing, and, due to the size of sampling involved, the study was unique in investigating various environmental influences that may shape the *Campylobacter* population within the flocks. Such environmental parameters included establishing the *Campylobacter* genotypes colonizing flocks reared on land free from contamination from chicken faeces, during the first crop rotation, and in contrast, the succession of *Campylobacter* genotypes colonizing flocks reared on land

contaminated with known genotypes in the following crop rotations. Chicken flocks were sampled shortly before depletion at 56 days, an age identified in Chapter 6 when they were highly likely to be colonized with multiple genotypes of *Campylobacter*. The much larger collection of isolates would allow more in depth and precise investigation into the population structure of *Camplobacter* colonizing free-range chickens. Detailed comparisons could be made with genotypes colonizing wild birds to determine whether they are a source of contamination and the cause of seasonal changes in domestic chicken flocks. In addition the study would give a detailed survey of the genotypes about to enter the first stage in free-range chicken meat preparation and potentially reach the human consumer.

7.2 Results.

A total of 2272 *Campylobacter* isolates were cultured from 78 free-range broiler flocks tested at depletion over a period of 18 months, using direct culture onto mCCDA plates. Of these, 881 isolates from the first 48 flocks were genotyped using the same MLST method and primers that were used in Chapter 6. As before, boiled cell suspensions were re-extracted using commercial Isoquick DNA extraction kits to enhance the quality of PCR reactions, and isolates were re-cultured and re-extracted if mixed sequencing traces were evident.

7.2.1 Shedding rate.

The shedding rate at depletion was consistently 80% or more of birds tested in all of the flocks, except flock 3a (70%), sampled in week seven in February, and flock 44 (72%) sampled in week 48, in late November (Figure 7.1). The shedding rates of flocks in the two study years were compared over 32 consecutive weeks, between

weeks five and 36 of the year. Over 70% (23 of 32) of flocks in the first year had 100% shedding rate amongst the birds tested compared to 37.5% (12 of 32) in the second year, although the average shedding rates were similar at 96.7% for the first year and 95.5% for the second year. A student's *t* test was calculated on the shedding rates of the two years matched by week, and gave a value of 0.75 and a *p* value of >0.1, giving evidence that the shedding rates over the 32 week period were not significantly different in the two different years.

There were small fluctuations in shedding rates of flocks throughout the year. Consistent levels of 100% shedding seen over a period of four weeks or more were apparent during weeks 14 to 21 (start of April to late May), and weeks 23 to 30 (start of June to late July) in the first year of the study. Levels fell to 80% for the first time in week 32 in mid August, and were seen until week 44 at the end of November, but they were interspersed by weeks where shedding levels were back to 90% or more. 100% shedding levels were not seen for a period of four weeks during the second year, but consistent rates of 95% shedding were apparent during weeks three to six (mid January to the start of February), eight to 12 (mid February to late March), 16-19 (mid April to mid May) and 24 to 30 (mid June to mid July) and 33-36 (mid August to the start of September). There were no times when shedding levels dropped consistently below 80%, although flocks were not tested after week 36 in September in the second year.

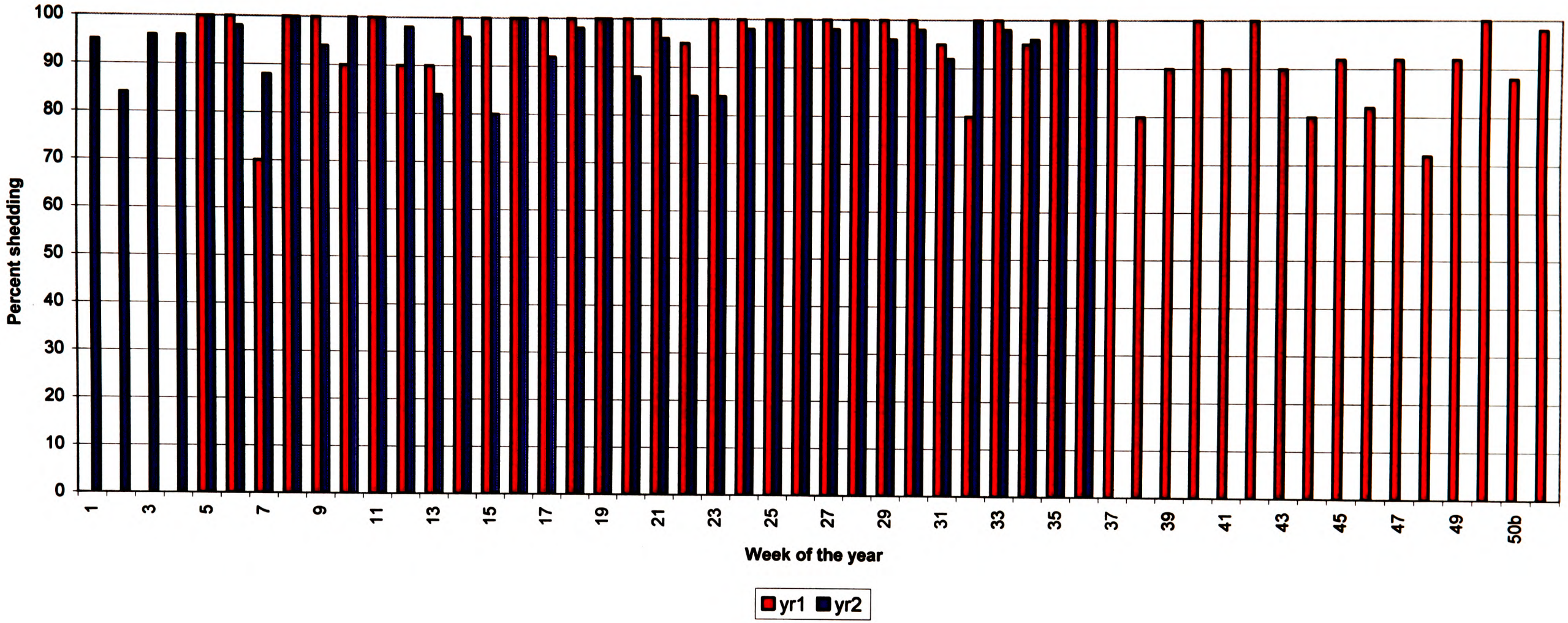

Figure 7.1. Shedding rate of *Campylobacter* species amongst consecutive free range broiler flocks over the period of 18 months.

Logistic regression analysis using sine and cosine models predicted the proportion of birds that would not be shedding *Campylobacter* if they were sampled over the course of a year (Figure 7.2). There is evidence to suggest that although proportions of birds shedding *Campylobacter* is high, the levels become significantly higher during spring and summer. The χ^2 value with two degrees of freedom indicating goodness of fit was 8.30, with a p value of 0.0158.

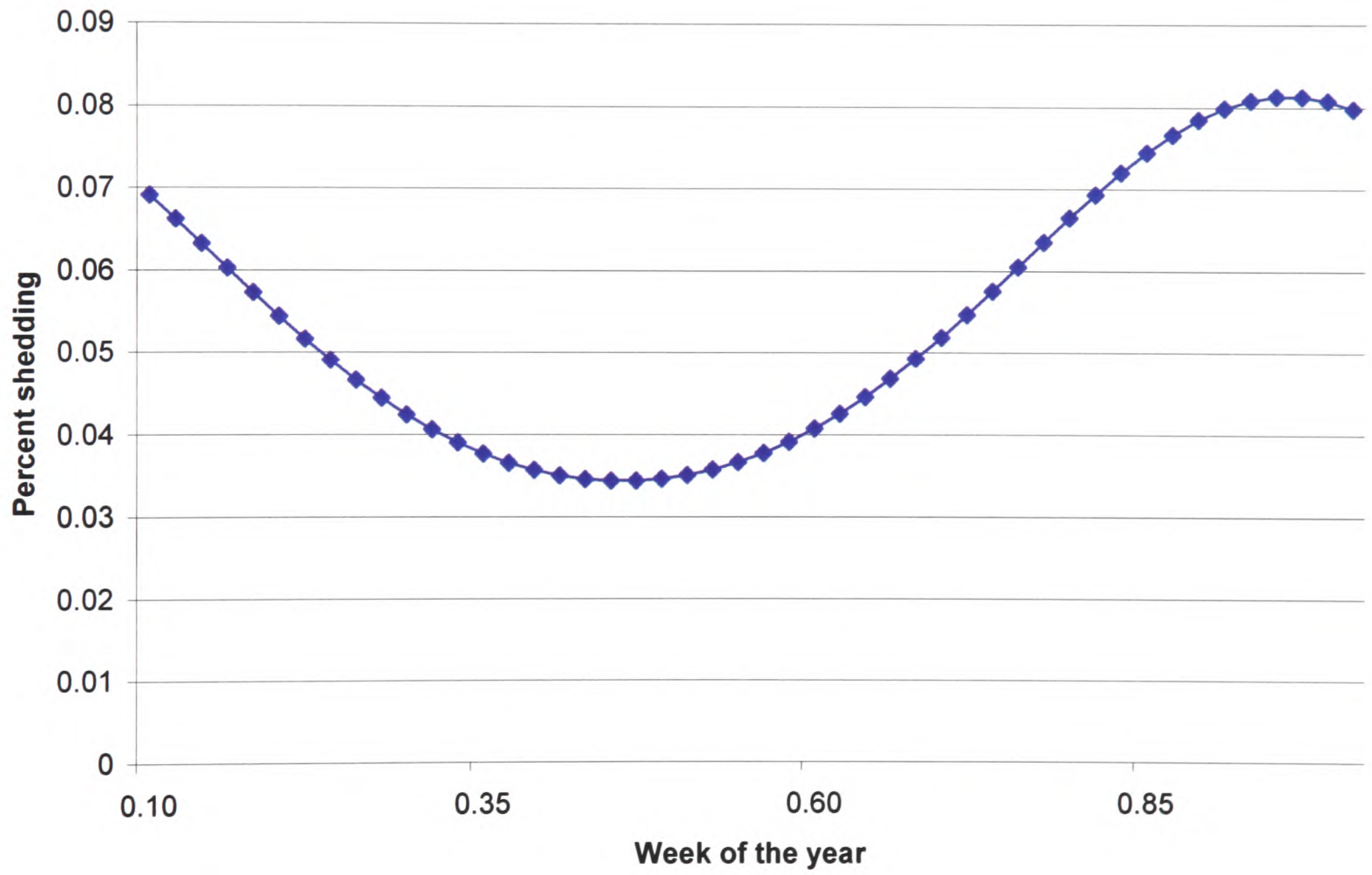

Figure 7.2. The predicted curve of the proportion of birds sampled being negative for *Campylobacter* over the course of a year, using the sine and cosine models of logistic regression analysis. A seasonal peak in the predicted number of birds shedding *Campylobacter* is seen between weeks 0.35 and 0.60, whilst the highest number of birds that are predicted not to be shedding *Campylobacter* are seen during the early and late weeks of the year. The χ^2 test gave evidence that the sine and cosine models indicative of cyclic trends were a good fit.

7.2.2 Distribution of clonal complexes.

The 881 isolates from the 48 flocks grouped into ten clonal complexes, eight *C. jejuni* and two *C. coli* (Figure 7.3). Nine clonal complexes had been previously identified, including ST-574 complex, ST-661 complex and ST-828 complex described in Chapter 6, but the ST-1150 *C. coli* complex was newly identified using data from this study, in combination with that archived on the *Campylobacter* MLST database. The ST-828 *C. coli* clonal complex was the most common, accounting for 346 of 881 (30.2%) isolates. ST-661 complex, ST-21 complex, ST-573 complex and unassigned isolates were isolated in roughly equal proportions of 10-14% of isolates, and the remaining clonal complexes accounted for less than 4% of isolates each. Seven of the ten clonal complexes were isolated from the longitudinal studies of flocks1 and 43 in Chapter 6, but three additional clonal complexes, ST-257 complex, ST-460 complex and the *C. coli* ST-1150 complex were isolated in this study testing a further 46 flocks.

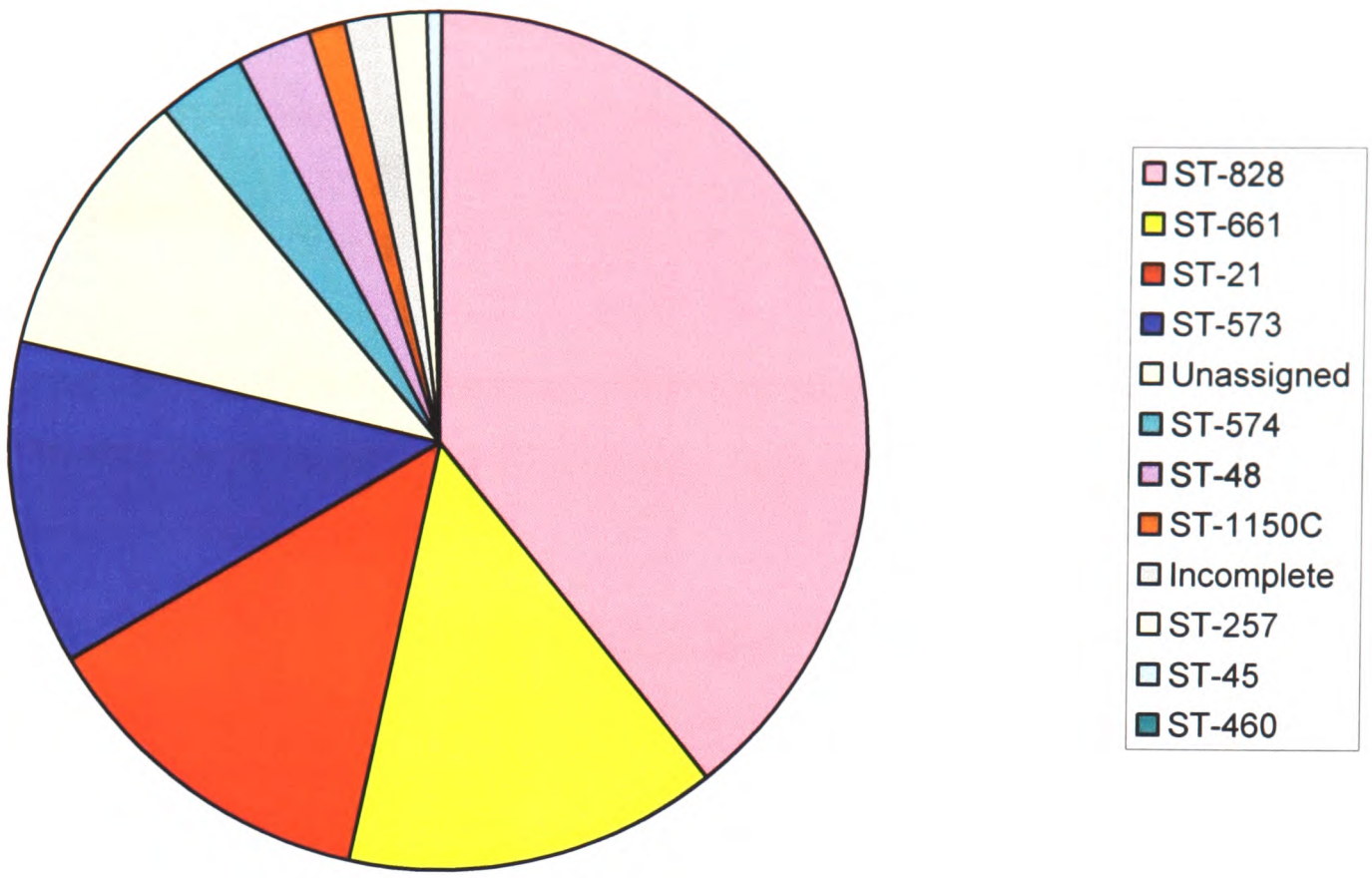

Figure 7.3. The *Campylobacter* clonal complexes isolated from 48 free-range flocks.
C= *C. coli.*

7.2.3 Distribution of STs.

There were 31 STs identified amongst isolates from the 48 flocks, of which 17 were *C. jejuni* and 14 *C. coli* (Table 7.1). ST-855 (828 complex), ST-573 (ST-573 complex) and ST-814 (ST-661 complex) were the dominant STs in terms of frequency of isolates, the number of flocks colonized and period of isolation. Only three STs, two *C. jejuni* and one *C. coli* could not be assigned to a clonal complex and they were completely unrelated to one another. The central genotype was isolated from five clonal complexes, but in the others only genetic variants were observed. The *C. coli* ST-828 complex was the largest in terms of number of variants, having 12. ST-21 complex was the next largest having five variants. The remaining clonal complexes were represented by only one or two variants. In all cases one variant was isolated much more frequently than the others, and it was not necessarily the central genotype

of the complex. Two of the 12 STs in the *C. coli* ST-828 complex, ST-855 and ST-854, were isolated at least 3.5 times more than the next most common variant. In general, the central genotype was isolated over the longest time period whilst single locus variants were the most frequently isolated (Table 7.2).

Table 7.1 .The composition of clonal complexes identified amongst 881 *Campylobacter* isolates from 48 broiler flocks in terms of STs, genetic variant and isolation period over the study.

Clonal complex	No. of isolates	ST	Complex variant[a]	No. flocks colonised	Frequency (%)	Isolation period (d)[b]
21	115	19	SLV	18	84(9.5)	186
		53	SLV	2	2(0.2)	65
		141	SLV	1	1(0.1)	1
		262	SLV	8	27(3.1)	49
		264	DLV	1	1(0.1)	1
45	5	45	C	1	5(0.6)	1
48	23	813	DLV	1	2(0.2)	1
		475	TLV	12	21(2.4)	147
257	12	257	C	4	12(1.4)	36
460	1	1538	DLV	1	1(0.1)	1
573	107	573	C	20	106(12.0)	252
		817	SLV	2	1(0.1)	1
574	31	574	C	13	31(3.5)	224
661	124	814	SLV	23	112(12.7)	246
		958	DLV	8	12(1.4)	56
828[c]	346	828	C	13	30(3.4)	126
		829	SLV	2	3(0.3)	35
		854	SLV	7	104(11.8)	151
		902	SLV	9	28(3.2)	63
		830	DLV	1	1(0.1)	1
		855	DLV	35	132(15.0)	238
		1089	DLV	7	22(2.5)	42
		1614	DLV	1	1(0.1)	1
		860	TLV	4	10(1.1)	49
		871	TLV	6	13(1.5)	91
		1088	TLV	1	1(0.1)	1
		1615	TLV	1	1(0.1)	1
1150[c]	14	1487	SLV	8	14(1.6)	112
Unassigned	90	1023	Singleton	1	1(0.1)	1
		1357	Singleton	1	1(0.1)	1
		1090[c]	Singleton	8	88(10.0)	105
Incomplete	13				13(1.5)	

C= central genotype, SLV=single locus variant, DLV=double locus variant, TLV= triple locus variant.

[a]The relationship to the central genotype. [b]The greatest number of days between sampling occasions on which the ST was isolated. [c]= *C. coli.*

Table 7.2 Summary statistics for the genetic variants within the clonal complex isolated amongst 881 *Campylobacter* isolates from broiler flocks.

Complex variant[a]	Average isolation period (days)	Longest period of isolation (days)	Number of STs	Frequency of isolates
C	127.8	252	5	184
SLV	90.9	246	10	376
DLV	42.6	238	8	172
TLV	57.8	147	5	46
U	35.7	105	3	107

C= central genotype, SLV=single locus variant, DLV=double locus variant, TLV= triple locus variant.
[a]The relationship to the central genotype.

The *C. jejuni* and *C. coli* STs grouped into separate clusters using splits analysis and Neighbour Joining trees, which was well supported by boot strap analysis. The alleles were generally 80% different between the species using the BLAST search function on the *Campylobacter* MLST database (Figure 7.4a). There was evidence of recombination between the STs within *Campylobacter* sub species and also between sub species to give three 'hybrid' genotypes (ST-1487, ST-1090 and ST-1615) (Figure 7.4b and Table 7.3). The allelic profile of ST-1487 contains five *C. coli* alleles, the *aspA* 103 allele from *C. jejuni* and the *tkt* 164 allele which is a *C. jejuni* - *C. coli* hybrid (section 7.2.5). ST-1090 and ST-1615 both contain six *C. coli* alleles and the *aspA* 87 allele which is also a *C. jejuni* – *C. coli* hybrid (section 6.2.5). Two of the hybrid genotypes appeared to be stable with ST-1090 being isolated 88 times and ST-1487 being isolated 14 times.

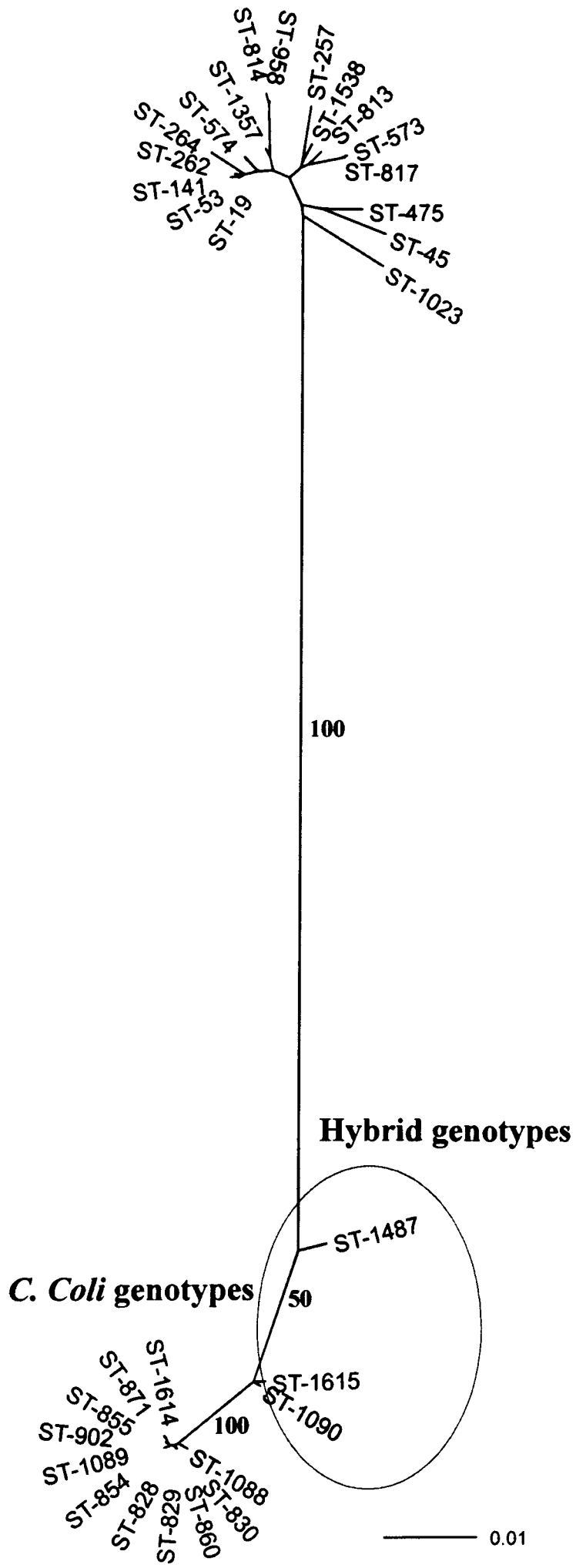

Figure 7.4a. A Neighbour Joining tree demonstrating the relationships between *C. jejuni*, *C. coli* and hybrid STs (circled) and giving bootstrap values (concatenated sequence).

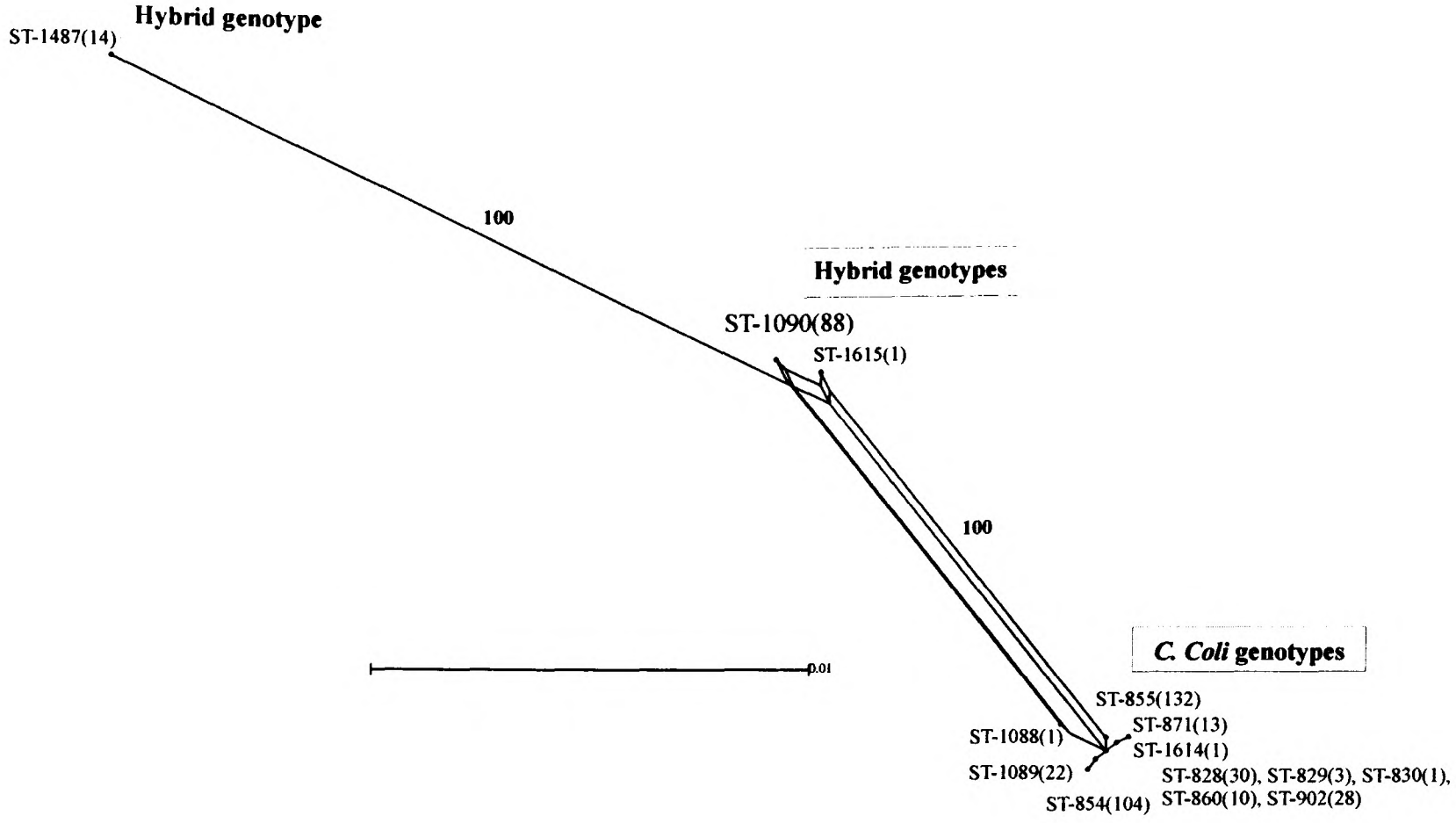

Figure 7.4b. Splits graph demonstrating recombination amongst *C. coli* and three hybrid STs. The frequency of isolation for each ST is given in brackets. Bootstrap values are given where they exceed 70%

Table 7.3. The allelic profiles of the hybrid genotypes with alleles from *C. jejuni*, *C. coli* and hybrid alleles from both species.

	aspA [a]	*glnA*	*gltA*	*glyA*	*pgm*	*tkt* [b]	*uncA*
ST-1487	103	110	103	140	188	164	79
ST-1090	87	39	30	82	189	44	74
ST-1615	87	39	30	79	104	35	17

[a]The *aspA* 103 allele originated in *C. jejuni*, the *aspA* 87 allele is a *C. jejuni-C. coli* hybrid.

[b]The *tkt* 164 allele is a *C. jejuni-C. coli* hybrid.

7.2.4 Succession of genotypes.

The ST-828 *C. coli* clonal complex was dominant in terms of frequency and number of genetic variants isolated over the year. It was also the most prevalent in colonizing the chicken

flocks at depletion, being isolated from 54 of the 64 flocks, including flock 1 (Figures7.5a and b). It was often present in high numbers, being isolated from more than 60% of the birds tested on 17 sampling occasions. Of the STs within the complex, ST-855 (ST-828 complex) was the dominant, and was isolated on 35 occasions from week 14 onwards (Figures 7.6a and 7.6b). On four of the 35 occasions, 60% or more of chickens tested were colonized by ST-855 (ST-828 complex). ST-854 (ST-828 complex) was the first ST from the complex to be isolated and it was seen on six occasions between weeks five to 11, and once again on week 25, after which it was not seen again. Some of the other STs within the complex could be isolated over several weeks, but then disappeared, for example ST-902 (ST-828 complex) was isolated only between weeks 12 to 21, and ST-1089 (ST-828 complex) was isolated during weeks 31 to 40 only (Figures 7.6a and b). STs such as ST-829 (ST-828 complex) and ST-830 (ST-828 complex) were seen sporadically and at low frequency.

The next most frequently isolated complexes were ST-661 complex colonizing 29 flocks, ST-21 complex colonizing 28 flocks, ST-573 colonising 21 flocks, ST-48 complex colonising 14 flocks, and ST-574 colonizing 13 flocks (Figures 7.5a and b). Despite being widespread, ST-661 complex was often present in small numbers, and there were only four occasions where it was isolated from more than 60% of the chickens tested. The complex was first seen at the end March and during April, but was more commonly detected after August and through the latter half of the year. Of the STs within the complex ST-814 (ST-661 complex) was the predominant ST, particularly through the beginning and end of the study period, and was identified nine times more often than the other complex variant, ST-958 (ST-661 complex) which was confined to weeks 32 to 40 (Figures 7.6a and b).

There were six occasions during the first part of the year, until May, where ST-21 complex, and predominantly ST-19 (ST-21 complex) was isolated from more than 60% of birds within a flock (Figures 7.5a and b and 7.6a and b). The ST-21 complex was rarely

detected during the summer months, but was seen in low numbers from the end of October where ST-262 (ST-21 complex) became the predominant ST and ST-19 (ST-21 complex) was absent.

ST-573 complex was first seen in April and it was present sporadically, and usually in small numbers, although during October and early November there were three occasions where it was isolated from more than 60% of birds tested (Figures 7.5a and b). The central genotype was the most common, with the other variant from the complex only being detected on one occasion (Figures 7.6a and b).

ST-48 complex was always present in small numbers and was seen only between March and August (Figures 7.5a and b). ST-574 (ST-574 complex) was first detected in May but was never isolated from more than 20% of birds tested. ST-257 complex was present in only four flocks and ST-45 and ST-460 complexes were detected in one flock each. None of these complexes was isolated from more than 50% of birds tested in a flock.

Variants from the same complex were not often isolated in the same flock. The exceptions were seen amongst the dominant complexes, and included ST-19 and ST-141 (ST-21 complex) isolated from flock 5, ST-53 and ST-264 (ST-21 complex) from flock 18b, ST-814 and ST-958 (ST-661 complex) from flocks 34 and 35, and members of the ST-828 *C. coli* complex with 19 of 66 possible combinations being isolated together (Table 7.4). Each of the ST-828 complex STs were co-isolated with at least one other ST from the complex, although the dominant STs 828 and 855 were the most common to be found in co-colonization.

Table 7.4. Number of occasions when co-colonization of a flock by *C. coli* STs occurred.

	ST											
ST	**828**	**829**	**830**	**854**	**855**	**860**	**871**	**902**	**1088**	**1089**	**1614**	**1615**
828	-	-	-	-	-	-	-	-	-	-	-	-
829	**2**	-	-	-	-	-	-	-	-	-	-	-
830	**1**	0	-	-	-	-	-	-	-	-	-	-
854	0	0	0	-	-	-	-	-	-	-	-	-
855	**8**	**2**	**1**	0	-	-	-	-	-	-	-	-
860	**3**	0	**1**	0	**2**	-	-	-	-	-	-	-
871	0	0	0	**1**	**4**	0	-	-	-	-	-	-
902	0	0	0	0	**7**	0	0	-	-	-	-	-
1088	0	0	0	0	0	0	0	0	-	-	-	-
1089	**4**	**1**	0	0	**2**	**2**	**1**	0	0	-	-	-
1614	0	0	0	**1**	0	0	**1**	0	0	0	-	-
1615	0	0	0	0	0	0	0	0	**1**	0	0	-

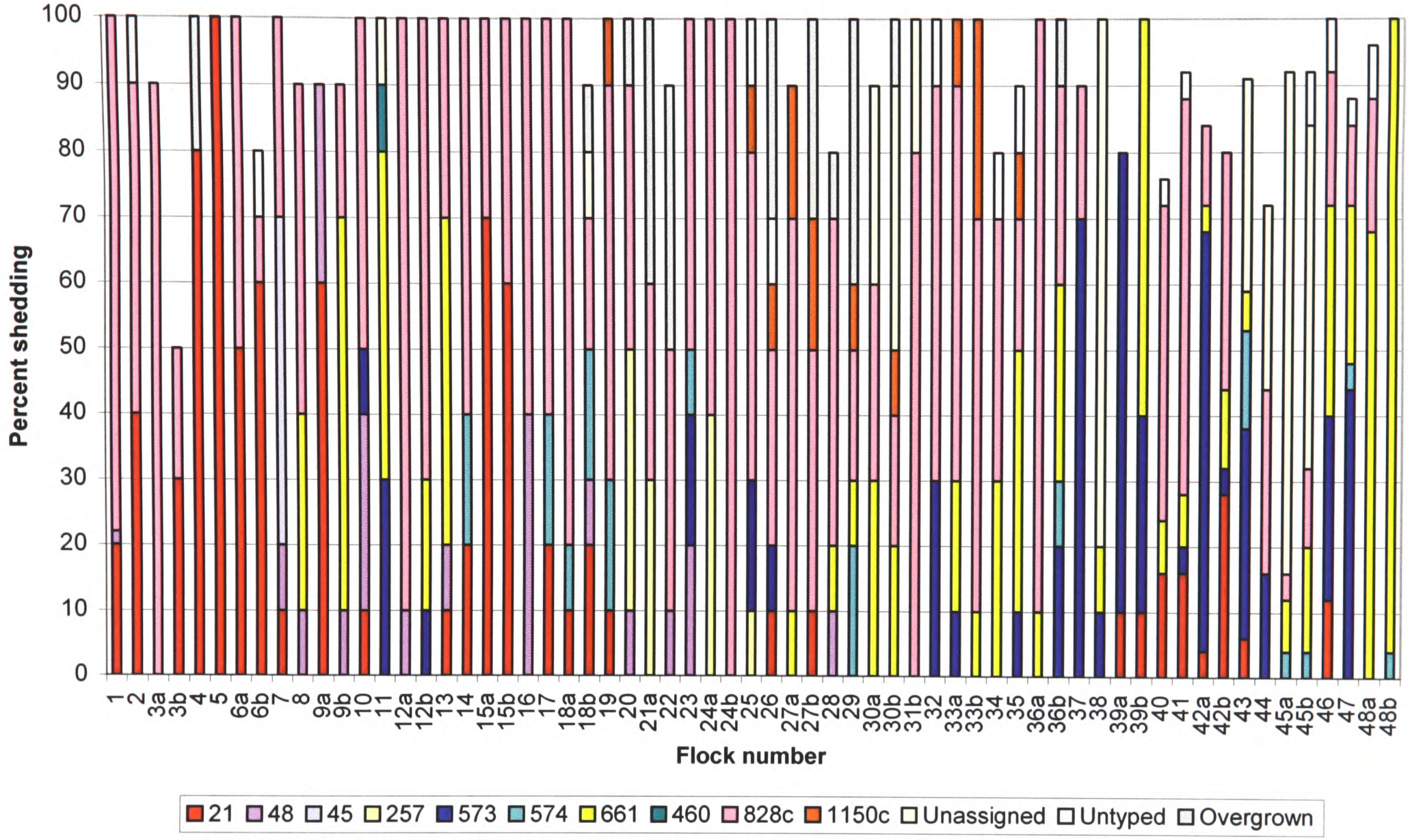

Figure 7.5a. ***Campylobacter*** **clonal complexes isolated from 48 free-range chicken flocks.** C=C. coli

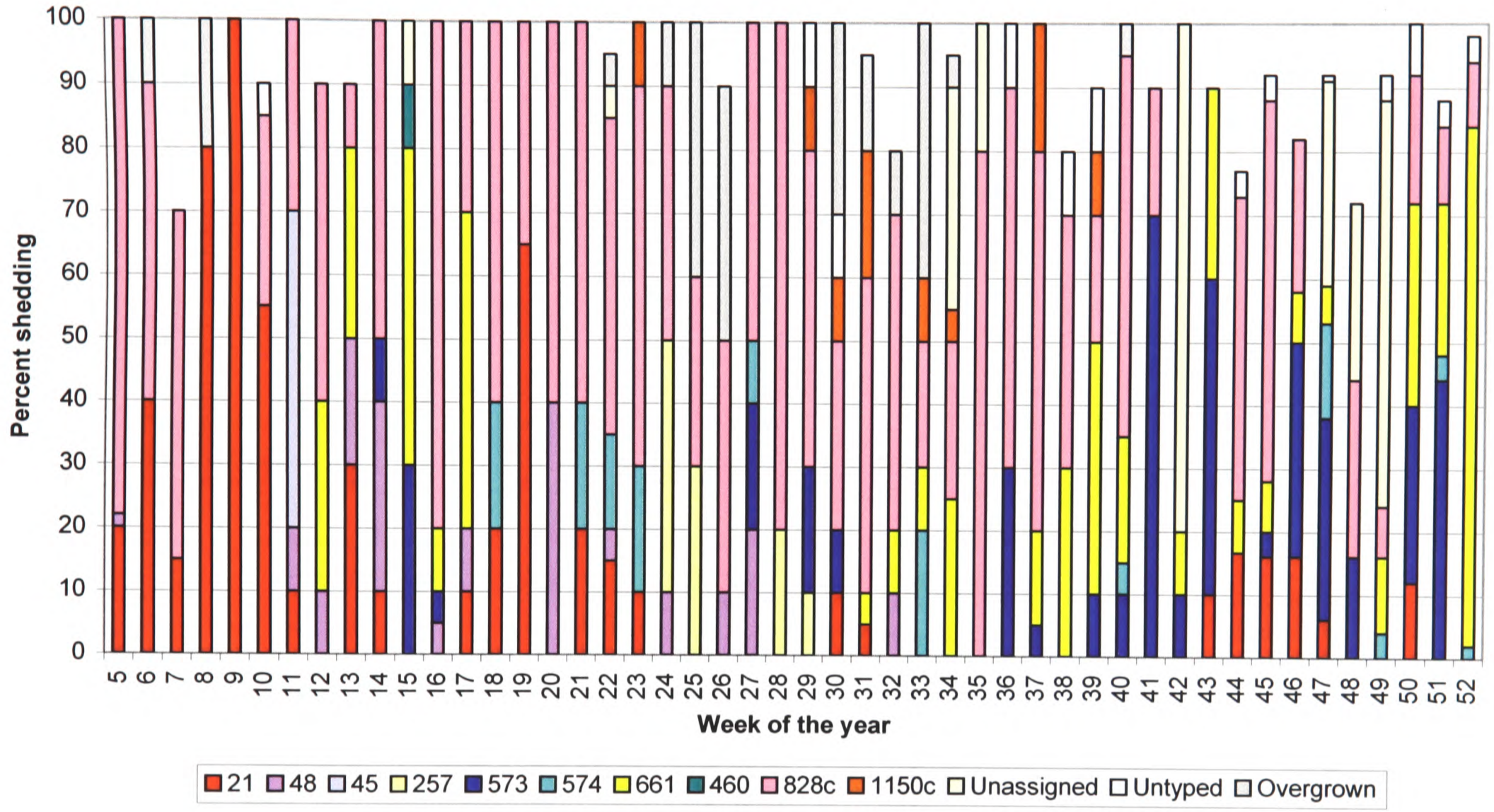

Figure 7.5b. ***Campylobacter*** **clonal complexes isolated during weeks of the year.** *C = C. coli.*

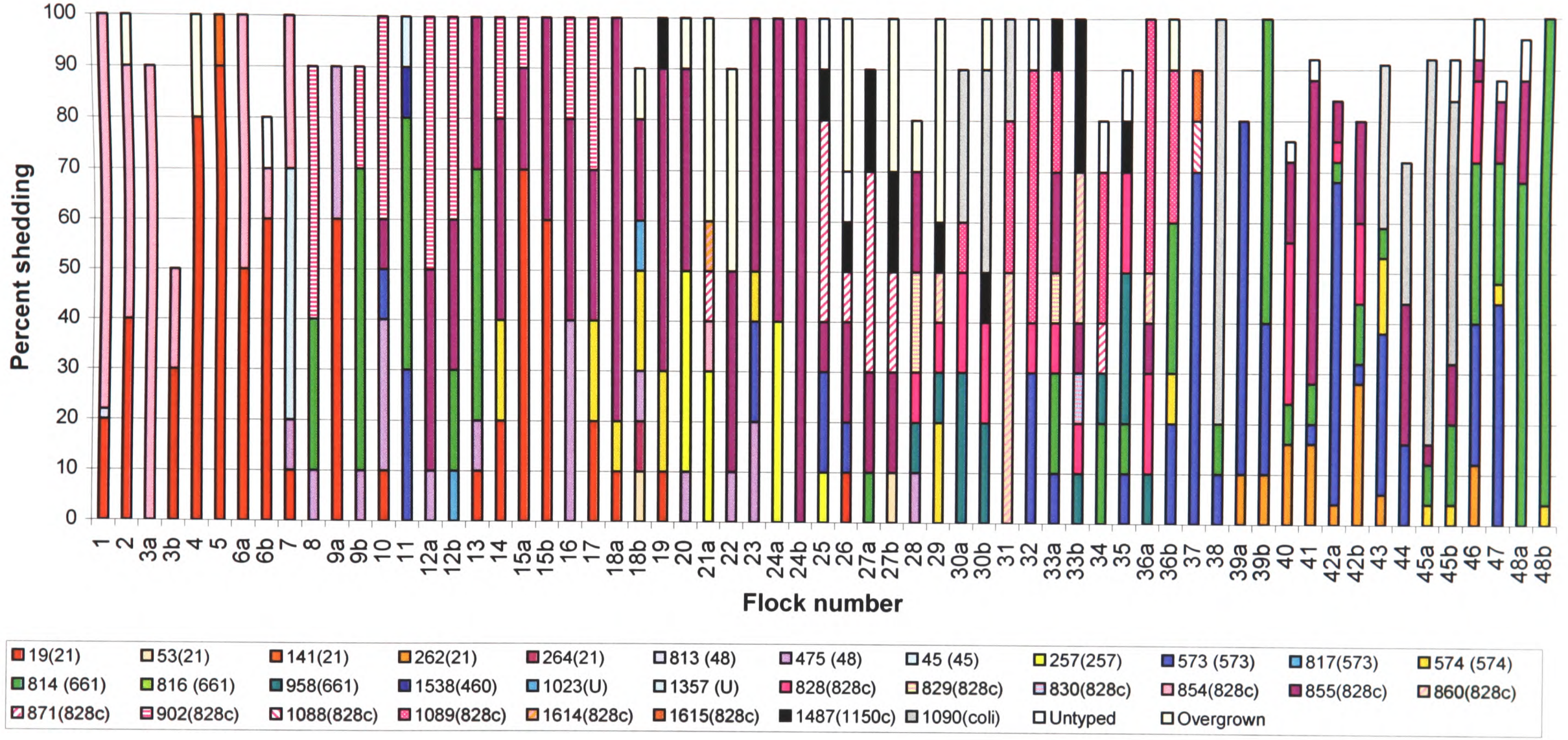

Figure 7.6a. *Campylobacter* STs isolated from 48 free-range chicken flocks. C = *C. coli.*

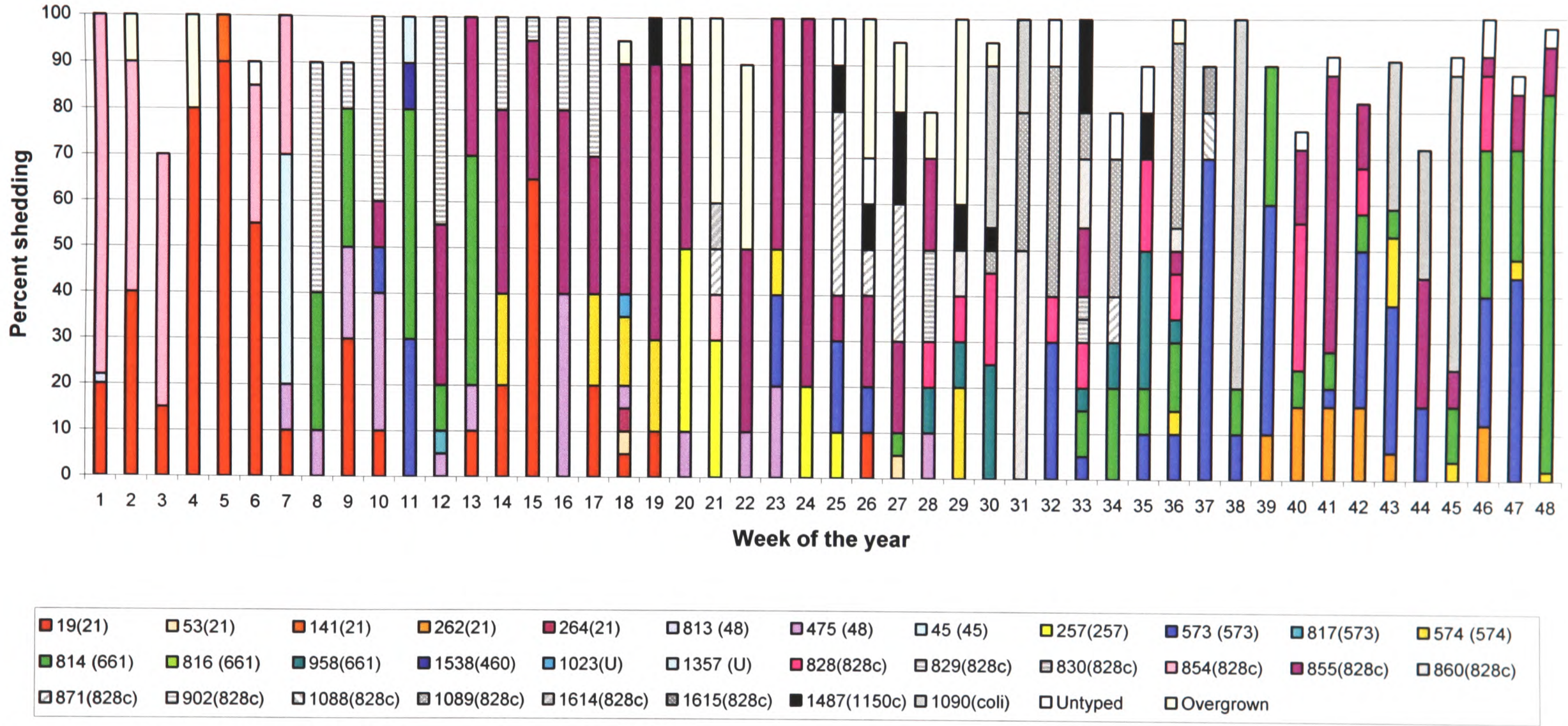

Figure 7.6b. *Campylobacter* STs isolated during weeks of the year. C = *C. coli*

7.2.5 Allelic diversity.

The hybrid *aspA* 87 allele identified amongst chicken isolates in Chapter 6 was also isolated amongst chicken isolates from this chapter as part of the *C. coli* ST-1090 (unassigned) and ST-1615 (ST-828 complex). Another hybrid allele *tkt* 164 was identified as part of the *C. coli* ST-1487 (Figures 7.7a and 7.7b). The *tkt* 164 allele has multiple polymorphic sites in common with both the *C. jejuni tkt* 5 allele and the *C. coli tkt* 43 allele, but it does not appear to be a simple mosaic between them both. ST-1487 also contained the *aspA* 103 allele thought to have originated in *C. jejuni*. The number of alleles at each locus ranged from nine at the *aspA* and *uncA* loci to 15 at the *tkt* locus when both *Campylobacter* species were combined (Table 7.5). The number of alleles amongst *C. jejuni* isolates ranged from six at the unc locus to ten at the *pgm* locus amongst *C. jejuni* isolates. The number of alleles ranged from two at the *aspA* locus to six at the *tkt* locus amongst *C. coli* isolates. The number of variable sites varied from 52 (12.9%) at the *gltA* locus to 98 (19.3%) at the *glyA* locus for both *Campylobacter* species. The number of variable sites varied from six (1.2%) at the *uncA* locus to 34 (6.8%) at the *pgm* locus amongst *C. jejuni* isolates. The number of variable sites varied from 2 (0.4%) at the *glyA* locus to 37 (7.8%) at the *aspA* locus (with divergent allele 87 included) amongst *C. coli* isolates. The d_N/d_S values were all considerably below 1 indicating the lack of positive selection at each of the loci.

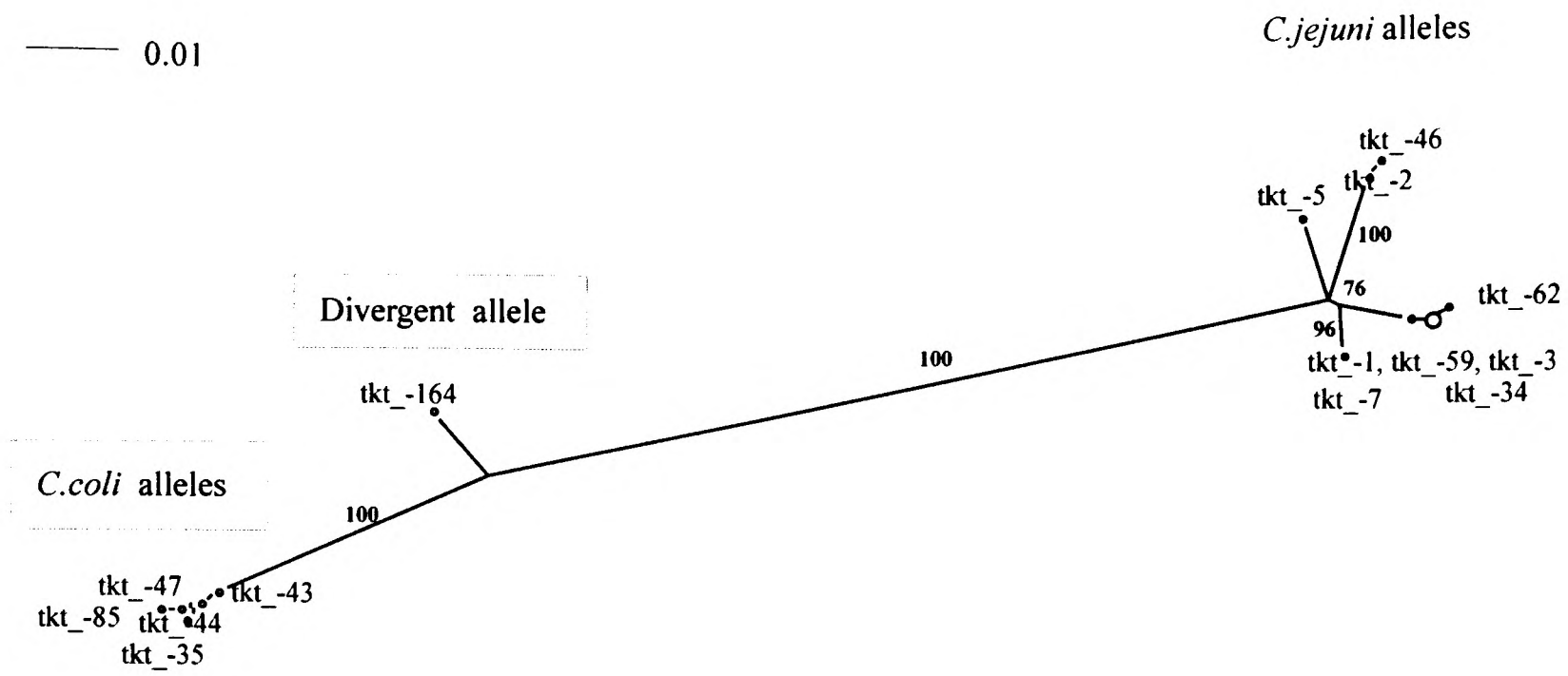

Figure 7.7a. A splits graph demonstrating the relationship of the divergent *tkt* allele 104 with other alleles identified amongst *C. jejuni* and *C. coli* isolates.

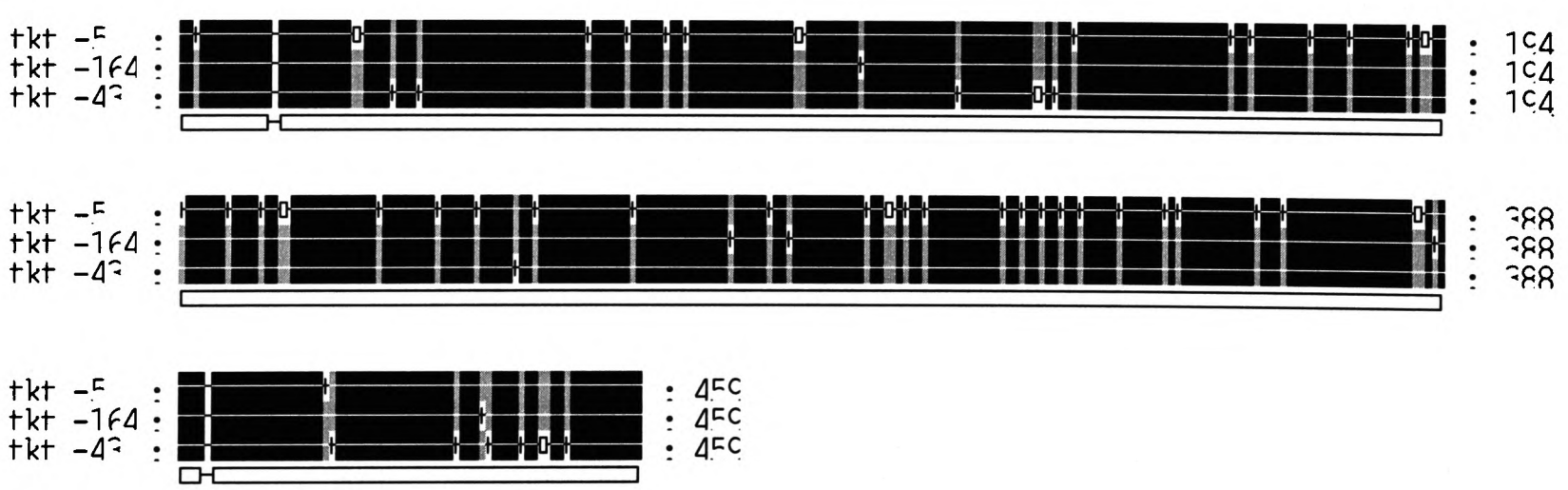

Figure 7.7b. Alignment of the *C. jejuni tkt* 5 alleles, the *C. coli tkt* 43 allele and the hybrid *tkt* 164 allele. Multiple polymorphic sites are apparent amongst the three alleles.

Table 7.5. Diversity of alleles amongst *Campylobacter* isolated from free-range poultry flocks at 56 days of age.

Locus	Fragment size (bp)	No. of alelles			No. of variable sites			% variable sites			d_N/d_S ratio		
		jej	*coli*	*total*	*jej*	*coli*	*total*	*jej*	*coli*	*total*	*jej*	*coli*	*total*
aspA[a]	477	7	2	9	14	37	62	2.9	7.8	13.0	0.011	0.080	0.044
glnA	477	9	4	13	15	3	63	3.1	0.7	13.2	0.013	0.003	0.063
gltA	402	8	3	11	13	2	52	3.2	0.5	12.9	0.011	0.003	0.059
glyA	507	9	3	12	31	2	98	6.1	0.4	19.3	0.024	0.003	0.090
pgm	498	10	4	14	34	13	91	6.8	2.6	18.3	0.028	0.013	0.086
tkt[b]	459	9	6	15	27	24	83	5.9	5.2	18.1	0.023	0.002	0.089
unc	489	6	3	9	6	4	74	1.2	0.8	15.1	0.005	0.005	0.083
all loci[c]	3309	17	14	31	140	106	523	4.2	0.3	15.8	0.013	0.008	0.080

[a] = *aspA* 103 was classified as *C. jejuni* although it was identified as part of the *C. coli* ST 1487. The hybrid *aspA* 87 allele is included in calculations for *C. coli* alleles.

[b] = the divergent *tkt* 164 allele was included in calculations for *C. coli* alleles.

[c] = The hybrid ST-1487 was included in calculations for *C. coli* alleles in the 'all loci' row

7.2.6 Antigenic diversity.

A total of 27 *flaA* SVR types were identified amongst *Campylobacter* isolates from the 48 flocks (Table 7.6). Of these, 12 were found only amongst *C. jejuni* isolates and another eight only with *C. coli* isolates. Seven *flaA* SVR types were associated with both *C. jejuni* and *C. coli* STs. Of the shared *flaA* SVR alleles, six predominated with a 71% share or more in one of the species over the other, with three being prevalent amongst *C. jejuni* and three being prevalent amongst *C. coli*. One, *flaA* SVR type 312-4, was isolated on two occasions, once in association with *C. jejuni* and once in association with *C. coli*.

Table 7.6. Distribution of *flaA* SVR alleles amongst *Campylobacter* species.

flaA SVR allele-peptide	Frequency *C. jejuni*	*C. coli*
8-1	112	-
32-1	7	-
34-1	1	-
36-1	85	6
37-1	1	-
40-1	-	1
41-1	2	-
66-1	1	198
89-1	-	1
105-1	22	9
325-1	-	30
415-1	-	1
453-1	-	1
21-2	9	-
312-4	1	1
15-5	4	-
67-8	1	-
314-8	7	-
17-11	-	4
30-11	1	56
16-12	12	-
319-15	1	100
86-18	1	-
100-33	-	2
191-33	123	5
80-48	1	-
351-111	-	12

The majority of *flaA* SVR types found in association with more than one ST were not linked with a particular clonal complex and were often spread over several, although three *flaA* SVR types were seen only in association with STS from the large *C. coli* ST-828 complex (Table 7.7). Some *flaA* SVR types were found in association with up to six different STs, although they were often not evenly distributed, and seen only at low frequency with some STs. For example *flaA* SVR type 8-1 was most frequently seen in association with ST- 573 (82 of 106 isolates) and ST-574 (29 of 31 isolates), but only once with ST-958 (one of 12 isolates), part of ST-661 complex. Nineteen of 31 (60%) STs were linked with only one *flaA* SVR type, but the remainder were found in association with up to eight *flaA* SVR types of varying peptide

as well as nucleotide type. For example ST-855 was associated with five different nucleotide variations of peptide one, one nucleotide variation of peptide 11 and two nucleotide variations of peptide 33.

Table 7.7. Distribution of *flaA* SVR types amongst *Campylobacter* STs isolated from the 48 free-range chicken flocks.

Clonal complex	ST	Complex variant[a]	Freq	*flaA* SVR allele-peptide (frequency)
21	19	SLV	84	36-1(83), 319-15(1)
	53	SLV	2	32-1(2)
	141	SLV	1	36-1(1)
	262	SLV	27	37-1(27)
	264	DLV	1	32-1(1)
45	45	C	5	15-5(4), 36-1(1)
48	813	DLV	2	41-1(2)
	475	TLV	21	105-1(21)
257	257	C	12	16-12(12)
460	1538	DLV	1	34-1(1)
573	573	C	106	8-1(82),314-8(7),191-33(11),32-1(4),67-8(1),86-18(1),
	817	SLV	1	32-1(1)
574	574	C	31	8-1(29), 66-1(1), 321-4(1)
661	814	SLV	112	191-33(111), 30-11(1)
	958	DLV	12	21-2(9), 105-1(2), 8-1(1)
828[C]	828	C	30	325-1(22), 66-1(5), 30-11(2), 312-4(1)
	829	SLV	3	17-11(3)
	854	SLV	104	319-15(100), 36-1(4)
	902	SLV	28	66-1(28)
	855	DLV	132	66-1(76),30-11(38),105-1(8),191-33(5),100-33(2),325-1(1),453-1(1),36-1(1)
	830	DLV	1	325-1(1)
	1089	DLV	22	415-1(21),40-1(1)
	1614	DLV	1	30-11(1)
	860	TLV	10	325-1(5), 30-11(2), 415-1(1),17-11(1), ,89-1(1)
	871	TLV	13	30-11(13)
	1088	TLV	1	66-1(1)
	1615	TLV	1	105-1(1)
1150[C]	1487	SLV	14	351-111(12), 36-1(1), 325-1(1)
Unassigned	1023	Singleton	1	80-48(1)
	1357	Singleton	1	191-33(1)
	1090[C]	Singleton	88	66-1(88)
Incomplete			13	Not tested

C= central genotype, SLV=single locus variant, DLV=double locus variant, TLV= triple locus variant.

[a]The relationship to the central genotype. C = *C. coli.*

7.2.7 Diversity and accumulation of STs on the farm sites.

Between one and seven STs were identified per flock, and the modified Simpson's Index of Diversity was calculated amongst the flocks and plotted per week of the year (Figure 7.8). The level of diversity varied from 0, in week eight in mid February to 0.95 in week 30 at the end of July. The level of diversity was generally below 0.8, and only exceeded this between weeks 29 (mid July) and 37 (mid September). Average levels of diversity per quarter were; February-March 0.47, April-June 0.68, July-September 0.74 and October-December 0.60. Logistic regression analysis gave a p value of 0.0045 giving evidence that there was variability in the *Campylobacter* diversity over the year. Predicted values using the sine and cosine models gave a peak in diversity at week 30 at the end of July (Figure 7.9)

Dividing the first year's flocks into quarters revealed that 13 new STs were seen in flocks 1 to 12, eight new STs were seen in flocks 13 to 24, seven new STs were seen in flocks 25 to 36 and four new STs were seen in flocks 37 to 48 (Figure 7.10). Introduction of new STs in the flocks appeared to be random events (Table 7.8). Up to three new *Campylobacter* genotypes were introduced at a time by particular flocks, but this did not appear to influence whether or not new STs may be seen in consecutive flocks. Eight flocks had 'rare' STs seen only once on the farm sites, with three flocks (flocks 8 and 22, both at Northmoor, and flock 37 at Wytham) having two new 'rare' STs at the same time.

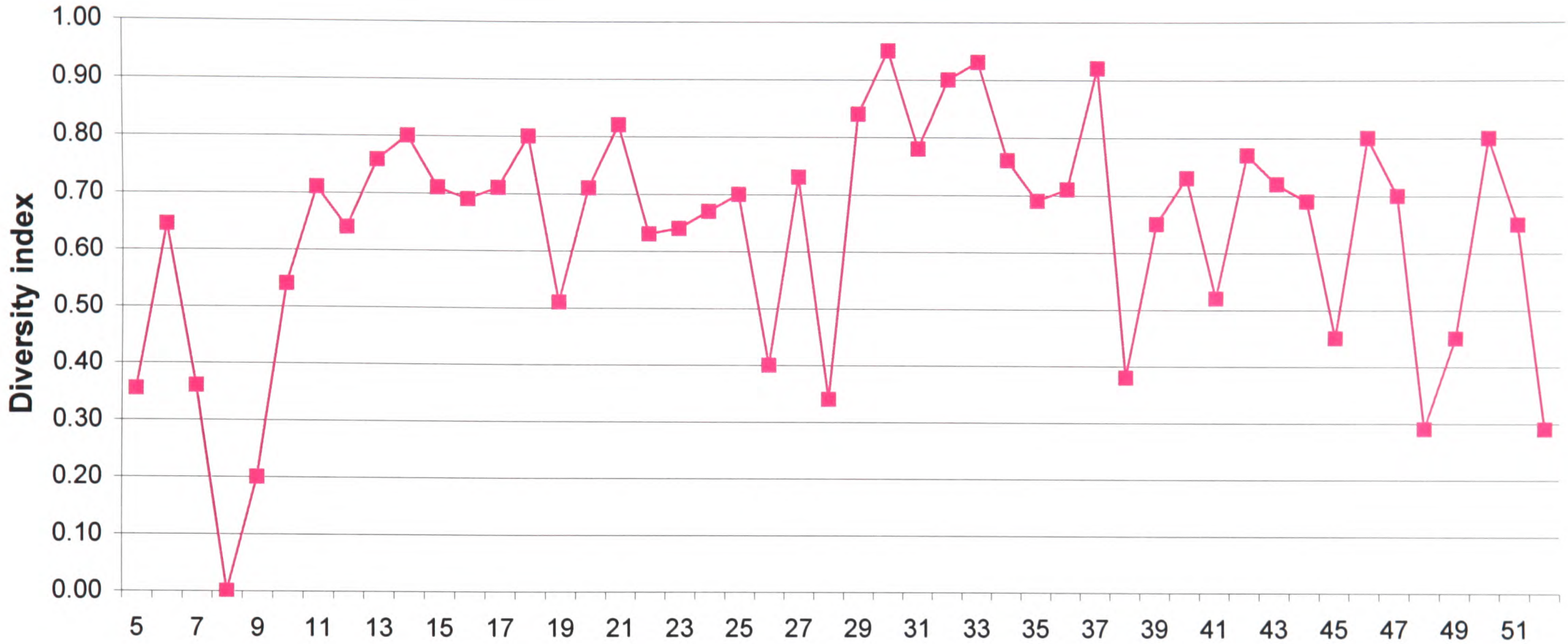

Figure 7.8. The genotype diversity per flock *vs* the week of the year, calculated using the modified Simpson's Index of Diversity.

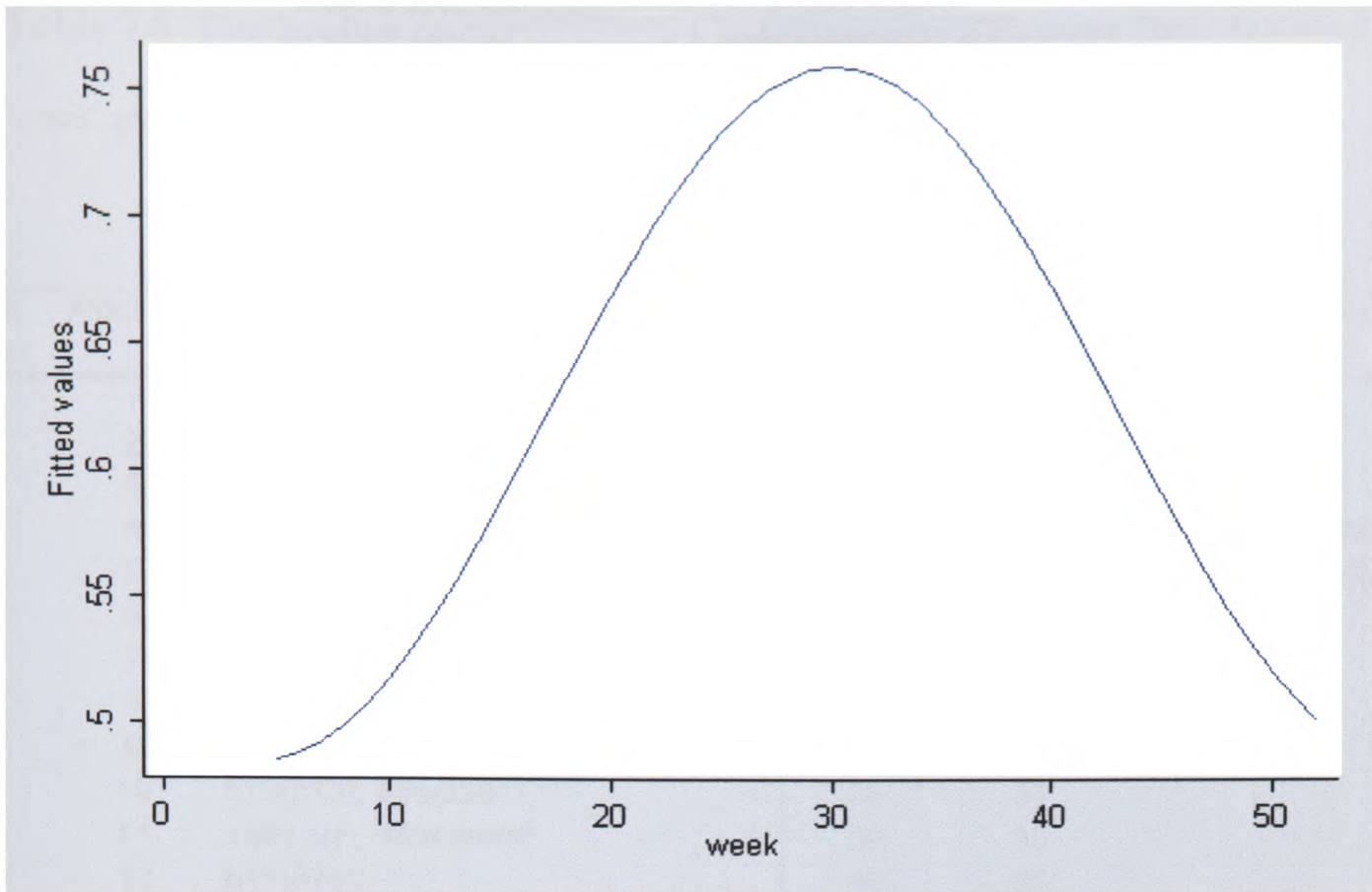

Figure 7.9. The predicted values of *Campylobacter* diversity using logistic regression analysis and the sine/cosine model. A seasonal peak in the predicted diversity of *Campylobacter* genotypes was seen at week 30 (July). The χ^2 test gave evidence that the sine and cosine models indicative of cyclic trends were a good fit.

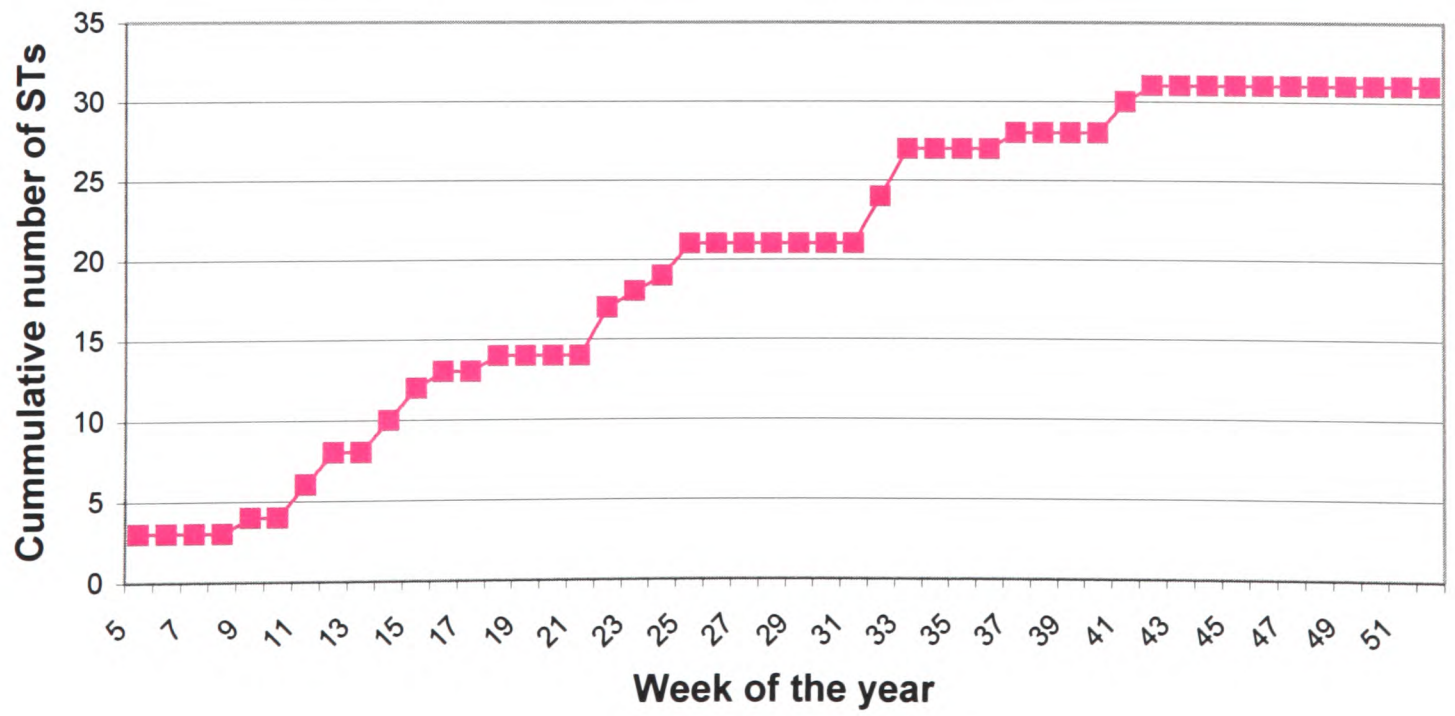

Figure 7.10. The accumulation of new STs amongst the 48 free-range broiler flocks. New STs were acquired by flocks in clusters.

Table 7.8 The broiler flocks in which *Campylobacter* STs were first detected.

[a] 'rare' genotypes isolated from one flock only. C = *C. coli.*

Week of year	Flock no	New STs acquired (clonal complex)	Week of year	Flock no	New STs acquired (clonal complex)
5	1	19(21), 813(661)[a], 854(828c)	29	25	
6	2		30	26	
7	3		31	27	
8	4		32	28	958(661), 828(828c), 829(828c)
9	5	141(21)[a]	33	29	860(828c), 1089(828c), 1090(coli)
10	6		34	30	
11	7	475(48), 45(45)[a]	35	31	
12	8	902(828c), 814(661)	36	32	
13	9		37	33	830(828c)[a]
14	10	573(573), 855(828c)	38	34	
15	11	1367(U)[a], 1538(460)[a]	39	35	
16	12	817(573)	40	36	
17	13		41	37	1615(828c)[a], 1088(828c)
18	14	574(574)	42	38	
19	15		43	39	262(21)
20	16		44	40	
21	17		45	41	
22	18	53(21), 264(21)[a], 1023(U)[a]	46	42	
23	19	1487(1150c)	47	43	
24	20	257(257)	48	44	
25	21	1614(828c)[a], 871(828c)	49	45	
26	22		50	46	
27	23		51	47	
28	24		52	48	

7.2.8 Comparison of *Campylobacter* colonization at the two farm sites, Wytham and Northmoor.

7.2.8.1 Shedding rates.

The average shedding rate over the year was 90.9% of birds tested per flock at Wytham, and 93.75% of birds tested per flock at Northmoor. When data was divided into successive rotations and the shedding rate averaged, rates were higher at Northmoor on five of eight occasions (Figure 7.11). However the differences in

Campylobacter shedding rate between the two sites was not statistically significant, $p = 0.1517$.

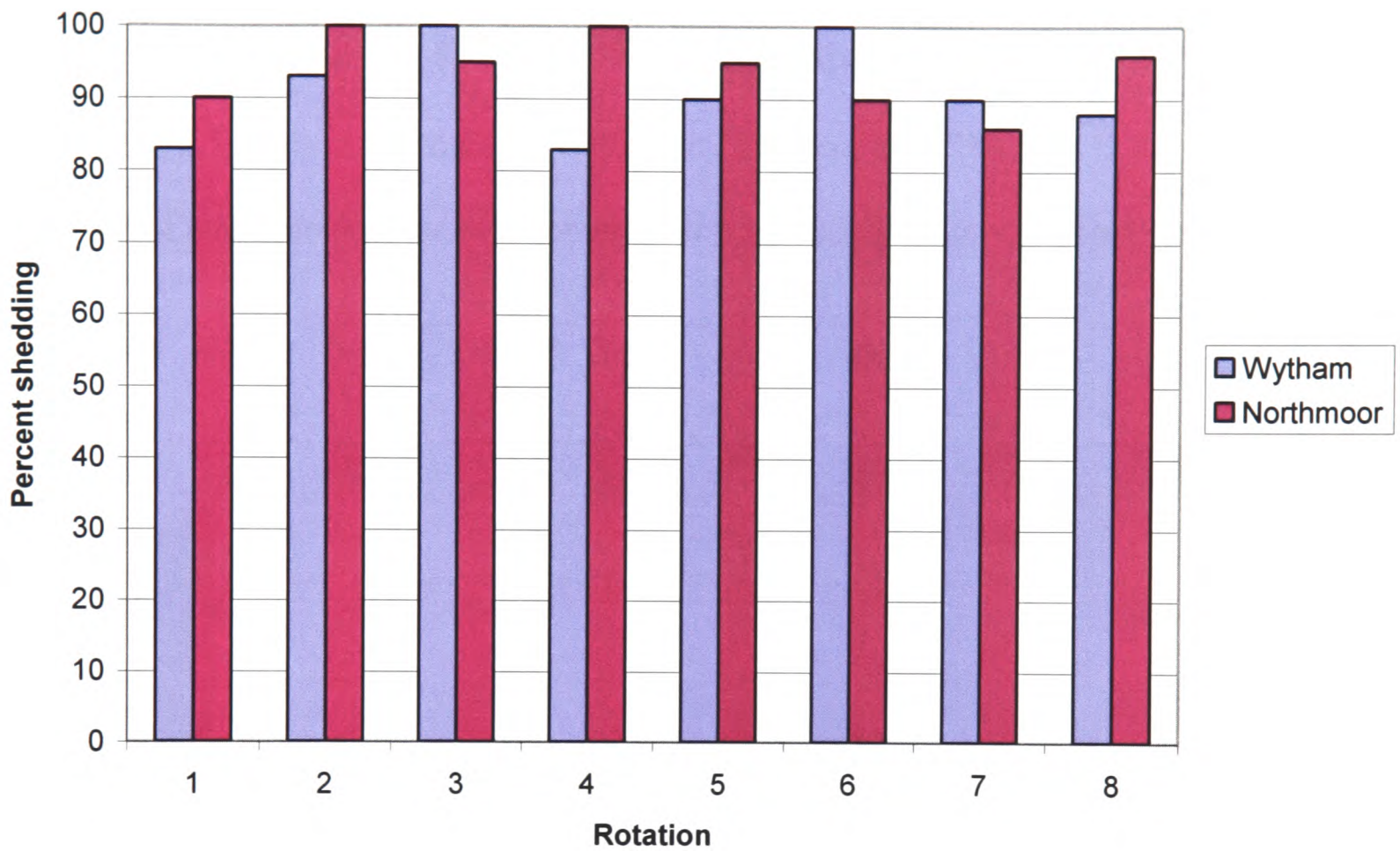

Figure 7.11. The percentage of birds within free-range broiler flocks at Wytham and Northmoor that were shedding *Campylobacter* species.

7.2.8.2 Clonal complex distribution.

Eight of the ten clonal complexes were identified at both sites. The remaining two complexes were isolated infrequently and at one farm site only, ST-45 complex being isolated only at Wytham and ST-460 complex being isolated only at Northmoor (Figure 7.12). The frequency of isolation for seven of the eight shared clonal complexes did not differ largely between farm sites, but ST-661 complex, as well as its individual STs, was isolated nearly three times more frequently from Northmoor

than from Wytham (Figure 7.13). In addition the unassigned isolates were isolated seven times more frequently at Wytham, largely as a result of a single ST, ST-1090 being much more common at Wytham. Although the ST-828 complex was isolated from both farm sites in high frequency, the proportions of ST-854 and ST-855 were varied greatly at both sites, ST-854 being 12.8 times more common at Wytham and ST-855 being 2.5 times as common at Northmoor. The overall differences in the distribution of clonal complexes between the two farm sites was not significant, p = 0.5.

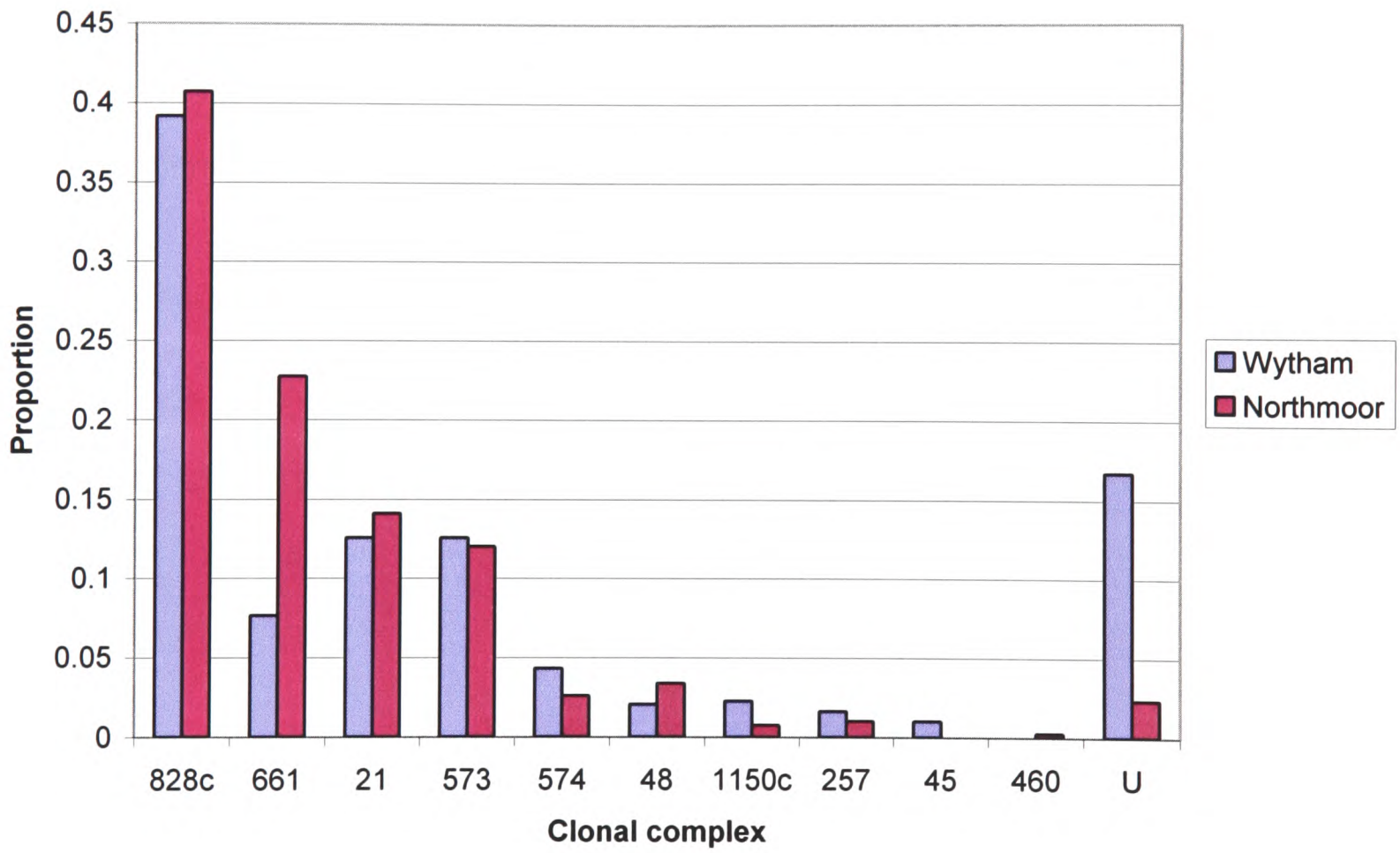

Figure 7.12. The distribution of clonal complexes at Wytham and Northmoor, shown as a proportion of the total number of *Campylobacter* isolates collected at each site. C = *C. coli.* U = isolates that could not be assigned to a clonal complex.

7.2.8.3 ST distribution.

Nineteen of 31 STs were detected at both sites, although six STs including ST-813 (ST-48 complex) and ST-45 (ST-45 complex) both *C. jejuni*, and ST-830, ST-1088, ST-1614 and ST-1615, all part of the ST-828 *C. coli* complex were unique to Wytham, and a further six, ST-141 (ST-21 complex), ST-264 (ST-21 complex), ST-817 (ST-573 complex), ST-1538 (ST-460 complex), ST-1023 (unassigned) and ST-1357 (unassigned), all *C. jejuni* were unique to Northmoor. All of these STs were rarely seen, and the majority were only isolated once. The overall differences in the distribution of STs between the two farm sites was not significant, $p = 0.5$. The distribution of individual STs appeared to be clustered in time rather than with farm site, with each rotation being a three week period (Table 7.9).

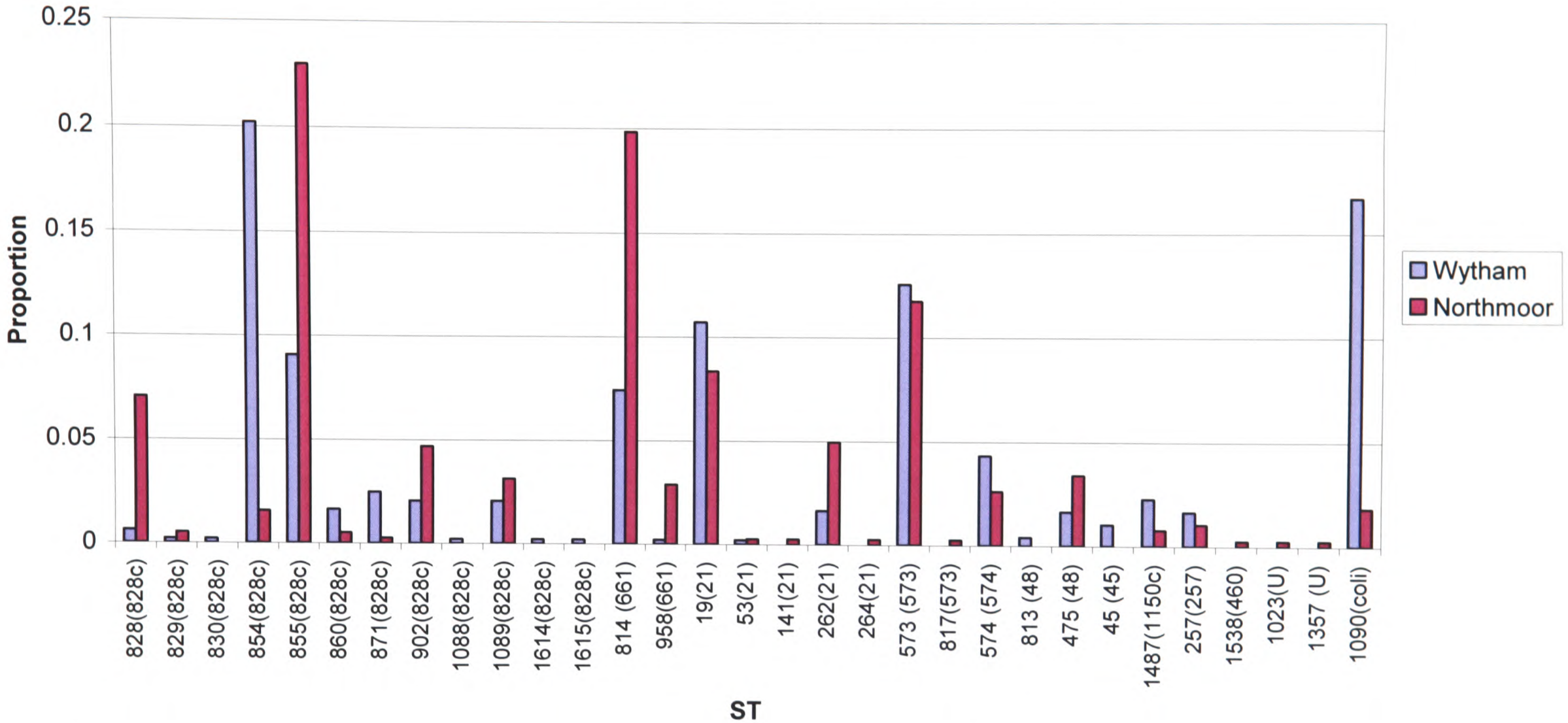

Figure 7.13. The distribution of *Campylobacter* STs at Wytham and Northmoor, shown as a proportion of the total number of *Campylobacter* isolates collected at each site. C = *C. coli.* U = isolates that could not be assigned to a clonal comple

Table 7.9. Frequency of *Campylobacter* STs at Wytham and Northmoor during the year. U = isolates that could not be assigned to a clonal complex.

ST(Clonal complex)	Rotation															
	W1	N1	W2	N2	W3	N3	W4	N4	W5	N5	W6	N6	W7	N7	W8	N8
19(21)	27	28	7	1	16	3	1	-	1	-	-	-	-	-	-	-
53(21)	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	-
141(21)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
262(21)	-	-	-	-	-	-	-	-	-	-	-	-	2	16	6	3
264(21)	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
813(48)	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
475(48)	-	-	6	4	1	5	1	3	-	1	-	-	-	-	-	-
45(45)	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-
257(257)	-	-	-	-	-	-	7	4	1	-	-	-	-	-	-	-
573(573)	-	-	-	4	-	-	-	2	3	-	4	3	18	18	36	18
817(573)	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
574(574)	-	-	-	-	2	4	2	1	-	2	-	1	-	-	17	2
814(661)	-	-	9	7	5	-	-	-	1	-	2	6	7	8	12	55
958(661)	-	-	-	-	-	-	-	-	-	7	1	4	-	-	-	-
1538(460)	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
1023(U)	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
1357(U)	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
828(828)	-	-	-	-	-	-	-	-	-	6	3	4	-	13	-	4
829(828)	-	-	-	-	-	-	-	-	-	2	1	-	-	-	-	-
830(828)	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
854(828)	94	6	3	-	-	-	1	-	-	-	-	-	-	-	-	-
855(828)	-	-	-	8	13	17	10	25	7	2	3	1	-	26	11	9
860(828)	-	-	-	-	-	-	-	-	-	1	8	1	-	-	-	-
871(828)	-	-	-	-	-	-	1	-	11	-	-	1	-	-	-	-
902(828)	-	-	7	13	3	5	-	-	-	-	-	-	-	-	-	-
1088(828)	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
1089(828)	-	-	-	-	-	-	-	-	-	1	10	11	-	-	-	-
1614(828)	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
1615(828)	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
1487(1190)	-	-	-	-	-	-	1	-	6	2	4	1	-	-	-	-
1090(U)	-	-	-	-	-	-	-	-	-	7	2	-	8	-	71	-

7.2.8.4 Gene flow (F_{ST}).

The F_{ST} value indicating gene flow between *Campylobacter* populations from the two farm sites for concatenated sequences was 0.0357 which was significant with a p value of <0.0001. The result indicated that the *Campylobacter* populations were less than 4% divergent at the farm sites, but this had not occurred by chance. When F_{ST} values were calculated for individual alleles they were less than 10% divergent at

the two farm sites, although only the value for the *aspA* locus was significant. The values were; *aspA* 0.017 $p<0.001$*, *glnA* -0.108 $p=0.99$, *gltA* -0.108 $p=0.99$, *glyA* -0.103 $p=0.99$, *pgm* -0.080 $p=0.99$, *tkt* -0.070 $p=0.95$ and *uncA* -0.129, $p=0.99$.

7.2.8.5 Antigenic diversity.

Seven of the 31 STs were associated with exactly the same *flaA* SVR allele at both farm sites (Table 7.10). A further ten STs had at least one *flaA* SVR allele in common at both sites and the differences were linked with flock number and time rather than farm site. For example ST-574 isolated from flocks reared at Wytham was linked with *flaA* SVR type 312-4 in isolates from flocks 14 and 19, and *flaA* SVR type 8-1 in isolates from flock 34. Only one ST had different *flaA* SVR types at both sites, ST-958 isolates were *flaA* SVR type 8-1 at Wytham and *flaA* SVR types 21-1 and 105-1 at Northmoor. Three *flaA* SVR types, 15-5, 41-4 and 67-8 were unique to Wytham and three, 34-1, 86-18 and 453-1 were unique to Northmoor. They were all identified in low frequency, and three were associated with rare STs seen only at one or other of the sites.

Table 7.10. The *flaA* SVR types associated with STs isolated from free-range chicken flocks at the Wytham and Northmoor farm sites.

Clonal complex	ST	*flaA* SVR allele-peptide (frequency)											
		Wytham						Northmoor					
21	19	36-1(61)	312-4(1)	319-15(1)	U(1)			36-1(32)					
	53	32-1(1)											
	141							36-1(1)					
	262	37-1(9)						37-1(19)					
	264							32-1(19)					
45	45	15-5(6)	36-1(2)										
48	475	105-1(8)						105-1(13)					
	813	41-4(2)											
257	257	16-12(8)						16-12(4)					
460	1538							34-1(1)					
573	573	8-1(45)	191-33(11)	314-8(4)	67-8(1)			8-1(36)	32-1(4)	314-8(3)	86-18(1)		
	817							32-1(1)					
574	574	8-1(16)	312-4(4)	66-1(1)				312-4(8)	8-1(2)	30-11(1)			
661	814	191-33(50)						191-33(73)	8-1(1)	30-11(1)			
	958	8-1(1)						21-1(8)	105-1(2)				
828coli	828	325-1(1)						325-1(19)	66-1(5)	30-11(2)	312-4(1)		
	829	17-11(1)						17-11(2)					
	830	325-1(1)											
	854	319-15(121)	36-1(4)					319-15(5)	36-1(1)				
	855	66-1(30)	30-11(11)	105-1(3)	36-1(1)	100-33(1)	191-33(1)	66-1(53)	30-11(27)	191-33(4)	105-1(1)	325-1(1)	453-1(1)	100-33(1)
	860	325-1(4)	89-1(1)	415-1(1)	17-11(1)	30-11(1)		325-1(1)	30-11(1)				
	871	30-11(12)						30-11(1)					
	902	66-1(15)						66-1(18)					
	1088	66-1(1)											
	1089	415-1(10)						415-1(12)	40-1(1)				
	1090	66-1(80)						66-1(7)					
	1614	30-11(1)											
	1615	105-1(1)											
1150coli	1487	351-111(9)	36-1(1)	325-1(1)				351-111(3)					
Unassigned	1023							80-48(1)					
	1357							191-33(1)					

7.2.9 Influence of arc reuse amongst broiler flocks on *Campylobacter* colonization.

7.2.9.1 Clonal complex distribution.

The eight sets of rearing arcs were moved between two adjacent plots and housed eight separate flocks of broilers during the first year of the study period (see section 2.1.4). The houses were fully disinfected after flock depletion and left empty for five days before restocking. When the *Campylobacter* populations colonizing all of the flocks in an ark were considered, between five (arcs G/H Wytham) and ten (arcs A/B Wytham) clonal complexes, in addition to unassigned isolates, were identified per arc (Table 7.11). There were occasions where the same clonal complexes were identified from consecutive flocks reared within the same arcs, but arc K at Northmoor was the only one in which the same clonal complex, ST-828 complex, was isolated from every flock.

7.2.9.2 ST distribution.

The *Campylobacter* STs isolated from all flocks reared within the same arcs were diverse, and ranged from 12 STs isolated from flocks reared in arcs G/H at Northmoor to18 STs isolated from flocks reared in arcs A/B at Wytham and arc L at Northmoor. The STs colonizing a flock could not be predicted by those colonizing a previous flock, although there were 44 occasions where at least one ST was found in consecutive flocks. The number of occasions where STs were isolated from consecutive flocks in an arc ranged from three in arcs C, D, F and L to ten in arcs G and H. There were no occasions where the same ST was isolated from every flock reared within an arc.

Table 7.11. ***Campylobacter*** **strains (ST;*flaA* SVR allele-peptide) isolated from each flock within a set of arcs.**

Arcs	Flock no	Highest frequency of isolation ----------> Lowest frequency of isolation						
Arcs A/B Wytham	1	ST-19;36-1	ST-813;41-4	ST-854;319-15	ST-854;36-1			
	7	ST-45;15-5	ST-854;319-15	ST-19;36-1	ST-45;36-1	ST-475;105-1		
	13	ST-19;36-1	ST-475;105-1	ST-814;191-33	ST-855;66-1			
	19	ST-855;66-1	ST-574;312-4	ST-19;36-1	ST-1487;351-111			
	25	ST-257;16-12	ST-573;8-1	ST-573;314-8	ST-855;191-33	ST-871;30-11	ST-1487;351-111	
	31	ST-1089;415-1	ST-1090;66-1	ST-860;17-11	ST-860;30-11	ST-860;89-1	ST-860;325-1	ST-860;415-1
	37	ST-573;8-1	ST-573;191-33	ST-1088;66-1	ST-1615;105-1			
	43	ST-1090;66-1	ST-573;8-1	ST-574;8-1	ST-262;37-1	ST-814;191-33	ST-573;86-18	
Arcs C/D Wytham	2	ST-19;36-1	ST-854;319-15					
	8	ST-902;661	ST-814;191-33	ST-475;105-1				
	14	ST-855;66-1	ST-19;36-1	ST-574;312-4	ST-902;66-1			
	20	ST-257;16-12	ST-855;66-1	ST-475;105-1				
	26	ST-871;30-11	ST-19;312-4	ST-855;100-33	ST-855;105-1	ST-573;314-8	ST-1487;351-111	
	32	ST-1089;415-1	ST-573;8-1	ST-573;67-8	ST-828;325-1			
	38	ST-1090;66-1	ST-573;8-1	ST-814;191-33				
	44	ST-855;30-11	ST-1090;66-1	ST-573;191-33	ST-573;8-1	ST-573;314-8		
Arc E Wytham	3a	ST-854;319-15						
	9a	ST-19;36-1	ST-475;105-1					
	15a	ST-19;36-1	ST-855;66-1	ST-902;66-1				
	21a	ST-257;16-12	ST-1614;30-11	ST-871;30-11	ST-854;3119-15			
	27a	ST-814;191-33	ST-855;105-1	ST-855;36-1	ST-871;30-11	ST-1487;351-111		
	33a	ST-855;105-1	ST-814;191-33	ST-1089;415-1	ST-829;17-11	ST-573;314-8	ST-1487;351-111	ST-828;325-1
	39a	S T-262;37-1	ST-573;8-1					
	45a	ST-574;661	ST-814;191-33	ST-855;30-11	ST-1090;66-1			
Arc F Wytham	3b	ST-19;36-1	ST-854;319-15					
	9b	ST-814;191-33	ST-902;66-1	ST-475;105-1				
	15b	ST-19;36-1	ST-855;66-1					
	21b	No data.						
	27b	ST-53;32-1	ST-855;105-1	ST-871;30-11	ST-1487;351-111			
	33b	ST-860;325-1	ST-1487;315-111	ST-958;8-1	ST-855;105-1	ST-1487;325-1	ST-828;325-1	ST-830;325-1
	39b	ST-262;37-1	ST-573;8-1	ST-814;191-33				
	45b	ST-574;8-1	ST-814;191-33	ST-855;30-11	ST-1090;66-1			

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Arcs	Flock no	Highest frequency of isolation ----------> Lowest frequency of isolation					
Arcs G/H Northmoor	4	ST-19;36-1					
	10	ST-902;66-1	ST-475;105-1	ST-573;32-1	ST-19;36-1	ST-855;66-1	
	16	ST-475;105-1	ST-855;66-1	ST-902;66-1			
	22	ST-855;66-1	ST-475;105-1				
	28	ST-475;105-1	ST-829;17-11	ST-855;66-1	ST-958;105-1	ST-828;325-1	
	34	ST-1089;415-1	ST-814;191-33	ST-958;21-2	ST-871;30-11		
	40	ST-828;66-1	ST-855;30-11	ST-262;37-1	ST-828;325-1	ST-814;191-33	
	46	ST-814;191-33	ST-573;8-1	ST-828;325-1	ST-262;37-1	ST-855;30-11	
Arcs I/J Northmoor	5	ST-19;36-1	ST-141;36-1				
	11	ST-814;191-33	ST-573;32-1	ST-1538;34-1	ST-1357;191-33		
	17	ST-855;66-1	ST-902;66-1	ST-19;36-1	ST-574;312-4		
	23	ST-855;66-1	ST-475;105-1	ST-855;191-33	ST-573;314-8	ST-574;312-4	ST-855;453-1
	29	ST-574;312-4	ST-958;21-2	ST-828;325-1	ST-860;325-1	ST-1487;351-111	
	35	ST-958;21-2	ST-828;325-1	ST-573;8-1	ST-814;191-33	ST-1487;351-111	
	41	ST-855;30-11	ST-855;66-1	ST-262;37-1	ST-573;8-1	ST-814;8-1	ST-814; 191-33
	47	ST-573;8-1	ST-814;191-33	ST-855;30-11	ST-574;8-1		
Arc K Northmoor	6a	ST-19;36-1	ST-854;319-15	ST-854;36-1			
	12a	ST-475;105-1	ST-855;66-1	ST-902;66-1			
	18a	ST-19;36-1	ST-574;312-4	ST-855;66-1			
	24a	ST-257;16-12	ST-855;66-1				
	30a	ST-1090;66-1	ST-958;21-2	ST-958;105-1	ST-828;312-4	ST-828;325-1	ST-1089;40-1
	36a	ST-958;21-2	ST-828;30-11	ST-855;105-1	ST-860;30-11	ST-1089;415-1	
	42a	ST-262;37-1	ST-573;8-1	ST-814;191-33	ST-828;325-1	ST-855;30-11	
	48a	ST-814;191-33	ST-855;30-11	ST-814;30-11			
Arc L Northmoor	6b	ST-19;36-1	ST-854;319-15				
	12b	ST-817;32-1	ST-814;191-33	ST-855;66-1	ST-902;66-1		
	18b	ST-53;32-1	ST-264;32-1	ST-475;105-1	ST-574;312-4	ST-1023;80-48	
	24b	ST-855;66-1	ST-855;100-33	ST-855;191-33			
	30b	ST-1090;66-1	ST-958;21-2	ST-828;325-1	ST-1487;351-111		
	36b	ST-573;8-1	ST-574;8-1	ST-814;191-33	ST-1089;415-1		
	42b	ST-262;37-1	ST-573;8-1	ST-814;191-33	ST-828;325-1	ST-855;30-11	ST-855;325-1 ST-855;66-1
	48b	ST-814;191-33	ST-574;30-11				

ST-21 complex ST-45 complex ST-48 complex ST-257 complex ST-460 complex ST-573 complex ST-574 complex ST-661 complex ST-828 complex ST-1150 complex Unassigned STs

7.2.9.3 Gene flow (F_{ST}).

Gene flow within arcs (concatenated sequence); the gene flow between *Campylobacter* populations colonizing flocks within the same arc was tested in order to determine whether the population isolated from a flock influenced that isolated from a subsequent flock. Of the eight arcs, six had significantly different *Campylobacter* populations that diverged by 20% or more within a least one pair of successive flocks, measured by F_{ST} (Table 7.12). None of the arcs had significantly different *Campylobacter* populations within every pair of successive flocks. The least amount of gene flow between subsequent flocks was evident in arc L at Northmoor, with *Campylobacter* populations from six of seven flock pairs being divergent by greater than 20%, and with five of the seven observations being significantly different.

Table 7.12. Gene flow (F_{ST}) values comparing *Campylobacter* populations between successive flocks within the same arcs (concatenated sequence).

Arc Id Location	Flock numbers	F_{ST}	*p* value (*=sig)	Arc Id Location	Flock numbers	F_{ST}	*p* value (*=sig)
Arcs A/B Wytham	1-7	0.370	0.009*	**Arcs G/H Northmoor**	4-10	0.437	0.009*
	7-13	-0.033	0.667		10-16	-0.086	0.541
	13-19	0.171	0.072		16-22	-0.073	0.369
	19-25	-0.094	0.739		22-28	-0.177	0.847
	25-31	0.243	0.009*		28-34	-0.104	0.297
	31-37	0.741	0.000*		34-40	-0.073	0.360
	37-43	0.000	0.315		40-46	0.289	0.000*
Arcs C/D Wytham	2-8	-0.089	0.604	**Arcs I/J Northmoor**	5-11	0.621	0.000*
	8-14	-0.087	0.604		11-17	0.519	0.000*
	14-20	-0.035	0.468		17-23	-0.074	0.423
	20-26	-0.052	0.477		23-29	0.120	0.667
	26-32	-0.133	0.622		29-35	-0.155	0.784
	32-38	0.051	0.279		35-41	0.075	0.252
	38-44	-0.033	0.514		41-47	0.416	0.000*
Arc E Wytham	3a-9a	0.316	0.054	**Arc K Northmoor**	6a-12a	0.256	0.018*
	9a-15a	0.204	0.036*		12a-18a	0.119	0.081
	15a-21a	0.019	0.333		18a-24a	0.008	0.117
	21a-27a	0.134	0.171		24a-30a	-0.052	0.405
	27a-33a	-0.050	0.532		30a-36a	0.106	0.135
	33a-39a	0.611	0.000*		36a-42a	0.689	0.000*
	39a-45a	0.795	0.000*		42a-48a	0.139	0.081
Arc F Wytham	3b-9b	-0.015	0.243	**Arc L Northmoor**	6b-12b	0.423	0.018*
	9b-15b	0.033	0.153		12b-18b	0.545	0.000*
	15b-21b	no data	no data		18b-24b	0.965	0.000*
	21b-27b	no data	no data		24b-30b	0.309	0.000*
	27b-33b	-0.113	0.748		30b-36b	0.205	0.081
	33b-39b	0.803	0.000*		36b-42b	-0.044	0.423
	39b-45b	0.642	0.000*		42b-48b	0.465	0.000*

Gene flow between arcs (concatenated sequence); *Campylobacter* populations from all eight flocks reared within a set of arcs were compared with those raised within every other set of arcs in order to determine whether *Campylobacter* population was dependent on arc (Table 7.13). All of the F_{ST} values indicated that the *Campylobacter* populations differed by less than 10% between the arcs, even when comparing those on the different farm sites. Significant results were achieved for 11

of the 28 comparisons indicating that although the differences were small, they did not occur by chance.

Table 7.13. Gene flow (F_{ST}) values comparing *Campylobacter* populations isolated from different arcs (concatenated sequence). Significant *p* values are marked with an asterisk.

Arc Id	Farm site		Arc Id/Farm site AB Wytham	CD Wytham	E Wytham	F Wytham	GH N-moor	IJ N-moor	K N-moor	L N-moor
AB	Wytham	F_{ST}	-	-	-	-	-	-	-	-
		p value	-	-	-	-	-	-	-	-
CD	Wytham	F_{ST}	0.002	-	-	-	-	-	-	-
		p value	0.306	-	-	-	-	-	-	-
E	Wytham	F_{ST}	0.001	-0.006	-	-	-	-	-	-
		p value	0.297	0.568	-	-	-	-	-	-
F	Wytham	F_{ST}	0.001	-0.004	-0.007	-	-	-	-	-
		p value	0.234	0.387	0.505	-	-	-	-	-
GH	Northmoor	F_{ST}	0.013	0.040	0.020	0.005	-	-	-	-
		p value	0.117	0.027*	0.045*	0.225	-	-	-	-
IJ	Northmoor	F_{ST}	0.046	0.089	0.058	0.030	0.002	-	-	-
		p value	0.000*	0.018*	0.000*	0.090	0.270	-	-	-
K	Northmoor	F_{ST}	0.005	0.036	0.010	0.000	-0.005	0.084	-	-
		p value	0.189	0.018*	0.153	0.306	0.559	0.000*	-	-
L	Northmoor	F_{ST}	0.054	0.096	0.054	0.064	0.014	0.007	0.018	-
		p value	0.000*	0.000*	0.000*	0.000*	0.153	0.198	0.081	-

7.2.9.4 Antigenic diversity.

The *flaA* SVR type was correlated with ST and broiler flock rather than arc. Of the 44 occasions where STs were identified in consecutive flocks, 42 were the same ST-*flaA* SVR strain. The number of occasions where identical ST-*flaA* SVR strains were isolated from consecutive flocks in an arc ranged from three in arcs C, D, F and L to nine in arcs G and H.

7.2.10 Influence of flock rotation onto the same plots of land on *Campylobacter* colonization.

7.2.10.1 Clonal complex distribution.

There were 16 separate plots of land on which the birds ranged, eight at Wytham and eight at Northmoor (section 2.1.4). Four broiler flocks were raised on each plot of land over the study period, with a fallow period of seven weeks between each flock. Only two of ten clonal complexes, the *C. coli* ST-828 complex and the *C. jejuni* ST-573 complex were isolated from all four flock rotations on the same plot (Table 7.14). ST-828 complex was consistently isolated from seven of the 16 plots, but the STs differed in six of them. ST-573 complex was consistently isolated from one of the 16 plots, plot 14 in Northmoor, and the ST was also consistent on each occasion.

Table 7.14. ***Campylobacter*** **strains (ST;*flaA* SVR allele-peptide) isolated in each plot of land during four rotations of chicken flocks.**

Rotation		Highest frequency of isolation ----------> Lowest frequency of isolation					
Plot1 Wytham	1	ST-19;36-1	ST-813;41-4	ST-854;319-15	ST-854;36-1		
	2	ST-19;36-1	ST-475;105-1	ST-814;191-33	ST-855;66-1		
	3	ST-257;16-12	ST-573;8-1	ST-573;314-8	ST-855;191-33	ST-871;30-11	ST-1487;351-111
	4	ST-573;8-1	ST-573;191-33	ST-1088;66-1	1615;105-1		
Plot2 Wytham	1	ST-19;36-1	ST-854;319-15				
	2	ST-855;66-1	ST-19;36-1	ST-574;312-4	ST-902;66-1		
	3	ST-871;30-11	ST-19;312-4	ST-855;100-33	ST-855;105-1	ST-573;314-8	ST-1487;351-111
	4	ST-1090;66-1	ST-573;8-1	ST-814;191-33			
Plot3 Wytham	1	ST-854;319-15					
	2	ST-19;36-1	ST-855;66-1	ST-902;66-1			
	3	ST-814;191-33	ST-855;105-1	ST-855;36-1	ST-871;30-11	ST-1487;351-111	
	4	S T-262;37-1	ST-573;8-1				
Plot4 Wytham	1	ST-19;36-1	ST-854;319-15				
	2	ST-19;36-1	ST-855;66-1				
	3	ST-53;32-1	ST-855;105-1	ST-871;30-11	ST-1487;351-111		
	4	ST-262;37-1	ST-573;8-1	ST-814;191-33			
Plot5 Northmoor	1	ST-19;36-1					
	2	ST-475;105-1	ST-855;66-1	ST-902;66-1			
	3	ST-475;105-1	ST-829;17-11	ST-855;66-1	ST-958;105-1	ST-828;325-1	
	4	ST-828;66-1	ST-855;30-11	ST-262;37-1	ST-828;325-1	ST-814;191-33	
Plot6 Northmoor	1	ST-19;36-1	ST-141;36-1				
	2	ST-855;66-1	ST-902;66-1	ST-19;36-1	ST-574;312-4		
	3	ST-574;312-4	ST-958;21-2	ST-828;325-1	ST-860;325-1	ST-1487;351-111	
	4	ST-855;30-11	ST-855;66-1	ST-262;37-1	ST-573;8-1	ST-814;8-1	ST-814; 191-33
Plot7 Northmoor	1	ST-19;36-1	ST-854;319-15	ST-854;36-1			
	2	ST-19;36-1	ST-574;312-4	ST-855;66-1			
	3	ST-1090;66-1	ST-958;21-2	ST-958;105-1	ST-828;312-4	ST-828;325-1	
	4	ST-262;37-1	ST-573;8-1	ST-814;191-33	ST-828;325-1	ST-855;30-11	
Plot8 Northmoor	1	ST-19;36-1	ST-854;319-15				
	2	ST-53;32-1	ST-264;32-1	ST-475;105-1	ST-574;312-4	ST-1023;80-48	
	3	ST-1090;66-1	ST-958;21-2	ST-828;325-1	ST-1487;351-111		
	4	ST-262;37-1	ST-573;8-1	ST-814;191-33	ST-828;325-1	ST-855;30-11	ST-855;325-1	ST-855;66-1

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Rotation		Highest frequency of isolation ----------> Lowest frequency of isolation						
Plot9 Wytham	1	ST-45;15-5	ST-854;319-15	ST-19;36-1	ST-45;36-1	ST-475;105-1		
	2	ST-855;661	ST-574;312-4	ST-19;36-1	ST-1487;351-111			
	3	ST-1089;415-1	ST-1090;66-1	ST-860;17-11	ST-860;30-11	ST-860;89-1	ST-860;325-1	ST-860;415-1
	4	ST-1090;66-1	ST-573;8-1	ST-574;8-1	ST-262;37-1	ST-814;191-33	ST-573;86-18	
Plot10 Wytham	1	ST-902;661	ST-814;191-33	ST-475;105-1				
	2	ST-257;16-12	ST-855;66-1	ST-475;105-1				
	3	ST-1089;415-1	ST-573;8-1	ST-573;67-8	ST-828;325-1			
	4	ST-855;30-11	ST-1090;66-1	ST-573;191-33	ST-573;8-1	ST-573;314-8		
Plot11 Wytham	1	ST-19;36-1	475;105-1					
	2	ST-257;16-12	ST-1614;30-11	ST-871;30-11	ST-854;3119-15			
	3	ST-855;105-1	ST-814;191-33	ST-1089;415-1	ST-829;17-11	ST-573;314-8	ST-1487;351-111	ST-828;325-1
	4	ST-574;661	ST-814;191-33	ST-855;30-11	ST-1090;66-1			
Plot12 Wytham	1	ST-814;191-33	ST-902;66-1	ST-475;105-1				
	2	No data.						
	3	ST-860;325-1	ST-1487;315-111	ST-958;8-1	ST-855;105-1	ST-1487;325-1	ST-828;325-1	ST-830;325-1
	4	ST-574;8-1	ST-814;191-33	ST-855;30-11	ST-1090;66-1			
Plot13 Northmoor	1	ST-902;66-1	ST-475;105-1	ST-573;32-1	ST-19;36-1	ST-855;66-1		
	2	ST-855;66-1	ST-475;105-2					
	3	ST-1089;415-1	ST-814;191-33	ST-958;21-2	ST-871;30-11			
	4	ST-814;191-33	ST-573;8-1	ST-828;325-1	ST-262;37-1	ST-855;30-11		
Plot14 Northmoor	1	ST-814;191-33	ST-573;32-1	ST-1538;34-1	ST-1357;191-33			
	2	ST-855;66-1	ST-475;105-1	ST-855;191-33	ST-573;314-8	ST-574;312-4	ST-855;453-1	
	3	ST-958;21-2	ST-828;325-1	ST-573;8-1	ST-814;191-33	ST-1487;351-111		
	4	ST-573;8-1	ST-814;191-33	ST-855;30-11	ST-574;8-1			
Plot15 Northmoor	1	ST-475;105-1	ST-855;66-1	ST-902;66-1				
	2	ST-257;16-12	ST-855;66-1					
	3	ST-958;21-2	ST-828;30-11	ST-855;105-1	ST-860;30-11	ST-1089;415-1		
	4	ST-814;191-33	ST-855;30-11	ST-814;30-11				
Plot16 Northmoor	1	ST-817;32-1	ST-814;191-33	ST-855;66-1	ST-902;66-1			
	2	ST-855;66-1	ST-855;100-33	ST-855;191-33				
	3	ST-573;8-1	ST-574;8-1	ST-814;191-33	ST-1089;415-1			
	4	ST-814;191-33	ST-574;30-11					

ST-21 complex | ST-45 complex | ST-48 complex | ST-257 complex | ST-460 complex | ST-573 complex | ST-574 complex | ST-661 complex | ST-828 complex | ST-1150 complex | Unassigned STs

7.2.10.2 ST distribution.

All plots were diverse in terms of ST. The average number of STs isolated per plot was 10.7, and ranged from seven to 15 at Northmoor and eight to 14 at Wytham. Of the 31 STs identified, ST-573 (ST-573 complex) and ST-855 (ST-828 complex) were the only two to be isolated from all four flock rotations on a single plot. ST-573 (ST-573 complex) was isolated from all flock rotations on plot 14 in Northmoor and ST-828 (ST-828 complex) was isolated from all flock rotations on plot 15 also in Northmoor. ST-19 (ST-21 complex) was isolated from the first two flock rotations in six of the 16 plots and appeared to be influenced by time rather than plot number. The distribution of the remaining STs could not be predicted with regards to flock rotation and plot number.

7.2.10.3 Gene flow (F_{ST}).

Gene flow within the free-range plots (concatenated sequence); the gene flow between *Campylobacter* populations colonizing flocks reared on the same plot of land was tested in order to determine whether the population isolated from a flock influenced that isolated from a subsequent flock (Table 7.15). Of the 16 plots, 12 contained pairs of subsequent flocks whose *Campylobacter* populations differed by more than 20%, with all but two of the F_{ST} values being significant. Two plots, plot 3 at Wytham and plot 16 at Northmoor, had chicken flocks with significantly different *Campylobacter* populations in every rotation. None of the smaller differences in F_{ST} observed between subsequent flocks within a plot were significant and may have occurred by chance.

Table 7.15. Gene flow (F_{ST}) values comparing *Campylobacter* populations isolated from different plots (concatenated sequence). Significant *p* values are marked with an asterisk.

Plot Location	Rotation numbers	F_{ST}	*p* value (*=sig))	Plot Location	Rotation numbers	F_{ST}	*p* value (*=sig))
Plot 1 Wytham	1-2	0.398	0.000*	**Plot 9 Wytham**	1-2	0.182	0.072
	2-3	0.179	0.108		2-3	0.204	0.027*
	3-4	0.242	0.045*		3-4	0.490	0.000*
Plot 2 Wytham	1-2	0.154	0.063	**Plot 10 Wytham**	1-2	-0.055	0.306
	2-3	-0.131	0.829		2-3	0.001	0.180
	3-4	-0.054	0.613		3-4	-0.032	0.378
Plot 3 Wytham	1-2	0.333	0.000*	**Plot 11 Wytham**	1-2	0.389	0.027*
	2-3	0.262	0.036*		2-3	-0.036	0.360
	3-4	0.687	0.000*		3-4	0.124	0.072
Plot 4 Wytham	1-2	0.251	0.072	**Plot 12 Wytham**	1-2	0.618	0.009*
	2-3	0.789	0.000*		2-3	0.047	0.108
	3-4	No data	No data		3-4	No data	No data
Plot 5 Northmoor	1-2	0.554	0.000*	**Plot 13 Northmoor**	1-2	0.019	0.378
	2-3	-0.097	0.478		2-3	-0.048	0.207
	3-4	-0.090	0.703		3-4	0.166	0.081
Plot 6 Northmoor	1-2	0.556	0.000*	**Plot 14 Northmoor**	1-2	0.407	0.009*
	2-3	-0.100	0.613		2-3	-0.004	0.405
	3-4	0.003	0.288		3-4	0.105	0.261
Plot 7 Northmoor	1-2	0.096	0.054	**Plot 15 Northmoor**	1-2	0.139	0.081
	2-3	-0.006	0.28		2-3	0.141	0.054
	3-4	0.427	0.009*		3-4	0.573	0.000*
Plot 8 Northmoor	1-2	-0.049	0.162	**Plot 16 Northmoor**	1-2	0.217	0.000*
	2-3	0.253	0.063		2-3	0.621	0.000*
	3-4	0.112	0.117		3-4	0.454	0.000*

Gene flow between the free-range plots (concatenated sequence); the *Campylobacter* populations isolated from all flock rotations within a plot were compared with those isolated from all flock rotations from each of the 15 other plots in order to determine whether gene flow occurred between the plots, despite their being no mixing of the chickens (Table 7.15). Of the 120 combinations of plots, four had flocks with *Campylobacter* populations differing by more than 20%, three of which were significant. These comparisons were all between plots at different farm sites. The highest F_{ST} value of 0.858 was seen when plot 2 at Wytham was compared with plot 7 at Northmoor. The remaining plot comparisons, including those between

plots at different farm sites, had flocks with *Campylobacter* populations differing by less than 20%, of which 44 values were significant.

Table 7.16. Gene flow (F_{ST}) values comparing *Campylobacter* populations between plots of land (concatenated sequence). Significant *p* values are asterisked. w=wytham, n=northmoor. Between farm site comparisons are shaded in grey.

Plot		Plot 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1	F_{ST}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w
	p value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
2	F_{ST}	0.003	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w
	p value	0.252	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
3	F_{ST}	0.050	0.008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w
	p value	0.036*	0.324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
4	F_{ST}	0.162	0.090	0.007	-	-	-	-	-	-	-	-	-	-	-	-	-	w
	p value	0.000*	0.009*	0.252	-	-	-	-	-	-	-	-	-	-	-	-	-	
5	F_{ST}	0.040	0.006	-0.018	0.006	-	-	-	-	-	-	-	-	-	-	-	-	n
	p value	0.054	0.306	0.676	0.279	-	-	-	-	-	-	-	-	-	-	-	-	
6	F_{ST}	0.210	0.228	-0.017	0.001	-0.016	-	-	-	-	-	-	-	-	-	-	-	n
	p value	0.000*	0.153	0.685	0.207	0.613	-	-	-	-	-	-	-	-	-	-	-	
7	F_{ST}	0.146	0.858	0.005	-0.017	0.046	0.010	-	-	-	-	-	-	-	-	-	-	n
	p value	0.000*	0.027*	0.234	0.532	0.090	0.207	-	-	-	-	-	-	-	-	-	-	
8	F_{ST}	0.120	0.054	-0.006	-0.022	0.001	-0.012	-0.005	-	-	-	-	-	-	-	-	-	n
	p value	0.000*	0.018*	0.423	0.667	0.279	0.441	0.333	-	-	-	-	-	-	-	-	-	
9	F_{ST}	0.153	0.079	0.021	-0.001	0.041	0.026	-0.000	0.003	-	-	-	-	-	-	-	-	w
	p value	0.000*	0.027*	0.099	0.243	0.027*	0.090	0.270	0.297	-	-	-	-	-	-	-	-	
10	F_{ST}	0.004	-0.016	0.014	0.098	0.012	0.035	0.082	0.067	0.084	-	-	-	-	-	-	-	w
	p value	0.279	0.613	0.189	0.036*	0.198	0.072	0.009*	0.018*	0.000*	-	-	-	-	-	-	-	
11	F_{ST}	0.032	-0.009	0.056	0.144	0.0516	0.077	0.136	0.103	0.110	-0.001	-	-	-	-	-	-	w
	p value	0.108	0.532	0.063	0.000*	0.099	0.036*	0.000*	0.001*	0.000*	0.387	-	-	-	-	-	-	
12	F_{ST}	0.046	-0.009	0.006	0.050	0.006	0.015	0.059	0.026	0.045	-0.006	0.0046	-	-	-	-	-	w
	p value	0.063	0.423	0.207	0.081	0.189	0.189	0.063	0.144	0.009*	0.414	0.216	-	-	-	-	-	
13	F_{ST}	0.145	0.086	0.009	-0.003	0.018	0.009	-0.015	-0.008	0.006	0.081	0.132	0.051	-	-	-	-	n
	p value	0.000*	0.027*	0.252	0.324	0.153	0.243	0.559	0.477	0.198	0.009*	0.000*	0.045*	-	-	-	-	
14	F_{ST}	0.262	0.194	0.083	-0.004	0.101	0.076	0.014	0.034	0.033	0.188	0.207	0.135	0.004	-	-	-	n
	p value	0.000*	0.009*	0.009*	0.414	0.000*	0.036*	0.171	0.081	0.117	0.000*	0.000*	0.018*	0.360	-	-	-	
15	F_{ST}	0.036	0.011	0.002	0.043	-0.004	0.009	0.045	0.029	0.061	0.004	0.043	0.004	0.048	0.115	-	-	n
	p value	0.005	0.288	0.234	0.090	0.468	0.189	0.036*	0.090	0.000*	0.297	0.045*	0.270	0.036*	0.000*	-	-	
16	F_{ST}	0.188	0.129	0.047	-0.008	0.054	0.040	0.014	0.016	0.033	0.125	0.172	0.076	-0.009	-0.000	0.050	-	n
	p value	0.000*	0.009*	0.009*	0.405	0.063	0.054	0.162	0.216	0.036*	0.000*	0.000*	0.018*	0.577	0.297	0.045*	-	
		w	w	w	w	n	n	n	n	w	w	w	w	n	n	n	n	

7.2.10.4 Antigenic diversity.

Antigenic typing provided little evidence that identical *Campylobacter* strains were carried over between flocks raised on the same plot of land (Table 7.14). ST-573 isolates in plot 14 were associated with *flaA* SVR type 32-1 in the first flock, *flaA* SVR type 314-8 in the second, a mixture of *flaA* SVR types 8-1 and 314-8 in the third and 8-1 in the fourth. With the exception of the fourth flock, none of these *flaA* SVR types were isolated from preceding flock rotations, even in association with other STs. ST-855 isolates in plot 15 were *flaA* SVR type 66-1 in the first flock, a mixture of *flaA* SVR types 66-1, 191-33 and 100-33 in the second flock, *flaA* SVR type 105-1 in the third flock, and *flaA* SVR type 30-11 in the fourth flock. There was one ST-475 isolate in the first flock associated with *flaA* SVR type 105-1, and ST-828 and ST-860 isolates in the third flock associated with *flaA* SVR type 30-11, but no other similarities were detected.

In general, *flaA* SVR type was correlated with *Campylobacter* ST rather than plot number. There was also evidence that particular *Campylobacter* strains were correlated with flock rotation number and therefore time of year, rather than plot number. For example, ST-19;36-1 was isolated from ten plots, all of which were identified in the first or second flock rotations. Similarly ST-262;37-1 was isolated from the fourth rotation of the four plots it was identified from, and ST-1487; 351-111 was isolated from ten plots, nine of which were in the third rotation and one in the second rotation. Often the identical strains were isolated from successive plot numbers, also demonstrating correlation with time, but this was not always the case.

7.2.11 Are flocks divided at 21 days of age colonized by the same *Campylobacter* genotypes?

7.2.11.1 Comparison of *Campylobacter* colonization between flocks stocked at high and low densities.

The average number of birds shedding *Campylobacter* amongst the high density stocked birds was 95%, and the average number amongst low density stocked birds was 93.6%. There was no evidence that the shedding rates differed between the flocks stocked at the two densities, $p = 0.717$. The average measure of diversity of *Campylobacter* genotypes, using Simpson's Index of Diversity, was 0.64 for the flocks stocked at high density, and 0.62 for the birds stocked at low density. There was no evidence that the diversity of genotypes differed between the flocks stocked at the two densities, $p = 0.595$.

7.2.11.2 Clonal complex distribution.

Of the 15 flocks that were divided in half when they were moved on to the ranges at day 21, four (flocks 6, 15, 42 and 45), had exactly the same *Campylobacter* clonal complexes in both halves and in similar proportions (Table 7.17). Five clonal complexes, ST-21 complex, ST-573 complex, ST-574 complex, ST-661 complex and ST-828 complex, together with the unassigned ST-1090 were isolated from both halves of the identical divided flocks. The clonal complex distribution could not be predicted in the halves of the remaining divided flocks, although there were many similarities between them. ST-828 complex was the clonal complex most commonly isolated in two halves of a flock, occurring in both halves of 11 of the 15 divided flocks, followed by ST-661 complex, occurring in both halves of six of the 15 divided flocks. ST-257 complex was the least likely to be isolated from two halves of a flock.

7.2.11.3. ST distribution.

Every flock had at least one ST in common between the two halves. Of the four flocks that had identical distribution of *Campylobacter* clonal complexes in both halves, four had identical STs in similar proportions also. The number of STs isolated in the flock halves varied between one and seven, but there but there was no significant difference in the diversity of STs in both halves of the divided flocks p = 0.09. ST-855 (ST-828 complex) was the ST most commonly isolated from two halves of a flock, occurring in both halves of six of the 15 divided flocks. ST-53 (ST-21 complex), ST-257 (ST-257 complex), ST-829 (ST-828 complex), ST-830 (ST-828 complex) and ST-860 (ST-828 complex) were the least likely to be isolated from two halves of a flock.

7.2.11.4 Gene flow (F_{ST}).

Gene flow (F_{ST}) values were calculated using concatenated sequence to determine whether the saine *Campylobacter* populations were isolated from both flock halves 35 days after they were divided (Table 7.18). The *Campylobacter* populations were 20% divergent or more, in seven of the 15 flocks, with six of the values being significant. The remainder did not have significantly different *Campylobacter* populations.

7.2.11.5 Antigenic diversity.

Two flocks, flock 6 and flock 42, had identical STs in both halves, but had up to two additional *flaA* SVR types. Of the STs that were shared, the majority had the same *flaA* SVR type in both flock halves, although in some cases additional *flaA* SVR types were detected. There was only one occasion when a ST was identified in two

flock halves, but with different *flaA* SVR type; ST-574 in flock 45 that was *flaA* SVR type 66-1 in one half and *flaA* SVR type 8-1 in the other. The *flaA* SVR alleles differed by eight nucleotides that were scattered throughout the sequence.

Table 7.17. ***Campylobacter*** **STs and associated** ***flaA*** **SVR type isolated from free-range broiler flocks divided in half at 21 days of age.**

Flock	Highest frequency of isolation ----------->Lowest frequency of isolation ST; *flaA* SVR allele-peptide							
3a	ST-854;319-15							
3b	ST-19;36-1	ST-854;319-15						
6a	ST-19;36-1	ST-854;319-15	ST-854;36-1					
6b	ST-19;36-1	ST-854;319-15						
9a	ST-19;36-1	ST-475;105-1						
9b	ST-814;191-33	ST-902;66-1	ST-475;105-1					
12a	ST-475;105-1	ST-855;66-1	ST-902;66-1					
12b	ST-817;32-1	ST-814;191-33	ST-855;66-1	ST-902;66-1				
15a	ST-19;36-1	ST-855;66-1	ST-902;66-1					
15b	ST-19;36-1	ST-855;66-1						
18a	ST-19;36-1	ST-574;312-4	ST-855;66-1					
18b	ST-53;32-1	ST-246;32-1	ST-475;105-1	ST-574;312-4	ST-1023;80-48			
24a	ST-257;16-12	ST-855;66-1						
24b	ST-855;66-1	ST-855;100-33	ST-855;191-33					
27a	ST-814;191-33	ST-855;105-1	ST-855;36-1	ST-871;30-11	ST-1487;351-111			
27b	ST-53;32-1	ST-855;105-1	ST-871;30-11	ST-1487;351-111				
30a	ST-1090;66-1	ST-958;31-2	ST-958;105-1	ST-828;312-4	ST-828;325-1			
30b	ST-1090;66-1	ST-958;21-2	ST-828;325-1	ST-1487;351-111				
33a	ST-855;105-1	ST-814;191-33	ST-1089;415-1	ST-829;17-11	ST-573;314-8	ST-1487;351-111	ST-828;325-1	
33b	ST-860;325-1	ST-1487(1150)	ST-958;8-1	ST-855;105-1	ST-1487;325-1	ST-828;325-1	ST-830;325-1	
36a	ST-958;21-2	ST-828;30-11	ST-855;105-1	ST-860;30-11	ST-1089;415-1			
36b	ST-573;8-1	ST-574;8-1	ST-814;191-33	ST-1089;415-1				
39a	ST-262;37-1	ST-573;8-1						
39b	ST-262;37-1	ST-573;8-1	ST-814;191-33					
42a	ST-262;37-1	ST-573;8-1	ST-814;191-33	ST-828;325-1	ST-855;30-11			
42b	ST-262;37-1	ST-573;8-1	ST-814;191-33	ST-828;325-1	ST-855;30-11	ST-855;325-1	ST-855;66-1	
45a	ST-574;66-1	ST-814;191-33	ST-855;30-11	ST-1090;66-1				
45b	ST-574;8-1	ST-814;191-33	ST-855;30-11	ST-1090;66-1				
48a	ST-814;191-33	ST-855;30-11	ST-814;30-11					
48b	ST-814;191-33	ST-574;30-11						

Clonal complexes; ST-21 ST-48 ST-257 ST-573 ST-573 ST-661 ST-828 ST-1150 Unassigned STs

Table 7.18. The F_{ST} values comparing the *Campylobacter* populations in both halves of the split flocks (concatenated sequence).

Flock numbers	F_{ST}	*p* value (*=sig)
3a and b	0.62	0.02*
6a and b	0.14	0.31
9a and b	0.23	0.00*
12a and b	0.02	0.26
15a and b	-0.09	0.59
18a and b	0.32	0.05
24a and b	0.06	0.06
27a and b	-0.14	0.99
30a and b	-0.09	0.86
33a and b	0.01	0.25
36a and b	0.57	0.02*
39a and b	0.40	0.00*
42a and b	0.20	0.04*
45a and b	-0.01	0.36
48a and b	0.20	0.03*

7.2.12 Comparison with *Campylobacter* genotypes isolated from wild birds.

A total of 20 clonal complexes, plus unassigned isolates, were identified amongst *Campylobacter* isolates from the chicken flocks in this chapter and wild geese and Starlings in Chapters 4 and 5 (Figure 7.14). The four clonal complexes found to overlap amongst the chicken and wild bird sources in Chapter 6, namely ST-21 complex, ST-45 complex, ST-574 complex, and ST-48 complex were also found to overlap in this study. Only one additional overlapping complex, ST-257 complex was identified in this chapter. Clonal complexes were most often shared between chickens and Starlings and no STs were shared between chickens and geese. Only ST-21 complex and ST-45 complex were identified amongst isolates from chickens, geese and Starlings. Four STs, ST-45 (ST-45 complex), ST-574 (ST-574 complex), ST-257 (ST-257 complex) and ST-1023 (unassigned) were isolated from chickens and Starlings. STs within ST-21 complex that were isolated from geese and Starlings

differed at between one and 20 nucleotides from those STs isolated from the chickens. STs, other than ST-45, within the ST-45 complex that were isolated from geese and Starlings differed at between two and 22 nucleotides from those STs isolated from the chickens. ST-38 (ST-48 complex) isolated from Starlings differed at nine and 31 nucleotides compared to those STs within ST-48 complex isolated from the chickens. The Neighbour Joining tree gave evidence that *Campylobacter* genotypes isolated from chickens were no more closely related to those isolated from Starlings than those isolated from geese, despite having more STs in common (Figure 7.15).

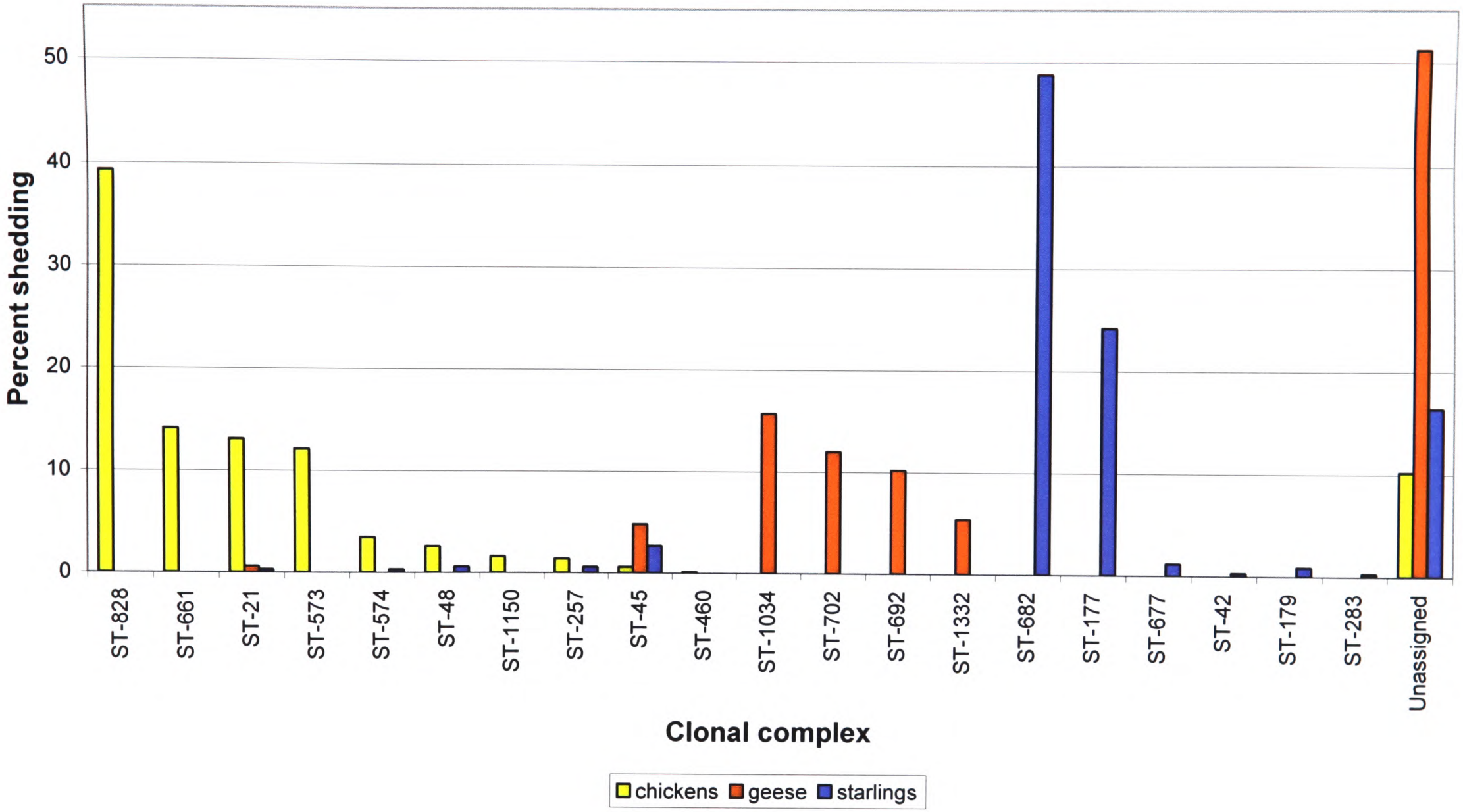
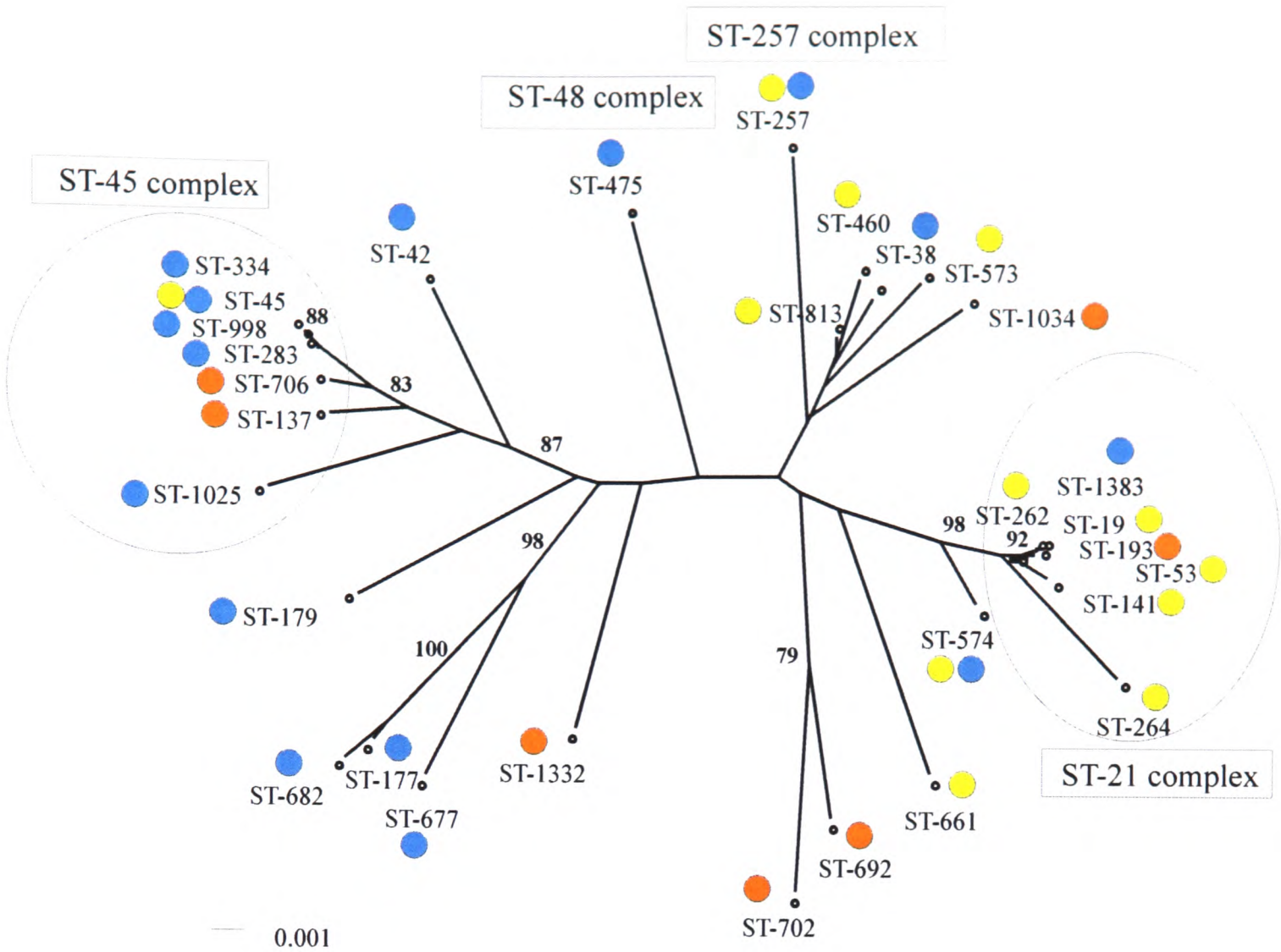


Figure 7.14. The *Campylobacter* clonal complexes isolated from free-range broiler chickens, wild geese and Starlings at Wytham, Oxfordshire.



Key: yellow=chicken isolates, blue=Starling isolates, orange=geese isolates

Figure 7.15. A Neighbour Joining tree showing the relationships of *Campylobacter* genotypes isolated from free-range broiler chickens, Starlings and geese (concatenated sequence). All STs from the overlapping ST-21, ST-45, ST-48 and ST-257 complexes are shown, otherwise only the central genotype of clonal complexes are shown.

Four STs, identified from Oxfordshire chickens were also isolated from the Oxfordshire Starlings (Table 7.19.). Of these, ST-275 (ST-275 complex), ST-574 (ST-574 complex) and ST-1023 (unassigned) were associated with the same *flaA*

SVR type in both host sources, although additional non-identical *flaA* SVR types were also present.

Table 7.19. The STs and associated *flaA* SVR types shared between starling and free-range broiler chicken sources in Oxfordshire. U=ST that could not be assigned to a clonal complex.

ST (complex)	*flaA* SVR allele-peptide	
	Starling	Chickens
45 (45)	21-2(2), 22-1(1), 2-27(1)	15-5(6), 36-1(2)
257 (257)	16-12(2)	16-12(12)
574 (574)	8-1(2)	8-1(29), 66-1(1), 15-5(1)
1023 (U)	80-48(7), 81-46(2)	80-48(1)

7.3 Discussion.

All of the free-range broiler chicken flocks tested were shedding *Campylobacter*, with at least 70%, and more usually 80% of individual birds tested being positive. This is consistent with findings of other studies where free-range and organic flocks have particularly high prevalence of *Campylobacter* (Heuer *et al.* 2001; Wittwer *et al.* 2005). Experience has shown that it is possible to raise *Campylobacter* negative flocks in a housed environment up until the point of slaughter using strict hygiene practices and good farm management (Berndtson *et al.* 1996b). Although bio-security measures applicable to free-range poultry, such as foot dips and restricted access for visitors, were employed on the Oxfordshire farm sites, it is likely that the flocks were constantly exposed to environmental sources of contamination. There were small fluctuations in shedding rate throughout the study period, which could be due to many different factors such as the age of bird when first colonized, health status, flock behaviours such as ranging, and environmental factors, particularly

temperature and sunlight hours (Corry and Atabay 2001; Bouwknegt *et al.* 2004; Patrick *et al.* 2004).

Statistical analysis gave evidence that there was a small but significant seasonal effect in common with that detected amongst wild birds and human disease, with more birds within the flocks shedding *Campylobacter* during the spring and summer months. Statistical analysis gave evidence that there was no year effect in sampling the flocks and that the seasonal variation was reproducible, between weeks five and 36 of the year. The sensitivity of the sampling was increased by taking samples from 25 rather than ten individual chickens per flock, but this did not appear to influence the results overall. The results are consistent with many other studies where increased carriage rates as well as numbers of *Campylobacters* have been noted during the late spring and early summer months (Wallace *et al.* 1997; Evans and Sayers 2000; Heuer *et al.* 2001; Refregier-Petton *et al.* 2001). Factors influencing the increased shedding were not obvious in this study, although as with the longitudinal study of flocks 1 and 43 described in Chapter 6, shedding rate did not appear to be directly related to *Campylobacter* genotype. There is some evidence from case control studies of human infection in England and Wales that *Campylobacter* incidence increases with increasing temperature (Louis *et al.* 2005). It is possible that the bacterial cells are better able to persist inside arcs with increased temperatures and humidity leading to greater colonization potential. In addition the birds were noticeably stressed during hot weather which could make them more susceptible to colonization. Levels of hormones such as Norepinephrine (Noradrenaline) in the gut are increased in stressed birds with effects on both the pathogen and host cells (Cogan *et al.* 2006). The hormone is thought to mediate removal of iron from host transferrins and causes increased growth of the pathogen *in vitro*. In addition

prolonged intracellular survival of *Campylobacter* within the epithelial cell may stimulate the production of IFNγ, which upregulates MHC class 1 expression on epithelial cells resulting in destruction of infected epithelial cells by cytotoxic $CD8^+$ T cells. The lower stocking density level amongst the flocks divided at 21 days of age did not affect the shedding rate, although the level was calculated for the entire plot rather than space within the arc, and so stocking densities would have been the same if birds remained within the arc. There is some discussion as to whether seasonal variation may be indirectly linked to activity of migratory birds, rodents or insects (Wallace *et al.* 1997). Comparison of the 19 genotypes isolated during the peak shedding period between weeks 18 in May and 31 in August, with those on the *Campylobacter* MLST database showed limited evidence of environmental influence or indeed any other particular source. Only one, ST-53 had been isolated from water and wild birds including Starlings (Colles *et al.* 2003) and there were no isolates from the environmentally associated ST-177 and ST-179 complexes (Dingle *et al.* 2002).

The proportion of flocks (84%) colonized by *C. coli* was higher than that seen in other studies, where levels of 4.5-43% were recorded amongst organic or free-range poultry (Heuer *et al.* 2001; El-Shibiny *et al.* 2005; Rivoal *et al.* 2005). Conventionally reared flocks are typically dominated by *C. jejuni* with between 2 and 22% of flocks being colonized by *C. coli* (Wallace *et al.* 1997; Hald *et al.* 2001; Petersen and Wedderkopp 2001). The high incidence of *C. coli* could be typical of colonization of chickens reared to an older age, as discussed in Chapter 6 (El-Shibiny *et al.* 2005). Alternatively it could reflect the sources extensively reared birds are subject to, and in this case may relate to farm specific sources.

A total of 31 STs, 17 *C. jejuni* and 14 *C. coli,* were isolated from 48 flocks over a period of ten months. They clustered into ten clonal complexes, eight *C. jejuni*

and two *C. coli* with three STs unassigned. Nine of the clonal complexes, including those newly described in Chapter 6 had been previously defined, but a new *C. coli* ST-1150 complex, associated with the *C. jejuni aspA* 103 allele, was identified amongst data from this chapter and that held on the *Campylobacter* MLST database. Miller *et al* also describe the complex but identified the central genotype to be ST-1161 and it may be that more sampling is required before the true central genotype can be confirmed (Miller *et al.* 2006). The *C. coli* ST-828 complex was the only complex to appear fully established within the poultry since the central genotype as well as many single, double and triple locus variants were identified. Two STs, ST-854 and ST-828 appeared to be dominant and perhaps represent local clonal expansion. The other complexes were much more restricted in terms of STs and variants. The fact that not many STs were unassigned suggests that much of the diversity amongst poultry isolates may have been accounted for. However, relatively few STs were identified amongst the isolates from chickens in this study, in contrast to *C. jejuni* isolates from Starlings where 75 STs were identified amongst 293 isolates, and it is possible that a more extensive *Campylobacter* population may be identified if more chicken flocks are sampled. The difference in diversity amongst the chickens and wild bird isolates seen in this study may be due to the fact that all chickens within a flock become colonized with a limited number of genotypes, whilst wild birds are likely to become colonized on an individual basis.

Whilst a range of clonal complexes were identified, the frequency of individual STs did not appear to follow any particular pattern. There is some discussion as to whether or not *C. coli* is as genetically diverse as *C. jejuni* (Dingle *et al.* 2005; Miller *et al.* 2006). The fact that almost equal numbers of STs clustered into ten *C. jejuni* clonal complexes and only two *C. coli* clonal complexes suggests that the

C. coli genotypes colonizing the chickens were less diverse than *C. jejuni*, but the chicken flocks may effectively be treated as one unit, in which case the sample was fairly small and rarer genotypes may not have been encountered. The single ST-1487 isolated from the *C. coli* ST-1150 complex was divergent from the other *C. coli* STs and reflects the fact that the *aspA* 103 allele appears to have been acquired from *C. jejuni.*

In addition to the seasonal peak in the number of birds shedding *Campylobacter* within a flock, there was statistical evidence that a peak in the diversity of *Campylobacter* genotypes colonizing flocks occurred during the early spring/late summer months also. The lower stocking density level of the flocks divided at 21 days of age did not affect the levels of diversity, although the differences in stocking density may be minimal in practice, as mentioned previously. As with the peak in shedding, there appeared to be no link with the distribution of individual STs. Most were clustered throughout the year, and their presence was neither consistent nor predictable and presumably reflects the varying sources with which the chickens have come in contact. The *C. coli* ST-828 complex was dominant amongst the flocks, but individual STs within the complex varied. Similarly, ST-21 complex was present during the start and end of the year, but the initial genotype to be identified, ST-19, was later replaced by ST-262. The absence of the complex over 11 weeks suggests that the STs originated from different sources, rather than occurring by genetic drift through recombination.

The majority of flocks were co-colonized by up to seven different genotypes, with only four of 48 flocks being colonized by one genotype only. There was no evidence that colonization by one genotype or *Campylobacter* species would exclude another. In addition there appeared to be a succession of genotypes amongst the

flocks as a whole, over the study period. This is consistent with multiple sources of colonization that may change over time, as might be expected with free-range chickens. The succession of genotypes may also reflect the changing dynamics of bacterial populations within the chicken gut, or the predator-prey relationship between *Campylobacter* specific phages (Atterbury *et al.* 2005). The accumulation of new STs onto the farm sites occurred in clusters that could not be predicted. Up to three new types were isolated from particular flocks, suggesting that they had been exposed to different sources compared to previous flocks, but they did not appear to influence whether or not the following flocks were colonized by them – indeed many of them were only seen once.

There was evidence that *Campylobacter* genotypes varied in colonization potential. Four STS, ST-19 (ST-21 complex), ST-573 (ST-573 complex), ST-814 (ST-661 complex) and ST-855 (ST-828 *C. coli* complex) accounted for half of the isolates from poultry indicating that they are particularly successful colonizers of chickens. They were also the STs that colonized the greatest number of flocks, including those divided at 21 days of age, suggesting they easily gain access to them and are able to persist within the chicken's environment. In contrast ST-45 (ST-45 complex) and ST-257 (ST-257 complex) were isolated only rarely, and in low numbers, suggesting they were poor colonizers of the birds, or were unable to persist within the chicken's environment. The results were inconsistent with other studies that identified ST-45 and ST-257 to show a host bias with chickens, chicken meat and human disease sources (Dingle *et al.* 2002; Colles *et al.* 2003; Bull *et al.* 2006). The differences were most likely due to the fact that the samples in this study were a population sample from live chickens, whilst many of the others were isolated from meat or environmental samples that are likely to select for a different sub-population

of Campylobacters. In addition there were discrepancies in the sampling regimes and flock management systems that were studied.

The two farm sites showed a slight difference in shedding rate of *Campylobacter*, with 90.9% of birds testing positive at Wytham and 93.8% of birds testing positive at Northmoor, although differences in shedding rates were not significant over all. Eight of ten (80%) of the clonal complexes and 19 of 31 (60%) of STs were isolated at both farm sites, and the overall distribution of *Campylobacter* genotypes was not significantly different between them. The genotypes that did differ between the farm sites were rare. Analysis of gene flow (F_{ST}), also gave evidence that the *Campylobacter* populations were not significantly different at both sites, with less than 4% divergence between genotypes isolated from the farm sites. Divergence was slightly higher amongst the loci when they were considered separately, although only the value for the *aspA* locus was significant. However there were some differences in genotype distribution between the farm sites, with six STs being unique amongst flocks at Wytham, and a further six being unique amongst flocks at Northmoor. It is possible the unique types were specific to the farm sites, or the results may have arisen through sampling error introduced by small sample numbers, particularly since the unique types were rare (Newell and Fearnley 2003). The overall trend suggests that contamination from environmental sources may not be as important as previously believed (Berndtson *et al.* 1996b; Newell and Fearnley 2003; Bouwknegt *et al.* 2004). Instead the chickens may be acquiring *Campylobacter* genotypes, particularly those adapted to survival amongst chicken sources, from other chickens.

The arcs used to house the birds at night were shared between adjacent plots in this study, but were disinfected and left empty for five days prior to restocking. Although some *Campylobacter* genotypes were isolated amongst consecutive flocks

within an arc, no ST was isolated from every flock within an arc. Antigenic typing revealed that although *Campylobacter* ST-*flaA* SVR strains did reoccur in flocks reared within the same arc, but *flaA* SVR type was correlated with ST and flock rather than the arcs. Gene flow (F_{ST}) values indicated that the genotype distribution throughout the study period was similar in all arcs, even when comparing those at different farm sites. In some cases *Campylobacter* populations were more different within arcs than between them. The results were consistent with studies of housed birds identifying that carry over of *Campylobacter* genotypes between flocks is unlikely assuming that thorough disinfection procedures have been followed (Shreeve *et al.* 2002).

It is unknown whether *Campylobacters* are able to persist in land used for extensively rearing poultry long enough to influence *Campylobacter* populations in subsequent flocks. Unlike housing, the land that is likely to become heavily contaminated whilst stocked with birds, can not be disinfected, and can only be left fallow for a period before restocking. The first rotation of birds in this study were readily colonized despite being reared on land that had never been previously used for chicken rearing, and had been fallow for a number of months following growth of crops. The number of STs isolated from each plot was very similar, although only the *C. coli* ST-828 complex and *C. jejuni* ST-573 complex and ST-855 and ST-573, were isolated from a plot on all four flock rotations. Antigenic typing gave evidence that although *Campylobacter* ST-*flaA* SVR strains did reoccur in flocks raised on the same plots, *flaA* SVR type was correlated with ST and flock rather than the plots. Gene flow (F_{ST}) analysis indicated that there were some differences in *Campylobacter* populations amongst flocks isolated on the same plot, but there was little difference between the *Campylobacter* populations isolated from flocks reared on the different

plots, even when comparing those on the different farm sites. Thus the results indicated that the carry over of genotypes between flocks was unlikely when the plots were left fallow for a period of seven weeks between flocks, although the *Campylobacter* populations were often similar. In addition the small differences in environmental factors such as aspect and exposure that may be encountered across a large field had no effect on *Campylobacter* population.

A total of 16 flocks (data from 15) were divided in half at 21 days and reared at a lower density on separate plots, but situated on the same farm site. There was no evidence that the lower density affected shedding rate or genotypic diversity of *Campylobacter*, although in practical terms the density rate may be better calculated with regards to the house area rather than total plot size. The *Campylobacter* genotypes isolated from both halves of the flocks were often similar, and in similar proportions, but they could not be predicted. Gene flow (F_{ST}) values indicated that *Campylobacter* populations were more than 20% divergent in seven of the 15 (46.7%) flocks. It seems that despite the flocks being divided at an age where they are unlikely to be shedding *Campylobacter*, they are subject to similar influences that result in both halves being colonized by comparable populations of the organism. More data are required to confirm the findings since the small sample numbers may introduce statistical error.

A total of five clonal complexes and four STs were found to overlap between the chicken isolates in this chapter and wild bird isolates in Chapters 4 and 5. The overlapping STs were only identified amongst the Starlings and poultry suggesting that they may have more interaction than geese and poultry – a situation that is more likely in practice also. It is also possible that Starlings have a diet more similar to poultry, an important factor noted by Waldenstrom *et al.* (2002). The Neighbour

Joining tree gave evidence that the *Campylobacter* genotypes isolated from Starlings were not necessarily more closely related to chicken genotypes in comparison to those isolated from geese however. None of the overlapping genotypes were predominant amongst either of the host sources and so the direction of transmission can not be identified. It is possible that both wild birds and poultry acquire similar genotypes independently, from an external source. Evidence from this study suggests that whilst wild birds can not be ruled out as a source of *Campylobacter* for free-range poultry flocks they are not likely to be of major importance. There was no evidence that the spring peak in shedding and genotypic diversity amongst the poultry was directly due to the wild bird species. The high frequency of *C. coli* amongst the chicken isolates contrasted very strongly with the very low frequency amongst wild birds and it is even more unlikely that wild birds would act as a potential reservoir of infection for *C. coli*.

In conclusion, a high percentage of birds in all of the free-range flocks were shedding *Campylobacter* and there was a small but significant increase both in shedding rates and in genetic diversity during the spring and summer months. A variety of genotypes, both *C. jejuni* and *C. coli* were isolated and all but three STs clustered into ten clonal complexes. The high levels of *C. coli* may reflect the colonization pattern of older chickens or relate to a specific source of colonization amongst free-range poultry. A succession of *Campylobacter* genotypes was isolated over the study period suggesting that sources of the bacterium may change over the year. There was little evidence to suggest that *Campylobacter* populations within flocks were directly correlated with environmental sources, at least within the study area, since there was little influence by farm site or land plot. In addition *Campylobacter* genotypes common amongst poultry were rare amongst wild birds, a

potential source of contamination. Since the plots of land did not appear to influence the *Campylobacter* populations within subsequent plots it may be concluded that the fallow period of seven weeks was sufficient as a bio-security measure.

Future work should include sampling of more chicken flocks in different areas of the UK and under different management regimes in order to determine whether or not the *Campylobacter* population structure amongst chickens has been fully elucidated. The genotypes overlapping wild bird and chicken sources identified in this study should be tested using additional housekeeping genes and antigenic loci in order to determine the extent to which the *Campylobacter* strains are identical. In addition *Campylobacter* strains from the wild birds and chickens could be used in competitive exclusion studies to determine whether strains associated with wild birds can become established within the chicken host. The genotypes isolated from poultry at depletion could be compared with those isolated from carcases immediately after slaughter, in order that genotypes capable of surviving the processing plant environment are recognised and preventative steps may be taken in the future. Further work is required to identify the most likely source of *Campylobacter* for poultry and should include sampling of 'chicken sources' such as broiler breeder flocks, hatcheries, where the organism could be endemic.

Abstract

Chapter 8: Local and national distribution of *Campylobacter* genotypes colonizing farm animals and wild birds.

The aim of this study was to determine whether the same strains of *Campylobacter* identified by ST and *flaA* SVR type occurred within the two relatively intensively sampled areas of the UK discussed in previous chapters. The Lancashire isolates were sampled 10 years previous to those in Oxfordshire giving an interesting time comparison also. Results are speculative since low numbers of isolates were obtained from some sources and sampling regimes differed, for example some were large population studies from a single farm, and others were slaughterhouse samples encompassing several farms.

In common with another study in Finland, some *Campylobacter* ST-*flaA* SVR strains did occur on a national scale and were stable over a decade. It is not certain whether the ST and *flaA* SVR combinations arose by chance or represent a clone that has successfully spread across the country. As noted in other studies, ST-21, ST-45 and ST-828 clonal complexes were widely dispersed amongst different host animals and may be best adapted for colonising multiple host types.

A total of six clonal complexes were shared between the sources tested in Lancashire and Oxfordshire. Of these, nine STs isolated from animals were amongst the 23 most commonly isolated from human disease (41% isolates). The farm thus appears to be the origin of genotypes entering the food chain, but although they are common amongst human disease, they are less common amongst livestock or wild birds, suggesting a bottleneck occurs in the slaughtering and food processing systems.

There were lots of shared polymorphisms amongst the *C. jejuni* genotypes, with fixed differences only apparent between *C. jejuni* and *C. coli* genotypes. There were subtle host association effects, with greater numbers of polymorphisms being shared amongst cattle, free-range poultry, human disease and chicken meat, and least polymorphisms being shared between Starlings and geese. The *flaA* SVR type was a useful marker for short term or local epidemiology and was loosely but not exclusively linked with ST, rather than animal source.

Chapter 8: Local and national distribution of *Campylobacter* genotypes colonizing farm animals and wild birds.

8.1 Introduction.

Previous chapters have concentrated on detailed analysis of *Campylobacter* populations in single host species. The analyses in this chapter aim to bring together the work of the previous chapters and compare and contrast the *Campylobacter* populations colonizing animal hosts in greater detail. Knowledge of the population genetics of *Campylobacter* on a flock, farm and national level, as well as individual host animals is essential if strategic intervention policies are to be designed in the hope of reducing human infection. Additional pilot studies into cattle and pigs on the University farm in Oxfordshire have been included in this chapter in order to give an insight into the *Campylobacter* population structure on the farm as a unit. The survey of genotypes isolated from animals in Lancashire during the mid 1990's allows a geographical and temporal comparison with those isolated from Oxfordshire almost a decade later. All of the isolates can be compared with genotypes isolated from human disease and with retail chicken meat held on the *Campylobacter* MLST database to determine if there are any potential epidemiological connections.

8.2 Results.

8.2.1. The diversity of *Campylobacter* genotypes isolated from farm animals, wild birds, human disease and retail chicken meat.

The number of STs isolated from a host source ranged from four (Lancashire poultry and Starlings) to 649 (human disease), and the number of polymorphisms ranged from 11 (Lancashire Starlings) to 647 (human disease). The modified Simpson's index was used to measure genotypic diversity amongst the different host sources. Values ranged from 0.61 to 0.99 for STs and from 0.14 to 0.93 for *flaA* SVR types. Diversity was greatest amongst chicken meat, human disease and geese and lowest amongst Lancashire Poultry and Starlings.

Table 8.1. The diversity of *Campylobacter* genotypes isolated from farm animals, wild birds, human disease and retail chicken meat.

Animal host	Number of		Diversity	
	STs	Polymorphisms	STs	*flaA SVR*
Geese[O] n=166	38	155	0.9533	0.9346
Starlings[O] n=293	75	240	0.9174	0.9248
Poultry[O] n=868	31	523	0.9056	0.8751
Cattle[O] n=31	14	486	0.8409	0.8145
Poultry[L] n-28	4	92	0.6138	0.1402
Cattle[L] n=33	17	184	0.9375	0.8554
Sheep[L] n=42	17	154	0.9175	0.8791
Starlings[L] n=9	4	11	0.8056	0.7222
Humans[DBa] n=1358	649	647	0.9893	Not tested
Chicken meat[DBa] n=144	122	538	0.9913	Not tested

[O]=Oxfordshire, [L]= Lancashire, [DBa]=all isolates listed on the *Campylobacter* MLST database with a worldwide distribution.

8.2.2. The distribution of *Campylobacter* genotypes amongst host sources in Oxfordshire.

8.2.2.1 Clonal complex distribution.

A total of 21 clonal complexes were identified amongst the five different animal sources on the University farm sites at Wytham and Northmoor (Table 8.2). Of these, eight (38.1%) were isolated from more than one source and ST-21 and ST-45 complexes were the most diverse being identified amongst all sources except pigs. The *C. coli* ST-828 complex was isolated from three sources and the remaining complexes were isolated from two sources or fewer. Starlings and chickens shared six complexes with other sources, and most commonly between themselves. Cattle shared four clonal complexes with other sources, geese shared two, ST-21 and ST-45 complexes and pigs shared only ST-828 complex with other sources. Thirteen of 21 (61.9%) clonal complexes were not shared amongst host sources. Geese and Starlings had four unique complexes each, chickens had three and cattle had two.

8.2.2.2 Distribution of STs.

A total of 128 STs were identified amongst the different sources on the farm sites, of which 8 (6.25%) were shared between one or more sources (see appendices). Four STs (ST-45, ST-257, ST-574 and ST-1023) were shared between chickens and Starlings, two STs (ST-19 and ST-262) were shared between chicken and cattle, two STs (ST-42 and ST-45) were shared between Starlings and cattle and two STs (ST-137 and ST-696) were shared between geese and sheep.

8.2.2.3 Allelic diversity.

Strains indistinguishable by ST and *flaA* SVR and shared between one or more host on the same farm site in Oxfordshire were identified on eight occasions (Table 8.3.). ST-1; 36-1 and ST-262;37-1 were isolated from cattle and chickens, ST-257;16-12 and ST-1023;80-48 were isolated from Starlings and chickens, ST-42;274-42 and ST-436;413-131 were isolated from Starlings and cattle and ST-137;85-24 and ST-696;15-5 were isolated from geese and sheep.

Table 8.2. The percentage distribution of *Campylobacter* clonal complexes amongst animal sources, human disease and retail chicken meat. O=Oxfordshire, L= Lancashire ^{DBa}=all isolates from *Campylobacter* MLST database with a worldwide distribution

Clonal complex	Geese^{O} n=166	Starlings^{O} n=293	Chickens^{O} n=868	Cattle^{O} n=31	Sheep^{O} n=4	Pigs^{O} n=8	Poultry^{L} n=28	Cattle^{L} n=33	Sheep^{L} n=42	Starlings^{L} n=9	Humans^{DBa} n=1358	Chicken^{DBa} n=144
21	0.6	0.34	13.25	19.35			3.7	36.36	26.2	22.2	21.34	26.39
22								9.09	2.4		3.68	0.69
41											1.33	
42		0.34		16.13				3.03	23.8		2.65	
45	4.82	2.73	0.58	3.23	0.25		96.4	9.09		11.1	8.47	17.36
48		0.68	2.65					18.18	7.1		8.25	2.78
49				6.45							1.47	0.69
52									4.8		1.62	1.39
61				6.45				3.03	30.1		3.83	0.69
177		24.23								33.3	0.22	
179		1.02									0.07	
206								12.12			3.46	1.39
257		0.68	1.38					3.03		33.3	4.49	6.94
283		0.34									1.1	2.08
353											4.12	0.69
354											1.91	5.56
362											0.88	
403											4.27	
433											0.44	2.08
443											1.77	0.69
446											0.59	
460											0.66	0.69
508											3.61	
573			12.33								0.22	3.47
574		0.34	3.57								0.74	1.39
607											0.81	
658											1.91	
661			14.29								0.22	1.39
677		1.37									0.07	
682		48.81										
692	10.24											
702	12.05											
828			39.86	41.94		100					5.08	7.64
1034	15.66										0.15	
1150			1.61								0.37	
1332	4.82				0.25							
Unassigned	51.2	19.11	10.37	6.45	0.5		6.06				10.16	15.97

Table 8.3. Distribution of identical *Campylobacter* strains amongst animal hosts. [O]=Oxfordshire, [L]= Lancashire

Clonal Complex	ST	*flaA* SVR allele-peptide (percentage of isolates within a host source)								
		Geese[O] n=166	Starlings[O] n=293	Chickens[O] n=868	Cattle[O] n=31	Sheep[O] n=4	Poultry[L] n=28	Cattle[L] n=33	Sheep[L] n=42	Starlings[L] n=9
21	19			36-1(10.7)	36-1(3.2)				36-1	
	21				412-49(3.2)				198-4	
	53			32-1(0.2)			22-1(3.6)	32-1 (3.0)	32-1(2.3)	32-1(22.2)
	262			37-1 (3.1)	37-1(6.4)			37-1(27.3)	37-1(7.1)	
22	22							108-64(6.1)	108-64(2.4)	
42	42		274-42(0.3)		274-42(9.7)			239-9(3.0)	239-9(19.0)	
45	45		22-1 (0.6)	15-5 (0.7)	70-5(3.2)		22-1(89.2)			
			21-2 (0.6)	36-1 (0.2)						
	137	85-24(4.2)				85-24(0.25)				16-12(11.1)
48	38		41-4(0.6)					41-4(6.1)	227-4(2.4)	
	48							32-1(6.1)	32-1(4.8)	
61	61				177-74(6.5)			42-14(3.0)	42-14(7.1)	
257	257		16-12(0.6)	16-12(1.4)			16-12(14.3)			16-12(33.3)
574	574		8-1(0.3)	8-1(2.0)						
1332	696	15-5(5.4)				15-5(0.25)				
Unassigned	436		413-131(0.6)		413-131(0.3)					
	1023		80-48 (2.4)	80-48(0.2)						

8.2.3. Comparison of genotypes between isolates from Lancashire and Oxfordshire.

8.2.3.1 Clonal complex distribution.

A total of 20 clonal complexes were identified amongst the animal sources from the different areas, six of which were common to both areas, three were isolated only in Lancashire and 11 were isolated only in Oxfordshire. ST-21 and ST-45 complexes were the most widespread being isolated from all sources except sheep from one of the two locations. ST-42 complex was isolated from Starlings and cattle in Oxfordshire and from cattle and sheep in Lancashire. ST-48 complex was isolated from Starlings and chickens in Oxfordshire and from cattle and sheep in Lancashire. ST-61 complex was isolated from cattle in Oxfordshire and cattle and sheep in Lancashire, and ST-257 complex was isolated from Starlings and chickens in Oxfordshire and from Starlings and cattle in Lancashire. Chi squared analysis gave evidence that the distribution of clonal complexes amongst animal host sources was non-random for 20 of the 24 clonal complexes identified (Table 8.4).

Table 8.4. Chi squared analysis to establish whether or not distribution of clonal complexes amongst host sources was random.

Clonal complex	$\chi 2$	*p* value	Significant
21	65.90	0.001	Yes
22	44.50	0.000	Yes
42	167.64	0.000	Yes
45	6.72	0.082	No
48	19.76	0.000	Yes
49	22.21	0.000	Yes
52	22.21	0.000	Yes
61	179.74	0.000	Yes
177	315.86	0.000	Yes
179	12.07	0.007	Yes
206	44.50	0.000	Yes
257	1.85	0.603	No
283	3.99	0.262	No
573	62.30	0.000	Yes
574	14.53	0.002	Yes
661	73.30	0.000	Yes
677	16.10	0.001	Yes
682	649.01	0.000	Yes
692	248.44	0.000	Yes
702	293.00	0.000	Yes
828	222.14	0.000	Yes
1034	382.80	0.000	Yes
1150	7.07	0.070	No
1332	102.69	0.000	Yes

8.2.3.2 ST distribution.

Nine of 148 (6.1%) STs were isolated in both areas. They were ST-19 isolated from chickens and cattle in Oxfordshire and Sheep in Lancashire, ST-21 isolated from cattle in Oxfordshire and sheep in Lancashire, ST-53 isolated from chickens in Oxfordshire and from chickens, cattle, sheep and Starlings in Lancashire, ST-262 isolated from chickens and cattle in Oxfordshire and from cattle and sheep in Lancashire, ST-42 isolated from Starlings and cattle in Oxfordshire and from cattle and sheep in Lancashire, ST-45 isolated from Starlings, chickens and cattle in Oxfordshire and from chickens and cattle in Lancashire, ST-137 isolated from geese

and sheep in Oxfordshire and Starlings in Lancashire, ST-61 isolated from cattle in Oxfordshire and from cattle and sheep in Lancashire, and ST-257 isolated from Starlings and chickens in Oxfordshire and from cattle and Starlings in Lancashire.

8.2.3.3 Antigenic diversity.

A total of six identical ST *flaA* SVR strains were isolated from the two areas. ST-19;36-1 was isolated from chickens and cattle in Oxfordshire and from sheep in Lancashire, ST-53;32-1 was isolated from chickens in Oxfordshire and from cattle, sheep and Starlings in Lancashire, ST-262;37-1 was isolated from chickens and cattle in Oxfordshire and from cattle and sheep in Lancashire, ST-45;22-1 was isolated from Starlings in Oxfordshire and from poultry in Lancashire, ST-38;41-4 was isolated from Starlings in Oxfordshire and from cattle in Lancashire and ST-257;16-12 was isolated from Starlings and chickens in both Oxfordshire and Lancashire.

8.2.4 Comparison of genotypes isolated from animal sources with those isolated from cases of human disease and retail chicken meat.

8.2.4.1 Clonal complex distribution.

A total of 32 clonal complexes were identified amongst the 1358 isolates from human disease with worldwide distribution listed on the *Campylobacter* MLST database. Of these,20 (62.5%) were identified amongst the animal sources and accounted for 67.5% of the human isolates. Six clonal complexes were shared only with retail chicken meat and seven were unique to human disease (Table 8.2.). Clonal complexes were commonly shared between isolates from human disease and Starlings

(n=10, 34.1% of isolates), poultry (n=9, 89.8% of isolates) cattle (n=8, 92.2% of isolates), sheep (n=7, 89.1% of isolates) and geese (n=4, 21.1% of isolates).

A total of 19 clonal complexes were identified amongst 144 isolates from chicken retail meat. Of these 14 (73.6%) were isolated from the animal sources and accounted for 74.3% of the chicken meat isolates. Six clonal complexes were identified amongst human disease sources and none were isolated from the chicken meat only. Clonal complexes were commonly shared between cattle (n=12, 84.4% of isolates), poultry (n=10, 88.3% of isolates), Starlings (n=9, 6.7% of isolates), and sheep (n=6, 67.4% of isolates). Two clonal complexes, accounting for 5.42% of isolates of geese isolates, were shared between isolates from chicken meat and geese.

8.2.4.2 ST distribution.

A total of 649 STs were identified amongst the human disease isolates of which 30 (4.6%) were shared with the animal sources, accounting for 29.8% of the human isolates (appendices). They were most commonly shared between isolates from human disease and cattle (n=13, 65.6% of isolates), sheep (n=13, 80.4% of isolates), poultry (n=10, 23.4% of isolates), and Starlings (n=9, 5.6% of isolates). Only three STs, accounting for 5.4% of isolates were shared between isolates from human disease and geese (Table 8.4.).

A total of 122 STs were identified amongst the retail chicken isolates, of which seven (5.7%) were shared with animal sources, accounting for 7.7% of the chicken meat isolates. They were most commonly shared between cattle (n=6, 3.1% of isolates), sheep (n=4, 3.7% of isolates), poultry (n=3, 15.7% of isolates), Starlings (n=2, 2.0% of isolates). No STs were shared between isolates from geese and retail chicken meat.

Table 8.5. Percentage of isolates from each animal source with genotypes indistinguishable from those isolated from retail chicken meat and human disease. ST=sequnce type, CC=clonal complex.

	Percentage isolates				
	geese	Starlings	poultry	cattle	sheep
Chicken meat (CC)	5.42	6.7	88.3	84.4	67.4
Chicken meat (ST)	0	2	15.7	3.1	3.7
Human disease (CC)	21.1	34.1	89.8	92.2	89.1
Human disease (ST)	5.4	5.6	23.4	65.6	80.4

8.2.5. Distribution of alleles amongst host sources.

A total of 49 alleles potentially indicative of host source were identified when those accounting for 10% or more of isolates from a host source were considered. When the *Campylobacter* MLST database was interrogated, potential host-allele associations noted amongst isolates from this study, were upheld amongst geese, Starling, chicken and pig sources with the definition that potentially host associated alleles accounted for 30% or more of isolates from a single host source. (Table 8.5). The largest number of host associated alleles was found in geese (16), with between one and four at each locus except *aspA* where there did not appear to be host association. A total of 12 unique alleles were recognised amongst Starling isolates, with between one and three alleles at each locus. Five chicken associated alleles were identified, two *glnA*, two *tkt* and one *pgm* alleles. One *tkt* allele was associated with pigs. Some alleles demonstrated association with clonal complexes, which sometimes coincided with host association. Those alleles accounting for 50% or more of STs within a clonal complex on the *Campylobacter* MLST database were included in Table 8.4.

Table 8.6. Alleles demonstrating association with host source or clonal complex.

[a]Frequency calculated from isolates in this study rather than from the *Campylobacter* MLST database.

Locus	Allele	Frequency in host source[a]	Host source (% of isolates)[a]	Clonal complex (% of isolates)[a]
aspA	9	101	-	ST-257 (59.4%)
	17	35	Starling (54.3%)	ST-177 (74.3%)
	26	22	Starling (86.4%)	ST-682 (72.7%)
	35	11	Starling (81.8%)	Unassigned (60.0%)
	87	5	-	Unassigned (60.0%)
glnA	28	11	Chicken (72.8%)	ST-573 (81.8%)
	29	9	Geese (75.0%)	Unassigned (87.5%)
	43	17	Starling (94.1%)	ST-682 (52.9%)
	52	8	Geese (75.0%)	ST-692 (62.5%)
	61	9	Geese (88.8%)	ST-1034 100%)
	75	10	Chicken (80.0%)	ST-661 (80.0%)
gltA	8	35	Starling (62.9%)	ST-177 (77.1%)
	9	36	Starling (69.4%)	ST-682 (69.4%)
	10	24	-	ST-45 (96.1%)
	12	55	-	ST-21 (89.1%)
	57	5	Geese (60%)	ST-692 (100%)
glyA	5	64	Starling (42.2%)	ST-177 (46.9%)
	26	5	Geese (60%)	ST-692 (100%)
	48	26	Geese (57.7%)	-
	51	15	Starling (80%)	ST-682 (93.3%)
	62	80	-	ST-257 (80%)
	64	11	Geese (45.5%)	ST-1034 (72.7%)
	82	38	-	ST-828 (84.2%)
pgm	1	154	-	ST-45 (90.3%)
	4	90	-	ST-257(82.2%)
	8	52	Starling (55.7%)	ST-177 (46.2%)
	13	7	Geese (85.7%)	Unassigned (71.4%)
	17	26	Chicken (57.6%)	-
	121	16	Starling (100%)	ST-682 (72.5%)
	126	7	Geese (100%)	ST-1034
	127	3	Geese (100%)	-
	141	7	Chicken (100%)	ST-661 (57.1%)
tkt	5	112	-	ST-257 (67.0%)
	9	57	-	ST-42 (84.2%)
	22	4	Pig (100%)	ST-403 (75.0%)
	24	4	Geese (100%)	Unassigned (100%)
	25	31	Geese (32.3%)	Unassigned (48.4%)
	29	7	Geese (57.1%)	ST-692 (71.4%)
	34	25	Chicken (76%)	-
	35	8	Chicken (75.0%)	ST-828 (62.5%)
	43	14	-	ST-828 (85.7%)
	46	35	Starling (80%)	ST-682 (74.3%)
	56	6	-	ST-828 (83.3%)
	99	3	Geese (100%)	ST-702 (66.0%)
uncA	12	23	-	ST-443 (69.6%)
	21	38	Starling (86.8%)	ST-682 (63.2%)
	23	22	Geese (81.8%)	-
	57	10	Geese (80.0%)	Unassigned (90.0%)

8.2.6. Distribution of *flaA* SVR alleles amongst the animal sources.

A total of 108 *flaA* SVR alleles were identified amongst the animal sources, of which 23 were shared between more than one host species (Table 8.6.). Each animal source was associated with between two and 56 *flaA* SVR alleles, the majority of which were associated with less than 5% of isolates from a particular source. There were some more prominent associations, the highest of which were between sheep and *flaA* SVR 239-9 which accounted for 21.4% of isolates, Starlings and *flaA* allele 400-43 which accounted for 20.6% of isolates and cattle and *flaA* SVR allele 255-1 which accounted for 20.3% of isolates. Three alleles, 8-1, 32-1 and 37-1 were shared between four animal sources, excluding geese. The *flaA* SVR alleles were most commonly shared between Starlings and cattle (nine occasions), and Starlings and poultry (eight occasions). Cattle and geese were the only sources which had no *flaA* allele in common.

Table 8.7. Distribution of *flaA* SVR types amongst animal sources. Figures are the percentage of the total number isolates from a source.

flaA allele	peptide	Source				
		Geese	Starling	Poultry	Cattle	Sheep
2	**27**		0.7			
8	**1**		0.3	13.3	6.25	2.4
11	**11**				4.7	
14	**11**					2.4
15	**5**	5.4		0.7		
16	**12**		3.3	1.5	1.6	
17	**11**			1.7		
21	**2**		1	1.2		
22	**1**	5.4	0.7	2.9		
23	**1**				1.6	
30	**11**			4.7		
32	**1**		1.3	8.9	6.25	2.4
34	**1**			11.2		2.4
36	**1**			11	3.1	2.4
37	**1**		0.3	3.1	15.6	4.8
40	**1**			0.1		
41	**4**		0.7	2.2	4.7	
42	**14**				3.1	
43	**14**					2.4
56	**1**	7.8				7.1
57	**4**					4.8
62	**29**		3.8			
64	**1**	5.4				
66	**1**			13.7	1.6	
67	**8**			0.1		
68	**5**		0.7			
69	**15**	9.0				
70	**5**				4.7	
77	**56**		0.3			
80	**48**		3.4	0.1		
81	**46**		3.4			
82	**5**		0.7			
85	**24**	4.8	5.5			
86	**18**		7	0.1		
89	**1**	0.6		0.1		
96	**34**					2.4
100	**33**			0.3		
105	**1**			3.6		
108	**64**				4.7	4.8
124	**68**		4.8			
165	**85**			0.1		
177	**74**				3.1	
181	**94**		1.4			
185	**95**		0.3			
186	**95**		0.3			2.4
191	**33**			0.16		
198	**4**					2.4
199	**15**	10.8				
205	**1**	0.6				
206	**35**	1.8				

Continued over page.....

Table 8.7 *continued.*

flaA allele	peptide	Source Geese	Starling	Poultry	Cattle	Sheep
213	**15**	1.2				
219	**15**	0.6				
224	**35**	7.8				
225	**11**	6.6				
227	**4**					1.6
234	**3**		1.7			
239	**9**		0.7		1.6	21.4
252	**44**	0.6				
255	**1**				20.3	
264	**15**	4.2				
274	**42**		0.3		6.25	
275	**27**	0.6	0.3			
306	**93**		0.7			
312	**4**			1.3		
314	**8**			0.8		
319	**15**			0.14		
322	**19**		1.4			
323	**67**		0.3			
325	**1**			0.9		
341	**18**		1.4			
348	**15**		0.3			
349	**67**		1			
351	**111**			1.3		
352	**1**	1.2				
353	**1**	4.8				
354	**27**	3				
355	**1**	12				
372	**118**		0.3			
391	**125**	0.6				
399	**118**		0.3			
400	**43**		20.6			
403	**67**		2.7			
404	**128**		2.7			
405	**45**		1.7			
412	**49**				1.6	
413	**131**		0.7		1.6	
415	**1**			2.5		
432	**45**		0.7			
433	**3**		0.3			
434	**134**		0.3			
447	**136**		1.4			
448	**137**		0.3			
449	**18**		0.3			
450	**45**		4.5			
451	**138**		0.3			
452	**37**		0.3			
453	**1**			0.1		
454	**24**		1			
455	**139**		0.3			
456	**140**		3.4			
457	**141**		0.3			
459	**18**		0.3			
460	**142**		0.3			
461	**46**		0.7			
462	**143**		0.3			
504	**155**		0.3			
508	**18**		0.3			
514	**118**		0.3			

8.2.8 Gene flow (F_{ST}) amongst the different host sources.

The F_{ST} values were calculated using concatenated sequence to determine how differentiated the *Campylobacter* populations were amongst the animal sources (Tables 8.7a and 8.7b.). The majority of *Campylobacter* populations were significantly different amongst the different sources, even when considering the same host species but in different locations and time. When *C. jejuni* and *C. coli* genotypes were both included in the analyses, the most divergent populations (67.3%) were those isolated from cattle and Starlings in Oxfordshire, and the least divergent populations (0.5%) were those isolated from human disease and chicken meat in the UK. When *C. jejuni* genotypes only were included in the analyses the most divergent populations (60.4%) were those isolated from geese and Starlings and the least divergent (0.3%) were those isolated from cattle in Oxfordshire and cattle in Lancashire.

Those that had populations that were less than 20% divergent when *C. jejuni* and *C. coli* genotypes were included in the analyses included chicken and cattle in Oxfordshire, Starlings and cattle in Lancashire, Starlings and sheep in Lancashire, human disease isolates and all of the Lancashire isolates with the exception of poultry, chicken meat isolates and all of the Lancashire isolates, and human disease and chicken meat isolates. Those that had populations that were less than 20% divergent when only *C. jejuni* isolates were included in the analyses were Oxfordshire cattle and Lancashire cattle, Lancashire sheep and Oxfordshire cattle, Lancashire sheep and Lancashire cattle, Lancashire Starlings and Oxfordshire chickens, Oxfordshire cattle, Lancashire cattle and Lancashire sheep, human disease isolates and Oxfordshire chickens and cattle, and all Lancashire isolates except poultry, chicken meat isolates

and Oxfordshire chickens and cattle and all Lancashire isolates, and human disease isolates and chicken meat.

Table 8.8a. Differentiation (F_{ST}) of *C. jejuni* and *C. coli* populations amongst animal sources, human disease and chicken meat using concatenated sequence.

[O]=Oxfordshire, [L]= Lancashire, [DBUK]=all UK isolates listed on the *Campylobacter* MLST database. *=significant *p* value.

	Geese[O] n=166	**Starlings[O]** n=293	**Chickens[O]** n=868	**Cattle[O]** n=31	**Cattle[L]** n=33	**Sheep[L]** n=42	**Poultry[L]** n=28	**Starlings[L]** n=9	**Humans[DBa]** n=722	**Chicken[DBa]** n=130
Geese[O]	-	-	-	-	-	-	-	-	-	-
p value	-	-	-	-	-	-	-	-	-	-
Starlings[O]	0.60433	-	-	-	-	-	-	-	-	-
p value	0.00000*	-	-	-	-	-	-	-	-	-
Chickens[O]	0.36275	0.41885	-	-	-	-	-	-	-	-
p value	0.00000*	0.00000*	-	-	-	-	-	-	-	-
Cattle[O]	0.56928	0.67284	0.01046	-	-	-	-	-	-	-
p value	0.00000*	0.00000*	0.18018	-	-	-	-	-	-	-
Cattle[L]	0.29120	0.54773	0.36362	0.35445	-	-	-	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.00000*	-	-	-	-	-	-
Sheep[L]	0.34581	0.54467	0.29864	0.30989	0.09464	-	-	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.00000*	0.00901	-	-	-	-	-
Poultry[L]	0.58222	0.51594	0.42450	0.62733	0.40716	0.35973	-	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.00000*	0.00000*	0.00000*	-	-	-	-
Starlings[L]	0.36946	0.30540	0.30733	0.25642	0.15908	0.16638	0.59789	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.00000*	0.00000*	0.00000*	0.00000*	-	-	-
Humans[DBa]	0.17979	0.33165	0.36450	0.42958	0.00586	0.12508	0.25137	0.05219	-	-
p value	0.00000*	0.00000*	0.00000*	0.00000*	0.14414	0.00000*	0.00000*	0.01802*	-	-
Chicken[DBa]	0.20686	0.38874	0.28247	0.31737*	0.02539	0.07252	0.21020	0.03147	0.00554	-
	0.00000*	0.00000*	0.00000*	0.00000*	0.05405	0.00000*	0.00000*	0.00000*	0.02703*	-

Table 8.8b. Differentiation (F_{ST}) of *C. jejuni* populations amongst animal sources, human disease and chicken meat using concatenated sequence.

[O]=Oxfordshire, [L]= Lancashire, [DBUK]=all UK isolates listed on the *Campylobacter* MLST database. *=significant *p* value.

	Geese[O] n=166	Starlings[O] n=293	Chickens[O] n=868	Cattle[O] n=31	Cattle[L] n=33	Sheep[L] n=42	Poultry[L] n=28	Starlings[L] n=9	Humans[DBa] n=722	Chicken[DBa] n=130
Geese[O]	-	-	-	-	-	-	-	-	-	-
p value	-	-	-	-	-	-	-	-	-	-
Starlings[O]	0.60433	-	-	-	-	-	-	-	-	-
p value	0.00000*	-	-	-	-	-	-	-	-	-
Chickens[O]	0.23328	0.59544	-	-	-	-	-	-	-	-
p value	0.00000*	0.00000*	-	-	-	-	-	-	-	-
Cattle[O]	0.33675	0.52306	0.24248	-	-	-	-	-	-	-
p value	0.00000*	0.00000*	0.00000*	-	-	-	-	-	-	-
Cattle[L]	0.29120*	0.54773*	0.18590	-0.00290	-	-	-	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.44748	-	-	-	-	-	-
Sheep[L]	0.34581	0.54467	0.29474	0.04782	0.09464	-	-	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.00901*	0.00901*	-	-	-	-	-
Poultry[L]	0.58222	0.51594	0.56268	0.34377	0.40716	0.35973	-	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.00000*	0.00000*	0.00000*	-	-	-	-
Starlings[L]	0.36946*	0.30540*	0.05590	0.10393	0.15908	0.16638	0.59789	-	-	-
p value	0.00000*	0.00000*	0.00000*	0.04505*	0.00000*	0.00000*	0.00000*	-	-	-
Humans[DBa]	0.23406	0.41384	0.11931	0.05038	0.00132	0.09407	0.33592	0.09109	-	-
p value	0.00000*	0.00000*	0.00000*	0.00000*	0.27027	0.00000*	0.00000*	0.01802*	-	-
Chicken[DBa]	0.26891	0.47754	0.14960	0.03873	0.01913	0.13539	0.11515	0.08305	0.01059	-
	0.00000*	0.00000*	0.00000*	0.02703*	0.09910	0.00000*	0.00000*	0.00000*	0.00901*	-

8.2.9 Fixed and shared polymorphisms.

The majority of *Campylobacter* populations from the different sources had no fixed polymorphisms between them (Table 8.8). A total of ten combinations had between one and 404 fixed differences. Of these seven were associated with *Campylobacter* isolates from pigs, and the high number of fixed differences reflects the comparison of populations consisting entirely of *C. jejuni* with the pig isolates that were entirely *C. coli.* The other sources containing a mixture of *C. jejuni* and *C. coli* had no fixed differences when compared with the *C. coli* isolates from pigs. Comparisons between the Oxfordshire sheep and Oxfordshire cattle, and Lancashire sheep and Lancashire poultry gave one fixed difference on each occasion.

The number of shared polymorphisms ranged from one to 560. The greatest numbers of polymorphisms were shared between the human disease and chicken meat isolates taken from the *Campylobacter* MLST database (Figure 8.1a). The next highest numbers of polymorphisms were shared between Oxfordshire chickens and human disease and chicken meat isolates. Oxfordshire cattle also shared high numbers of polymorphisms with human disease, chicken meat and Oxfordshire cattle. The number of shared polymorphisms was significantly lower amongst the other combinations of *Campylobacter* isolates, with the least number seen amongst Oxfordshire chickens and pigs (Figure 8.1b).

Table 8.9. The fixed and shared polymorphisms amongst *Campylobacter* isolated from animals, human disease and chicken meat using concatenated sequence. [O]=Oxfordshire, [L]=Lancashire, [DBUK]=all UKisolates listed on the *Campylobacter* MLST databases. FD=fixed differences, SP= shared polymorphisms.

		Geese[O] n=166	Starlings[O] n=293	Chickens[O] n=868	Cattle[O] n=31	Sheep[O] n=4	Pigs[O] n=8	Poultry[L] n=28	Cattle[L] n=33	Sheep[L] n=42	Starlings[L] n=9	Humans[DBUK] n=722	Chicken[DBUK] n=130
Geese[O]	FD	-	-	-	-	-	-	-	-	-	-	-	-
	SP	-	-	-	-	-	-	-	-	-	-	-	-
Starlings[O]	FD	0	-	-	-	-	-	-	-	-	-	-	-
	SP	121	-	-	-	-	-	-	-	-	-	-	-
Chickens[O]	FD	0	0	-	-	-	-	-	-	-	-	-	-
	SP	129	127	-	-	-	-	-	-	-	-	-	-
Cattle[O]	FD	0	0	0	-	-	-	-	-	-	-	-	-
	SP	112	134	506	-	-	-	-	-	-	-	-	-
Sheep[O]	FD	0	9	0	1	-	-	-	-	-	-	-	-
	SP	67	96	74	69	-	-	-	-	-	-	-	-
Pigs[O]	FD	382	369	0	0	339	-	-	-	-	-	-	-
	SP	1	1	8	6	1	-	-	-	-	-	-	-
Poultry[L]	FD	0	0	0	0	1	404	-	-	-	-	-	-
	SP	83	92	92	85	50	1	-	-	-	-	-	-
Cattle[L]	FD	0	0	0	0	0	329	0	-	-	-	-	-
	SP	109	119	180	172	62	2	89	-	-	-	-	-
Sheep[L]	FD	0	0	0	0	1	399	0	0	-	-	-	-
	SP	78	92	153	152	46	1	68	153	-	-	-	-
Starlings[L]	FD	0	0	0	0	0	397	0	0	0	-	-	-
	SP	89	110	111	93	55	1	91	92	70	-	-	-
Humans[DBUK]	FD	0	0	0	0	0	0	0	0	0	0	-	-
	SP	146	214	549	509	98	9	92	186	155	112	-	-
Chicken[DBUK]	FD	0	0	0	0	0	0	0	0	0	0	0	-
	SP	136	161	540	507	78	6	90	183	152	102	560	-

Figure 8.1a. The ten combinations of *Campylobacter* isolates sharing the highest number of polymorphisms, derived from Table 8.9.

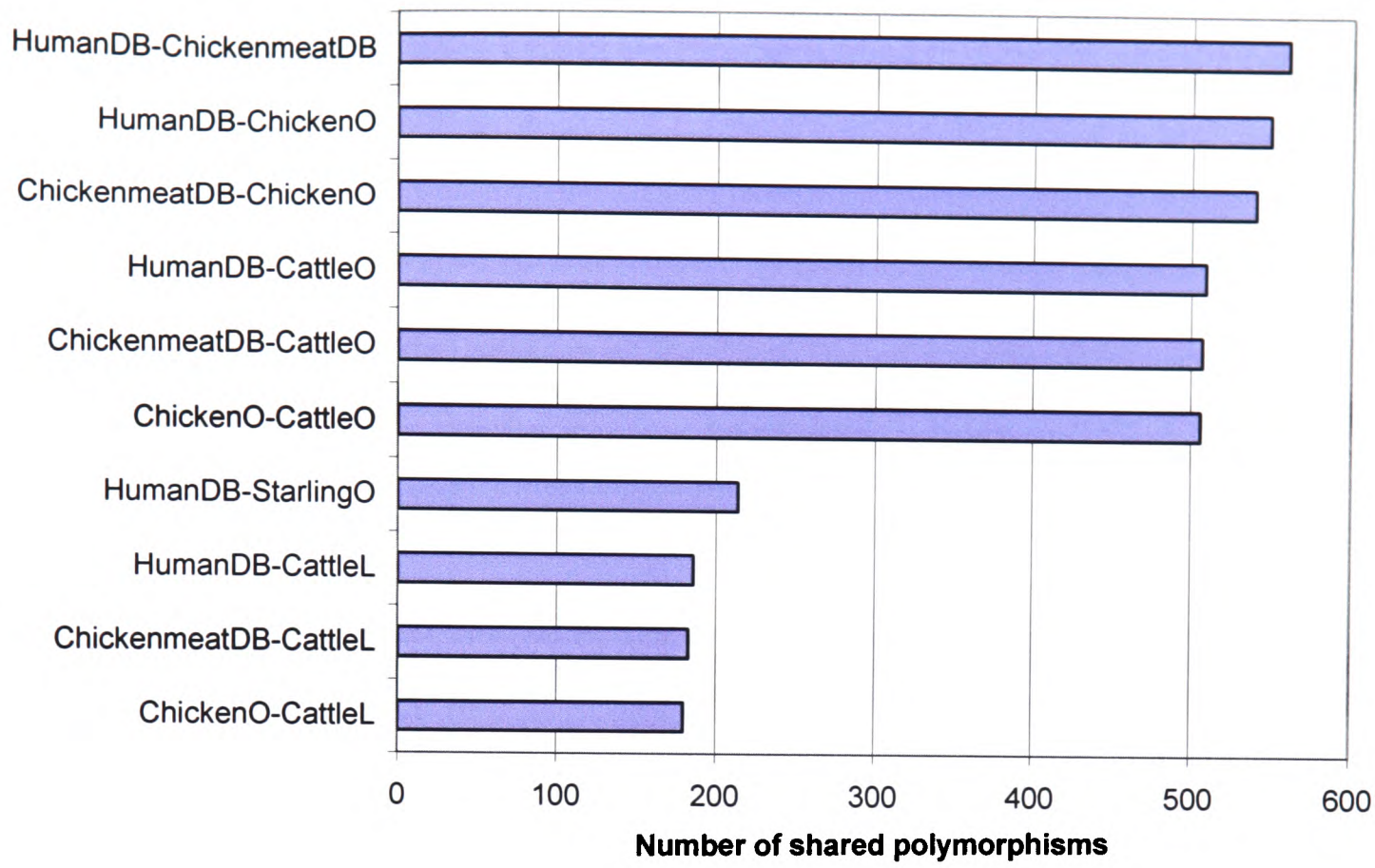
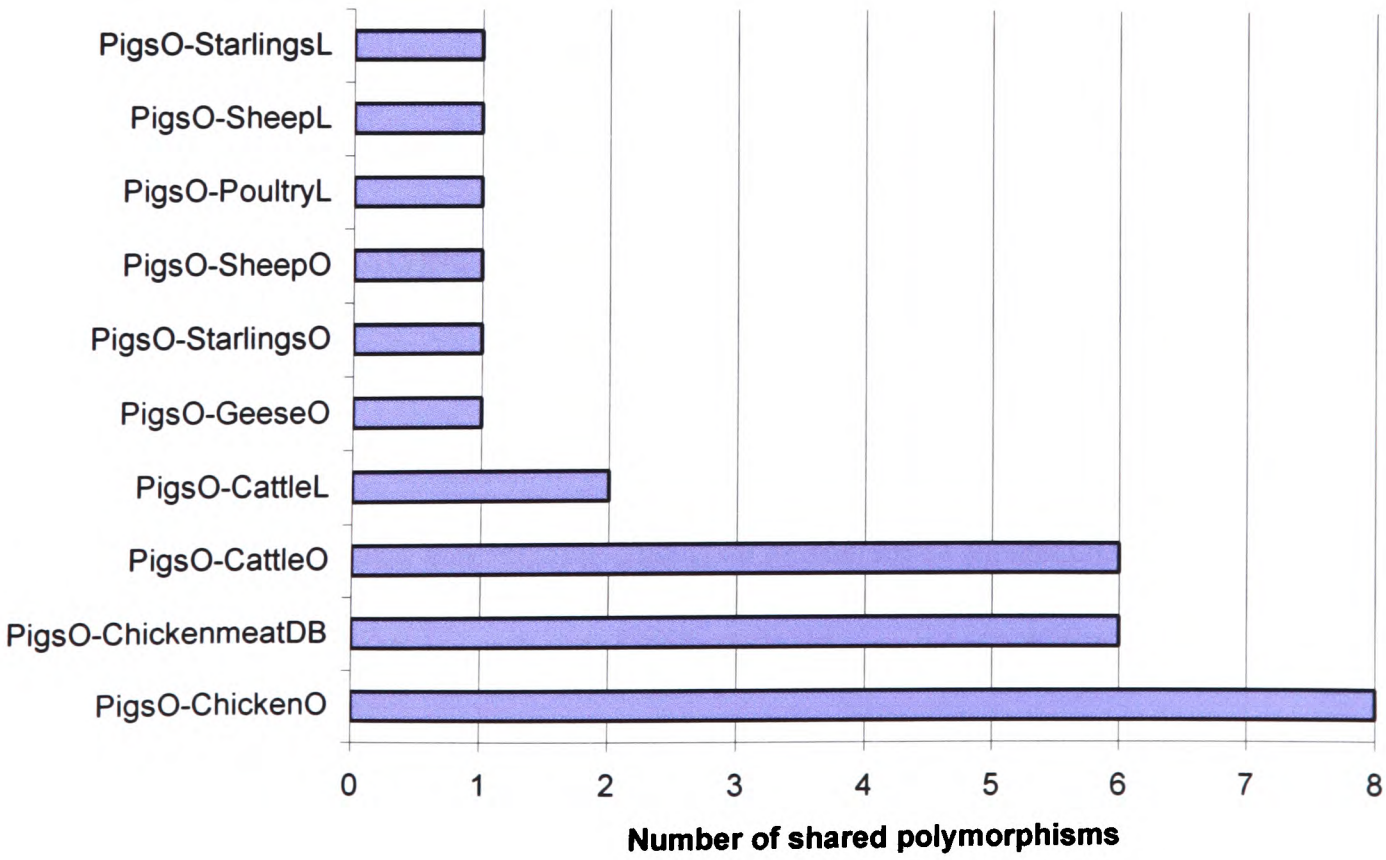


Figure 8.1b. The ten combinations of *Campylobacter* isolates sharing the least number of polymorphisms, derived from Table 8.9.



^{O}=Oxfordshire, ^{L}=Lancashire, ^{DB}=all UKisolates listed on the *Campylobacter* MLST databases

8.3 Discussion.

Although *Campylobacter* populations within individual animal sources were diverse, it was apparent through comparing the *Campylobacter* populations isolated from each of the host species at the University farm sites in Oxfordshire, that there was some overlap of genotypes between them. A total of 21 clonal complexes were identified amongst the animal sources on the farm, but only eight (38%) were isolated from more than one source. ST-21 complex and ST-45 complex were the most dispersed, being isolated from all of the Oxfordshire host sources except pigs. This supports findings from other studies that also identified the ST-21 and ST-45 complexes to have the most diverse sources of isolation and suggests they may be particularly well adapted to survival in multiple hosts and also for moving between hosts (Dingle *et al.* 2002; Colles *et al.* 2003). *C. coli* ST-828 complex was the next most dispersed, being isolated from Oxfordshire pigs, chicken and cattle. Despite some sharing of clonal complexes, the majority (13 of 21, 62%), were isolated from single host sources only suggesting they may be host adapted. The clonal complexes that were not shared amongst different hosts were most commonly isolated from Starlings and geese.

The genotypic diversity within clonal complexes may be further broken down into STs. A total of 128 STs were isolated amongst the animal sources on the Oxford farm sites of which only eight (6.25%) were isolated from more than one animal source. It was interesting that five of the eight shared STs, ST-19 (ST-21 complex), ST-262 (ST-21 complex), ST-42 (ST-42 complex), ST-137 (ST-45 complex) and ST-257 (ST-257 complex) were amongst the 23 genotypes most commonly isolated from human disease. The 23 most common genotypes accounted for 41.7% of the 1358 human disease isolates on the *Campylobacter* MLST database,

and the proportion is likely to be an underestimate since the database has a bias of unusual genotypes. The STs were not the most common amongst the animal sources which suggests that there could be bottlenecks in food production that promotes survival of these particular genotypes enabling them to reach the human consumer. There was an even spread of shared STs amongst the animal sources with almost equal numbers being shared between Starlings and cattle, Starlings and chickens, chickens and cattle and geese and lambs. Some host associations were still apparent, for example ST-257 and ST-574 associated with avian hosts were both isolated from Starlings and chickens (Dingle *et al.* 2002). It was interesting that despite the *C. coli* ST-828 complex being prevalent amongst Oxfordshire chickens, cattle and pigs none of the STs were shared amongst the different sources. The results suggest that *C. coli* genotypes may be less well adapted to living in different hosts and support evidence from other studies suggesting pigs have *C. coli* populations that are separate to those from other sources including humans, poultry and rodents (Rosef *et al.* 1985; Hopkins *et al.* 2004; Meerburg *et al.* 2006).

The *flaA* SVR typing gave evidence that some of the *Campylobacter* genotypes shared between the animal sources on the Oxfordshire farm were indistinguishable, indicating cross contamination of the *Campylobacter* strains between hosts may have occurred, or that the animals acquired them from the same external source. Of the eight STs shared amongst the Oxfordshire animals, seven were associated with the same *flaA* SVR type in more than one host source, although none were isolated from more than two host sources. The distribution of *Campylobacter* strains amongst the animal sources remained even, with chickens being as likely to share strains with cattle as Starlings. This occurred despite different farm staff looking after the chickens and cattle with appropriate bio-security

measures, and both species being free to interact with Starlings. The findings contrast with other studies that indicate *Campylobacter* genotypes are less likely to be shared between wildlife and domestic animal species than amongst domestic animals themselves (Petersen *et al.* 2001a; French *et al.* 2005). A possible explanation may be that the *Campylobacter* strains in question are STs that are not typically isolated from wild life. The strains were a small proportion of the total number of *Campylobacter* genotypes isolated from each source suggesting that sharing of genotypes occurred at low frequency. Gene flow analysis (F_{ST}) gave evidence that despite the small number of STs in common, the *Campylobacter* populations amongst domestic animal sources were at least 30% divergent and the *Campylobacter* populations amongst domestic animals and wild birds were generally even more differentiated.

The results from the Oxfordshire farm were replicated amongst the animals in the Lancashire area, despite them originating over a wider area with biased sampling. The number of *Campylobacter* clonal complexes, STs and ST-*flaA* SVR strains shared amongst animal sources were very similar. Six of 20 (30%) clonal complexes were isolated from more than one source, with ST-21 and ST-45 complexes being the most widely dispersed. Other clonal complexes were isolated from only one source, such as ST-177 isolated only from Starlings. Nine of 28 (32%) of STs were shared amongst the animal sources within Lancashire. All of the STs were amongst the 23 most commonly isolated from human disease, and four were the same as those identified on the Oxfordshire farm. Seven *Campylobacter* ST-*flaA* SVR strains were shared amongst different animal hosts, although this time strains were most commonly shared amongst cattle and sheep, with one isolated from Starlings also. One strain was isolated from poultry and Starlings and none were shared between poultry and ruminant animals. The most likely explanation is the greater distance

between the poultry and ruminant animals that were raised on entirely separate farms. This is supported by the measures of gene flow (F_{ST}) that were an order of magnitude lower when comparing *Campylobacter* populations amongst cattle and sheep in comparison to cattle and poultry or sheep and poultry. Additional reasons for the close relationship between cattle and sheep isolates, also could include similarities in physiology, farming practices and environmental sources (McCarthy *et al.* 2006).

A total of six clonal complexes, ten STs and six ST-*flaA* SVR strains were shared amongst more than one animal source in both Oxfordshire and Lancashire. As before, ST-21 and ST-45 complexes were the most widely dispersed. Nine of the ten shared STs were amongst the 23 STs most commonly isolated from human disease. The six *Campylobacter* ST-*flaA* SVR strains were not necessarily shared amongst the same animal species in the different locations, but the host associations noted in previous studies were consistent (Dingle *et al.* 2002; Colles *et al.* 2003). The ST-19;36-1, ST-53;32-1 and ST-262;37-1 strains were the most widely dispersed being isolated from chicken, cattle, sheep and Starlings, and all clustered into ST-21 complex. ST-38;41-4, part of ST-48 complex was isolated from cattle and Starlings and ST-257;16-12 (ST-257 complex) and ST-45;22-1 (ST-45 complex) were isolated from avian sources. The results suggest that some genotypes were common amongst farm animals and wild birds on a national level, and were stable over a time period of a decade. It is likely that they are particularly well adapted for survival in modern farming practices, or that they are common amongst reservoirs of infection. The national spread of identical *Campylobacter* strains has been noted in another study in Finland (Karenlampi *et al.* 2003). Further testing is necessary to determine if the strains have indeed spread across the UK in association with livestock movement for example, or whether they are common genotypes with ST and *flaA* SVR associations

that have arisen by chance (Fitzgerald *et al.* 2001). The nationally distributed strains accounted for 159 of 1482 (10.7%) isolates from farm animals and 71 of 772 isolates (9.2%) from human disease in the UK, suggesting that measures to control them in the future, for example by vaccination, could have a small but significant impact.

Some differences in *Campylobacter* genotypes were apparent between the same host sources in the different areas, most notably amongst chickens and Starlings. This is could be partly explained by the different sampling methods used in the two locations. The Oxfordshire isolate collections were from very much larger population studies designed to include those genotypes that are rare, whilst a smaller survey of Lancashire genotypes was used. The Oxfordshire samples were collected from fresh anal swabs or faeces whilst the Lancashire chicken samples were collected from intestinal contents at an abattoir and the Starling samples were faeces scraped from fence posts. None of the chicken and only one of the Starling adapted genotypes were identified amongst the Lancashire isolates which could be due to the different sample sizes, different locations, or selection for 'human-type' genotypes in abattoir samples or ageing faecal samples. Despite the limitations with sampling, there was evidence that some *Campylobacter* strains were localized, or farm specific, for example ST-42 was associated with *flaA* SVR 274-42 amongst isolates from Starlings and cattle in Oxfordshire and *flaA* SVR 239-9 amongst cattle and sheep isolates in Lancashire. In addition the gene flow (F_{ST}) values using concatenated sequence indicated that in most cases the *Campylobacter* populations from the same host species in different locations were significantly divergent. The high incidence of shared polymorphisms, and low incidence of fixed differences amongst *Campylobacter* populations isolated from the different hosts suggests that alleles are re-assorting, but there are localized bottlenecks in gene flow. Other studies have also found evidence of farm specific

strains, and strains that are persistent over six months amongst housed broiler flocks (Petersen and Wedderkopp 2001; Rivoal *et al.* 2005). Detailed sampling and comparison of the different farms could give an insight as to the different reservoirs of *Campylobacter* infection, although it is likely that large population samples would be needed in order that any markers for host specificity and epidemiological tracing are found.

The presence of genotypes that are potentially associated with a particular host may help to fingerprint the source of infection in cases of human disease. One study found that the host source of a good percentage of genotypes from chicken, cattle and sheep could be predicted when using the full allelic profile rather than ST or clonal complex which tended to loose information (McCarthy *et al.* 2006). The predictive accuracy could be much higher given allelic profiles from geese or Starlings since the majority (but not all) are very different to those isolated from human disease. In common with results form another study there also appeared to be some host association with particular alleles, most frequently those associated with isolates from wildlife (French *et al.* 2005). Often they were indicative of a host associated clonal complex, but sometimes they reflected host source and a range of clonal complexes. The observation may find application in a clinical laboratory where the single nucleotide polymorphism (SNPs) method could be used to rapidly identify a source of infection in an outbreak situation, although results would have to be confirmed to ensure they have not been affected by recombination events.

A total of 108 *flaA* SVR types were isolated amongst all of the animal sources, of which only 23 (21.3%) were shared. Each animal source had a range of *flaA* SVR types, with geese and Starlings having the greatest diversity and number, perhaps a reflection of their less intensive living conditions. The distribution of *flaA* SVR types

did not appear to occur at random, with some types being isolated from up to 20% of isolates from one source and others being linked, although not exclusively, to *Campylobacter* STs. Models presented by Gupta *et al*, and Meinersmann *et al* may explain the situation whereby multiple *flaA* SVR types are able to co-exist even within the same host. The models predict that the immune response limits those genotypes that are similar, but selection is not strong enough to promote competitive exclusion (Gupta *et al.* 1996; Gupta and Maiden 2001; Meinersmann *et al.* 2005). Although the rate of recombination within the locus is rapid the population appears to be partially clonal through limited migration of alleles thus maintaining some linkage to the source. Horizontal gene transfer is most likely to occur between nearest neighbours, for example between Starlings in the same flock, although it is interesting that geographical isolation is not a key factor since the same types have been isolated from different parts of the UK and even globally (Meinersmann *et al.* 2005). It is not known whether any *Campylobacter* genotype and antigen type can infect any host, but evidence from studies where cattle strains were infective for poultry suggests that some combinations at least probably are (Ziprin *et al.* 2002; Meinersmann *et al.* 2005).

All of the animal sources had clonal complexes in common with isolates from human disease and chicken meat. In the region of 90% of isolates from poultry, cattle and sheep were closely related to those from human disease and chicken meat, although figures were lower when comparing STs. Isolates from cattle and sheep still showed between 65.5 and 80.4% similarity, but only 23.4% of poultry isolates were 'human-type' STs. This is in contrast to findings from other studies where poultry typically show a large overlap with human disease and is likely to be a reflection of the different sampling protocols used in this study (Hudson *et al.* 1999; Kramer *et al.*

2000; Fitch *et al.* 2005). The Lancashire isolates in particular appeared to be dominated by 'human-type' genotypes which may have biased the comparisons. In addition the Oxfordshire chickens were free-range and make up a relatively small percentage of the market, and although distributed nationally, production was very limited in comparison to other companies. Genotypes from wild birds were much less similar to those from human disease and chicken meat in comparison to the farm animals in common with other studies (Petersen *et al.* 2001a; French *et al.* 2005). Between 21.1 to 34.1% of wild bird isolates were related to those from human disease and between 5.42 to 6.7% of isolates were related to those from chicken meat, although, as with the farm animals, the similarities were fewer when comparing STs rather than clonal compelx. The number of 'human-type' STs amongst both wild bird species was consistent at between 5.4 and 5.6% of isolates. A total of 2% of Starling isolates had STs identical to those isolated from chicken meat, but geese isolates had no STs identical to those isolated from chicken meat.

Although 'human-type' *Campylobacter* genotypes were common amongst some of the farm animal isolates, the STs isolated from animal sources in this project accounted for only 4.6% of STs and 29.8% of the isolates from human disease on the *Campyobacter* MLST website. The values are likely to be an underestimate of the true incidence since the human data used for comparison are not a population set. In addition it is unlikely that all of the diversity within genotypes colonizing food animals has been captured in this study. The *Campylobacter* populations amongst human disease isolates and those from chicken meat appeared much more similar in terms of clonal complex, and gene flow (F_{ST}) values, but again only 15 of 649 (2.3%) STs were found to overlap, accounting for 2.3% of the human disease isolates on the *Campylobacter* MLST database. A possible explanation is that clonal complexes may

give a good indication of the interactions between *Campylobacter* populations amongst sources, but that ST and *flaA* SVR typing are more applicable to local epidemiology and tracing infection to point source. Only 15.7% of poultry isolates were similar to those isolated from chicken meat suggesting that the *Campylobacter* population amongst poultry may not have yet been fully elucidated, and that slaughtering and processing plants may have a major influence on *Campylobacter* populations. The similarities between *Campylobacter* isolates from wild birds and other farm animals with those from chicken meat may be explained by the presence of 'common' strains that appear to be widely dispersed amongst different host sources.

In conclusion, evidence from this study, along with others, suggested that ST-21 and ST-45 complexes are widely dispersed amongst animal species, both wild and domesticated. Other clonal complexes were less widely dispersed with some being restricted to avian or ruminant hosts, and half of those isolated from the animal sources being isolated from one host species only. In contrast to *C. jejuni* genotypes, *C. coli* genotypes did not appear to colonize more than one host, despite being prevalent in many of them. Those *C. jejuni* STs that were commonly shared amongst the animal sources were amongst the most frequently isolated from human disease and it may be that they are the most adaptable to living within different hosts. Some *Campylobacter* strains identifiable by ST and *flaA* SVR type had localized distribution with animals that were closer in location being more likely to be colonized by similar types. A small number *Campylobacter* strains, accounting for approximately 10% of isolates from the farm animals were distributed nationally and were stable over a period of a decade, although more data is needed to confirm whether they arise by chance, or are associated with movement of livestock across the country. It may be that vaccination against these genotypes may be possible in the

future which could give a small but significant effect. A large percentage of *Campylobacter* isolates from human disease were related by clonal complex to those from poultry, cattle and sheep, but few were related to those from wild birds. The number of STs shared between human and animal sources was significantly lower, suggesting that the level of detail may be more applicable for local epidemiological investigation. Although some *fla* SVR types were more prevalent amongst particular hosts, relationships were not consistent. The *fla* SVR typing was most useful as an additional tool in identifying *Campylobacter* strains that were similar amongst the different hosts. Some alleles within clonal complexes that were host associated could also be useful markers of their origin for rapid and short term epidemiological investigation in a clinical setting. Much of the diversity in *Campylobacter* populations isolated from human disease, particularly in terms of ST still needs to be investigated.

Future work should include extensive sampling from animal sources, encompassing all systems of management, in order to establish the *Campylobacter* populations that colonize them more fully. In addition transmission of *Campylobacter* genotypes amongst animal sources and humans could be more accurately investigated using a large and contemporary matched population sample of *Campylobacter* samples from human disease. Further investigation into the movement of *Campylobacter* genotypes both locally and nationally is required and additional housekeeping and antigenic loci could be used to establish with greater detail how closely *Campylobacter* strains are related. Finally, differences in *Campylobacter* populations amongst live animals and meat should be investigated further to confirm whether abattoirs and food processing plants act as a bottleneck

promoting survival of those genotypes that are specifically adapted to colonizing multiple hosts, including humans.

Abstract

Chapter 9: Final discussion.

The main findings of the study are discussed in this chapter. The MLST method proved successful in typing genetic diversity of Campylobacters isolated from livestock and wild birds with few modifications. The data were convenient for genetic and evolutionary analyses giving an insight into the population biology of the human pathogen in its more natural setting. The *flaA* SVR type was useful in determining short term or local epidemiology. The clonal complex model appeared to fit the data well, and was valuable in comparing related genotypes from different sources. STs were useful as an added layer of differentiation, but may complicate the picture if the clonal complex structure is not appreciated initially.

There was a seasonal peak in shedding rate and diversity of *Campylobacter* genotypes isolated from both free-range broiler chickens and wild Starlings. Both appeared to have a rapid turnover of genotypes and/or multiple carriage of genotypes (not specifically tested), with no cross immunity inferred between *Campylobacter* species or between genotypes of the same species.

There were significant differences noted between the free-range chickens and wild birds in terms of onset of colonisation, shedding rates, and make-up of *Campylobacter* species and genotypes. Further investigation exploring the differences between hosts, for example genetic make-up and living conditions, as well as pathogenic virulence of the different *Campylobacter* genotypes is warranted. Evidence suggests that although transmission between different host sources on the farm may occur at low levels, the local environment may not be as important a source of contamination amongst poultry as first thought.

Some strains of *Campylobacter* identified by ST and *flaA* SVR type occurred on a national scale over long periods of time, although it can not be certain whether this has arisen by chance or is the spread of successful clones. Some genotypes appeared to be particularly well adapted to both colonising multiple host sources and surviving stresses in food processing plants. Further investigation should look to other likely reservoirs of contamination for chickens, particularly those occurring on a

large or national scale such as hatcheries and catching teams. Further loci could be tested to confirm the existence of successful *Campylobacter* clones.

Chapter 9: Final discussion.

The results from this study showed that the MLST method was very successful in cataloguing the diversity amongst *C. jejuni* and *C. coli* isolates from a variety of domestic and wild animals, with data lending themselves to population genetic and statistical analysis. Modifications from the methods published for both species were minor and consisted only of designing new primers for the *aspA* locus in Starling isolates, or optimising combinations of published primers for the chicken isolates. Sequencing of the *flaA* SVR, whilst less discriminatory than MLST on its own, added a valuable layer of differentiation between identical genotypes to enable short-term epidemiological investigation in more detail.

The clonal complex model of the population structure of *Campylobacter* appeared to fit the data from this study well. Often the central genotypes were the most readily isolated from all of the host sources and were isolated over longer periods, suggesting that they were the most 'fit' genotypes in terms of survival. The number of single, double and triple locus variants arising through local expansion or niche adaptation got progressively fewer the further removed from the central genotype they were indicating that they may be less fit in the long term. In common with other studies, some associations between host source and clonal complex were evident, for example ST-682 complex was only isolated from Starlings (Dingle *et al.* 2002; Colles *et al.* 2003; Waldenstrom *et al.* 2005a). However ST-45 and ST-257 complexes previously thought to be chicken associated were not common amongst the free-range broiler chickens in Oxfordshire and perhaps reflects differences between extensive and intensive production, and between live chicken and meat samples. In

addition ST-61 complex previously thought to be ruminant associated was not isolated from the lamb samples in Oxfordshire, but the number of isolates was very small.

Individual alleles were mixed amongst host sources with few fixed and many shared polymorphisms, but assortment of STs and some alleles, reflected by significant F_{ST} values, is likely to arise through local bottlenecks in gene flow. As in previous studies, ST-21 and ST-45 complexes were the most widely distributed whilst others were restricted to only one host source. Genotypes were most closely shared between the more closely related animal types, such as ruminant or avian species amongst the farm animals in Lancashire. However, the trend was not continued amongst isolates from the poultry and wild bird species in Oxfordshire, and probably reflects differences in ecology between the hosts.

The number of isolates from wild birds was sufficient to reveal an extensive population structure within *Campylobacter*, whilst a greater number of isolates from chickens gave a more limited structure. This is likely to reflect that fact that individual birds within flocks of intensively reared chickens become colonized with the same *Campylobacter* genotypes whilst individual wild birds are more widely dispersed in their environment and do not. In addition the free-range chickens in this study were genetically homogenous and represent a small fraction of the poultry industry. A more extensive population structure might be revealed if flocks of different origins and different management regimes were tested.

In all host sources variant genotypes of the host adapted clonal complexes were isolated most frequently, whilst non-host associated clonal complexes were often represented by the central genotype or one variant genotype only, presumably since they are best adapted for survival in multiple hosts. A large proportion of STs

isolated from wild birds could not be assigned to clonal complexes in contrast to those isolated from farm animals or poultry. This probably reflects the under representation of isolates from environmental origins on the *Campylobacter* MLST database upon which the clonal complexes are largely defined. It seems likely that increased sampling would help to resolve new clonal complexes amongst environmental isolates since many already form related clusters.

The shedding rate of *Campylobacter* was greater than 70% amongst all of the chicken flocks tested, but averaged between 37 and 50% in the wild bird species. The most likely explanation for this is their differing living conditions. The organism can rapidly spread amongst chickens reared in close proximity to each other via the faecal oral route, whilst this is perhaps less likely to occur amongst wild birds who are more widely dispersed. In addition chicken flocks consist entirely of young and immunologically immature individuals, whilst flocks of wild birds are of mixed age which may help to limit the spread of the organism.

There was evidence that carriage of *Campylobacter* amongst wild birds was transient, with a positive status having no apparent ill effect on the host. There was a rapid succession of genotypes amongst both poultry flocks and wild birds that apparently occurred at random. The role of the host immune system in effecting clearance of the genotypes was questionable since the *flaA* SVR locus capable of huge variation under immune selection was remarkably constant over a period of at least 17 weeks. In addition poultry appeared to be readily colonized by multiple *Campylobacter* genotypes that fluctuated both up and down in proportion over a number of weeks and it seems unlikely that the hosts exhibited an immune reaction strong enough to promote competitive exclusion. Colonization by one *Campylobacter* genotype did not appear to infer immunity to another and indeed it

was unclear in the first poultry flock whether or not birds became re-infected with the same genotype. Similarly colonization by one *Campylobacter* subspecies did not infer immunity to another species. Observation from the lambs grazing pasture contaminated with geese faeces suggests that animals could shed recently acquired *Campylobacter* genotypes, and perhaps colonization by a genotype adapted for survival in another animal host is transitory. One explanation for the rapid turnover of *Campylobacter* genotypes in the face of an apparent lack of strong host immune reaction could be that *Campylobacter* is unable to compete with resident gut flora and establish itself over a long period of time. *Campylobacter* genotypes may fluctuate due to their own predator-prey relationships with specific bacteriophages, thought to be common amongst the poultry environment. In addition the gut flora could differ in stages of maturity or may be modified through feeding of manufactured diets throughout the lifetime of the chicken.

Shedding of *C. coli* was significantly lower amongst the wild birds in comparison to the poultry, and similarly shedding of *C. lari*, the second most common species amongst Starlings, was not seen at all amongst the wild geese or poultry. This could be due to differing exposure amongst the hosts, although the lack of *C. coli* amongst geese is surprising when a water-adapted clone has recently been identified (Kemp *et al.* 2005). Alternatively it could reflect differences in host immunity, although this seems less likely in light of the evidence discussed above. Although not investigated specifically, mixed *Campylobacter* genotypes were notably more common amongst the poultry in comparison to wild birds, perhaps due to greater levels of exposure intensified by overcrowded living conditions.

A seasonal peak in shedding rate and genotypic diversity was apparent amongst *Campylobacter* isolates from both wild birds and poultry. The peak in the

free-range chickens and wild Starlings coincided with that typically seen amongst human Campylobacteriosis in late spring/early summer. The peak in geese may have occurred a bit later in the year, coinciding with that seen in gulls and is perhaps associated with clustering of birds in the autumn, although further sampling is required to confirm this (Broman *et al.* 2002). Although activity of migrational wild birds has been quoted as a contributing factor to the peak in poultry no direct links between the sources were identified in this study.

Many studies have looked for evidence of other environmental sources of contamination amongst chickens although it is almost impossible to sample the environment exhaustively and then determine the direction of transmission. Overall, results from the study showed an amazing lack of consistency with environmental contamination. The first six free-range chicken flocks had exactly the same *Campylobacter* populations despite being reared on six different plots of land at two different farm sites. Similarly results suggested that differences apparent amongst *Campylobacter* populations isolated from the free-range chickens at the different farm sites, rotations on the same plots of land, and split flocks throughout the study period were much smaller than might be expected if environmental sources were playing a significant role. Instead, the coincident seasonal peaks in *Campylobacter* colonization were more likely a result of environmental conditions favouring the survival of the organism and promoting its spread amongst individual animals. For example activity amongst potential vectors such as insects is generally greater in warmer months. High temperatures caused difficulties in managing heat stress amongst the broilers and may have made them more susceptible to infection by altering hormone levels which influence host immune response and pathogen growth. The variety of food sources

available for wild birds is greater when it is warmer and there are large numbers of young birds that appeared to be more prone to colonization.

The role of wild birds in transmission of *Campylobacter* amongst poultry and human disease could not be completely ruled out since approximately 5% of the genotypes isolated from wild birds were indistinguishable amongst the sources in this study. There was evidence that such genotypes are capable of surviving processing plants and entering the food chain via contaminated chicken meat. Starlings in particular were occasionally colonized by *Campylobacter* genotypes that were isolated from all of the farm animal species tested except pigs, and could act as a transmission vector for *Campylobacter* on farms. Cross contamination between wild birds and poultry appeared to occur at random since it was not the most common genotypes from either source that were transferred. It is not known whether poultry would be susceptible to all wild bird *Campylobacter* genotypes. The unassigned ST-1023 was isolated from both sources suggests that they may be, but it was surprising that the *C. coli* genotypes isolated from poultry were different to those isolated from pigs, a predominant source of *C. coli*, reared in the near vicinity. The majority of genotypes from wild birds were significantly different to those typically isolated from farm animals and human disease, with some alleles being host associated. It is likely that they could be easily be traced to source using assigning methods such as that developed by McCarthy *et al.* (2006) and would aid epidemiological investigation of human infection in a clinical setting.

The majority of genotypes isolated from the poultry appeared to be poultry associated and were presumably maintained by continual transmission amongst endemic populations within the poultry industry. It is possible that levels of shedding and diversity may be accentuated by other stresses put on the bird, for example the

chickens began shedding *Campylobacter* soon after moving to another location, and in the warmest months when levels of both shedding and diversity peaked, birds were uncomfortable due to heat stress. Some *Campylobacter* strains appeared to be farm site specific, suggesting environmental input, whilst others appeared to have spread nationally and even internationally being stable over at least a decade. Modern farming practises are likely to aid transmission of such widespread strains by using specialised hatcheries, teams of catchers and slaughter houses that cater for farms within large areas of the UK. In addition livestock travel can travel sizeable distances both nationally or even internationally.

Unlike housed birds, free-range chickens can not be kept *Campylobacter* free using strict biosecurity control measures, but it is difficult to identify preventative measures that could be taken at present. The study gave evidence that leaving plots of land fallow for a period of seven weeks was sufficient to prevent direct transmission of *Campylobacter* to subsequent flocks. Ensuring that contact between poultry and wild birds is eliminated, for example by keeping all food inside the sheds making it less accessible to wild birds, would also help to prevent colonization of poultry, although the impact is likely to be relatively small. In the future it may be possible to vaccinate against common host associated strains, as well as nationally distributed strains of *Campylobacter* although much more work is required first. It is possible that older birds, or those kept in less intensive conditions may be less susceptible to colonization, but it is likely that changes would have to be large to make an impact and would prove impractical for the industry. Minimising stressful situations such as moving locations, or keeping the temperature and ammonia levels constant within houses may help to contain levels of *Campylobacter* shedding. This could perhaps be extended to using local or on-farm slaughter houses.

Future work should include sequencing at more antigenic and housekeeping loci, together with sampling at more farms in other areas of the UK in order to confirm the presence of nationally distributed *Campylobacter* strains, as well as those that are transferred more locally. The persistence of poultry associated strains should be studied in order that preventative measures may be identified. In particular, the flow of genotypes amongst hatcheries, broiler breeder flocks and broiler flocks should be investigated. The interactions between *Campylobacter* and other gut flora should also be investigated further as well as influencing factors such as feed, health and immune status, effects of stress and inhibition of natural behaviours on host susceptibility. Isolates collected from a second year of free-range chicken production in Oxfordshire should be genotyped to see if the seasonal effects and succession of genotypes is repeated.

Further investigation is required to establish the differences between *Campylobacter* populations amongst samples from live animals and abattoirs in order to identify persistent strains that are able to survive the process from 'farm to fork'. Although poultry are considered to be a significant reservoir of human disease, many of the human disease genotypes are rare and cannot be clustered into clonal complexes or linked with any other source. Large population studies of potential reservoirs of infection such as ruminants and environmental waters may help to identify such genotypes and to resolve the large number of unassigned isolates from wildlife species that have been sampled to date. There was evidence that *C. coli* formed separate populations amongst different hosts and the significance of *C. coli* isolates amongst free-range poultry is unknown. It will be interesting to see whether the incidence of disease amongst humans becomes more frequent as free-range meat becomes more popular. Finally, a population sample of *Campylobacter* isolates from

human disease, matched for time and location, is required to give a more accurate estimation of the proportion of the *Campylobacter* genotypes colonizing chickens and wild birds (as well as other sources) that cause human disease.

References.

Ahmed, I. H., G. Manning, T. M. Wassenaar, S. Cawthraw, and D. G. Newell. 2002. Identification of genetic differences between two *Campylobacter jejuni* strains with different colonization potentials. Microbiology 148:1203-12.

Allos, B. 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. Clinical Infectious Diseases 2001:32:1201-6.

Altekruse, S. F., N. J. Stern, P. I. Fields, and D. L. Swerdlow. 1999. *Campylobacter jejuni* -an emerging foodborne pathogen. Emerging Infectious Diseases 5:28-35.

Ang, C. W., M. A. de Klerk, H. P. Endtz, B. C. Jacobs, J. D. Laman, F. G. A. van der Meché, and P. A. van Doorn. 2001. Guillain-Barré Syndrome and Miller Fisher Syndrome-associated *Campylobacter jejuni* lipopoplysaccharides induce anti-GM1 and anti-GQ1b antibodies in rabbits. Infection and Immunity 69:2462-2469.

Anonymous (2006). Biosecurity for housed broilers. Cleaner farms better flocks., Food Standards Agency.

Arzate Barbosa, P., R. Garcia Gonzalez, E. Ponce Nava and I. Nachamkin 1999. Comparison of two selective media for the isolation of *Campylobacter* species from a pediatric population in Mexico. Diagnostic Microbiology and Infectious Disease 34(4): 329-32.

Atterbury, R. J., E. Dillon, C. Swift, P. L. Connerton, J. A. Frost, C. E. Dodd, C. E. Rees, and I. F. Connerton. 2005. Correlation of *Campylobacter* bacteriophage with reduced presence of hosts in broiler chicken ceca. Applied and Environmental Microbiology 71:4885-7

Aydin, F., H. I. Atabay, and M. Akan. 2001. The isolation and characterization of *Campylobacter jejuni* subsp. *jejuni* from domestic geese (*Anser anser*). Journal of Applied Microbiology 90:637-42.

Axelsson-Olsson, D., J. Waldenstrom, T. Broman, B. Olsen, and M. Holmberg. 2005. Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni.* Applied and Environmental Microbiology 71:987-92.

Bates, C., K. L. Hiett, and N. J. Stern. 2004. Relationship of *Campylobacter* isolated from poultry and from darkling beetles in New Zealand. Avian Disease 48:138-47.

Baylis, C. L., S. MacPhee, K. W. Martin, T. J. Humphrey, and R. P. Betts. 2000. Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. Journal of Applied Microbiology 89:884-91.

Berndtson, E., U. Emanuelson, A. Engvall, and M.-L. Danielsson-Tham. 1996a. A 1-

year epidemiological study of Campylobacters in 18 Swedish chicken farms. Preventative Veterinary Medicine 26:167-185.

Berndtson, E., M. L. Danielsson-Tham and A. Engvall 1996b. *Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process. International Journal of Food Microbiology_32(1-2): 35-47

Berrang, M. E., R. J. Meinersman, R. J. Buhr, N. A. Reimer, R. W. Philips, and M. A. Harrison. 2003. Presence of *Campylobacter* in the respiratory tract of broiler carcases before and after commercial scalding. Poultry Science 82:1995-1999.

Biswas, D., K. Itoh, and C. Sasakawa. 2003. Role of microfilaments and microtubules in the invasion of INT-407 cells by *Campylobacter jejuni*. Microbiology and Immunology 47:469-73.

Boes, J., L. Nersting, E. M. Nielsen, S. Kranker, C. Enoe, H. C. Wachmann, and D. L. Baggesen. 2005. Prevalence and diversity of *Campylobacter jejuni* in pig herds on farms with and without cattle or poultry. Journal of Food Protection 68:722-7.

Bolton, F. J., S. B. Surman, K. Martin, D. R. A. Wareing, and T. J. Humphrey. 1999. Presence of *Campylobacter* and *Salmonellae* in sand from bathing beaches. Epidemiology and Infection 122:7-13.

Bouwknegt, M., A. W. van de Giessen, W. D. Dam-Deisz, A. H. Havelaar, N. J. Nagelkerke, and A. M. Henken. 2004. Risk factors for the presence of *Campylobacter* spp. in Dutch broiler flocks. Preventative Veterinary Medicine 62:35-49.

Boyd, Y., E. G. Herbert, K. L. Marston, M. A. Jones, and P. A. Barrow. 2005. Host genes affect intestinal colonisation of newly hatched chickens by *Campylobacter jejuni*. Immunogenetics 57:248-53.

Broman, T., H. Palmgren, S. Bergstrom, M. Sellin, J. Waldenstrom, M. L. Danielsson-Tham, and B. Olsen. 2002. *Campylobacter jejuni* in black-headed gulls (*Larus ridibundus*): prevalence, genotypes, and influence on *C. jejuni* epidemiology. Journal of Clinical Microbiology 40:4594-602.

Broman, T., J. Waldenstrom, D. Dahlgren, I. Carlsson, I. Eliasson, and B. Olsen. 2004. Diversities and similarities in PFGE profiles of *Campylobacter jejuni* isolated from migrating birds and humans. Journal of Applied Microbiology 96:834-43.

Brown, P. E., O. F. Christensen, H. E. Clough, P. J. Diggle, C. A. Hart, S. Hazel, R. Kemp, A. J. Leatherbarrow, A. Moore, J. Sutherst, J. Turner, N. J. Williams, E. J. Wright, and N. P. French. 2004. Frequency and spatial distribution of environmental *Campylobacter* spp. Applied and Environmental Microbiology 70:6501-11.

Bull, S. A., V. M. Allen, G. Domingue, F. Jorgensen, J. A. Frost, R. Ure, R. Whyte, D. Tinker, J. E. Corry, J. Gillard-King, and T. J. Humphrey. 2006. Sources of *Campylobacter* spp. Colonizing Housed Broiler Flocks during Rearing. Applied and Environmental Microbiology 72:645-52.

Butzler, J. P. 2004. *Campylobacter*, from obscurity to celebrity. Clinical Microbiology and Infectection 10:868-76.

Camarda, A., D. G. Newell, R. Nasti, and G. Di Modugnoa. 2000. Genotyping *Campylobacter jejuni* strains isolated from the gut and oviduct of laying hens. Avian Disease 44:907-12.

Cardarelli-Leite, P., K. Blom, C. M. Patton, M. A. Nicholson, A. G. Steigerwalt, S. B. Hunter, D. J. Brenner, T. J. Barrett, and B. Swaminathan. 1996. Rapid identification of *Campylobacter* species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. Journal of Clinical Microbiology 34:62-7.

Carvalho, A. C., G. M. Ruiz-Palacios, P. Ramos-Cervantes, L. E. Cervantes, X. Jiang, and L. K. Pickering. 2001. Molecular characterization of invasive and noninvasive *Campylobacter jejuni* and *Campylobacter coli* isolates. Journal of Clinical Microbiology 39:1353-9.

Chart, H., J. A. Frost, A. Oza, R. Thwaites, S. Gillanders, and B. Rowe. 1996. Heat-stable serotyping antigens expressed by strains of *Campylobacter jejuni* are probably capsular and not long-chain lipopolysaccharide. Journal of Applied Bacteriology 81:635-40.

Chuma, T., S. Hashimoto, and K. Okamoto. 2000. Detection of thermophilic *Campylobacter* from sparrows by multiplex PCR: the role of sparrows as a source of contamination of broilers with *Campylobacter*. Journal of Veterinary Medical Science 62:1291-5.

Cogan, T. A., A. O. Thomas, L. E. Rees, A. H. Taylor, M. A. Jepson, P. H. Williams, J. Ketley and T. J. Humphrey (2006). Norepinephrine increases the pathogenic potential of *Campylobacter jejuni*. Gut.

Coker, A. O., R. D. Isokpehi, B. N. Thomas, K. O. Amisu, and C. L. Obi. 2002. Human campylobacteriosis in developing countries. Emerging Infectious Diseases 8:237-44.

Colles, F. M., K. Jones, R. M. Harding, and M. C. Maiden. 2003. Genetic diversity of *Campylobacter jejuni* isolates from farm animals and the farm environment. Applied and Environmental Microbiology 69:7409-13.

Collins, C. H., P. M. Lyne, and J. M. Grange. 1995. Microbiological methods, 7th ed. Butterworth-Heinemann.

Connerton, P. L., C. M. Loc Carrillo, C. Swift, E. Dillon, A. Scott, C. E. Rees, C. E. Dodd, J. Frost, and I. F. Connerton. 2004. Longitudinal study of *Campylobacter jejuni* bacteriophages and their hosts from broiler chickens. Applied and Environmental Microbiology 70:3877-83.

Cooper, I. A., and K. J. Slee. 1971. Human infection by *Vibrio fetus*. The Medical Journal of Austria 1:1263-7.

Corry, J. E., and H. I. Atabay. 2001. Poultry as a source of *Campylobacter* and related organisms. Symposium Series Society for Applied Microbiology 90:96S-114S.

Corry, J. E., D. E. Post, P. Colin, and M. J. Laisney. 1995. Culture media for the isolation of campylobacters. International Journal of Food Microbiology 26:43-76.

Cox, N. A., N. J. Stern, K. L. Hiett, and M. E. Berrang. 2002a. Identification of a new source of *Campylobacter* contamination in poultry: transmission from breeder hens to broiler chickens. Avian Disease 46:535-41.

Cox, N. A., N. J. Stern, J. L. Wilson, M. T. Musgrove, R. J. Buhr, and K. L. Hiett. 2002b. Isolation of *Campylobacter* spp. from semen samples of commercial broiler breeder roosters. Avian Disease 46:717-20.

Craven, S. E., N. J. Stern, E. Line, J. S. Bailey, N. A. Cox, and P. Fedorka-Cray. 2000. Determination of the incidence of *Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens* in wild birds near broiler chicken houses by sampling intestinal droppings. Avian Disease 44:715-20.

Day, W. A., Jr., J. L. Sajecki, T. M. Pitts, and L. A. Joens. 2000. Role of catalase in *Campylobacter jejuni* intracellular survival. Infection and Immunity 68:6337-45

De Boer, P., B. Duim, A. Rigter, J. van Der Plas, W. F. Jacobs-Reitsma, and J. A. Wagenaar. 2000. Computer-assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli.* Journal of Clinical Microbiology 38:1940-6.

De Boer, P., J. A. Wagenaar, R. P. Achterberg, J. P. Putten, L. M. Schouls, and B. Duim. 2002. Generation of *Campylobacter jejuni* genetic diversity in vivo. Molecular Microbiology 44:351-9.

De, E., M. Jullien, G. Labesse, J. M. Pages, G. Molle, and J. M. Bolla. 2000. MOMP (major outer membrane protein) of *Campylobacter jejuni*; a versatile pore-forming protein. FEBS Letters 469:93-7.

Dekeyser, P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related *Vibrio*: first positive stool cultures. Journal of Infectious Disease 125:390-2.

Devane, M. L., C. Nicol, A. Ball, J. D. Klena, P. Scholes, J. A. Hudson, M. G. Baker, B. J. Gilpin, N. Garrett, and M. G. Savill. 2005. The occurrence of *Campylobacter* subtypes in environmental reservoirs and potential transmission routes. Journal of Applied Microbiology 98:980-90.

Dingle, K. E., F. M. Colles, D. Falush, and M. C. Maiden. 2005. Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. Journal of Clinical Microbiology 43:340-7.

Dingle, K. E., F. M. Colles, R. Ure, J. Wagenaar, B. Duim, F. J. Bolton, A. J. Fox, D.

R. A. Wareing, and M. C. J. Maiden. 2002. Molecular characterisation of *Campylobacter jejuni* clones: a rational basis for epidemiological investigations. Emerging Infectious Diseases 8:949-955.

Dingle, K. E., F. M. Colles, D. R. A. Wareing, R. Ure, A. J. Fox, F. J. Bolton, H. J. Bootsma, R. J. L. Willems, R. Urwin, and M. C. J. Maiden. 2001a. Multilocus sequence typing system for *Campylobacter jejuni*. Journal of Clinical Microbiology 39:14-23.

Dingle, K. E., N. van den Braak, F. M. Colles, L. J. Price, D. L. Woodward, F. G. Rodgers, H. P. Endtz, A. van Belkum, and M. C. J. Maiden. 2001b. Sequence typing confirms that *Campylobacter jejuni* strains associated with Guillain Barré and Miller Fisher syndromes are of diverse genetic lineage, serotype and flagella type. Journal of Clinical Microbiology 39:3346-3349.

Dominguez, C., I. Gomez, and J. Zumalacarregui. 2002. Prevalence of *Salmonella* and *Campylobacter* in retail chicken meat in Spain. International Journal of Food Microbiology 72:165-8.

Duim, B., C. W. Ang, A. van Belkum, A. Rigter, N. W. J. van Leeuwen, H. P. Endtz, and P. A. Wagenaar. 2000. Amplified fragment length polymorphism analysis of *Campylobacter jejuni* strains isolated from chickens and from patients with gastroenteritis or Guillain-Barré or Miller Fisher syndrome. Applied and Environmental Microbiology 66:3917-3923.

Duim, B., P. C. Godschalk, N. van den Braak, K. E. Dingle, J. R. Dijkstra, E. Leyde, J. van der Plas, F. M. Colles, H. P. Endtz, J. A. Wagenaar, M. C. Maiden, and A. van Belkum. 2003a. Molecular evidence for dissemination of unique *Campylobacter jejuni* clones in Curacao, Netherlands Antilles. Journal of Clinical Microbiology 41:5593-7.

Duim, B., P. C. Godschalk, N. van den Braak, K. E. Dingle, J. R. Dijkstra, J. van der Plas, F. M. Colles, H. P. Endtz, J. Wagenaar, M. C. Maiden, and A. van Belkum. 2003b. Molecular evidence for seasonal occurence of unique clonal groups of *Campylobacter jejuni* on the Carribean island of Curacao. Journal of Clinical Microbiology 41:5593-7.

Ekdahl, K., B. Normann, and Y. Andersson. 2005. Could flies explain the elusive epidemiology of campylobacteriosis? BMC Infectious Disese 5:11.

El-Shibiny, A., P. L. Connerton, and I.F. Connerton. 2005. Enumeration and diversity of campylobacters and bacteriophages isolated during the rearing cycles of free-range and organic chickens. Applied and Environmental Microbiology 71(3): 1259-66.

Elston, R. C., and W. D. Johnson. 1994. Essentials of Biostatistics. F.A. Davis Company.

Endtz, H. P., C. W. Ang, N. van den Braak, B. Duim, A. Rigter, L. J. Price, D. L. Woodward, F. G. Rodgers, W. M. Johnson, J. A. Wagenaar, B. C. Jacobs, H. A. Verbrugh, and A. van Belkum. 2000. Molecular characterization of *Campylobacter jejuni* from patients with Guillain-Barré and Miller Fisher syndromes. Journal of Clinical Microbiology 38:2297-2301.

Evans, S. J., and A. R. Sayers. 2000. A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. Preventative Veterinary Medicine 46:209-23.

Fearnhead, P., N. G. Smith, M. Barrigas, A. Fox, and N. French. 2005. Analysis of recombination in *Campylobacter jejuni* from MLST population data. Journal of Molecular Evolution 61:333-40.

Feil, E. J., and M. S. Chan. 1999. BURST program. http://www.mlst.net/new/data_analysis/burst/burst.htm.

Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. Journal of Bacteriology 186:1518-30.

Feil, E. J., M. C. Enright, and B. G. Spratt. 2000. Estimating the relative contributions of mutation and recombination to clonal diversification: a comparison between *Neisseria meningitidis* and *Streptococcus pneumoniae*. Research in Microbiology 151:465-9.

Fitch, B. R., K. L. Sachen, S. R. Wilder, M. A. Burg, D. W. Lacher, W. T. Khalife, T. S. Whittam, and V. B. Young. 2005. Genetic diversity of *Campylobacter* sp. isolates from retail chicken products and humans with gastroenteritis in Central Michigan. Journal of Clinical Microbiology 43:4221-4.

Fitzgerald, C., L. O. Helsel, M. A. Nicholson, S. J. Olsen, D. L. Swerdlow, R. Flahart, J. Sexton, and P. I. Fields. 2001. Evaluation of methods for subtyping *Campylobacter jejuni* during an outbreak involving a food handler. Journal of Clinical Microbiology 39:2386-90.

Fitzgerald, C., K. Stanley, S. Andrew, and K. Jones. 2001. Use of pulsed-field gel electrophoresis and flagellin gene typing in identifying clonal groups of *Campylobacter jejuni* and *Campylobacter coli* in farm and clinical environments. Applied and Environmental Microbiology 67:1429-36.

French, N., M. Barrigas, P. Brown, P. Ribiero, N. Williams, H. Leatherbarrow, R. Birtles, E. Bolton, P. Fearnhead, and A. Fox. 2005. Spatial epidemiology and natural population structure of *Campylobacter jejuni* colonizing a farmland ecosystem. Environmental Microbiology 7:1116-26.

Frost, J. A. 2001. Current epidemiological issues in human campylobacteriosis. Symposium Series Society of Applied Microbiology:85S-95S.

Frost, J. A., J. M. Kramer, and S. A. Gillanders. 1999. Phage typing of *Campylobacter jejuni* and *Campylobacter coli* and its use as an adjunct to serotyping. Epidemiology and Infection 123:47-55.

Frost, J. A., A. N. Oza, R. T. Thwaites, and B. Rowe. 1998. Serotyping scheme for *Campylobacter jejuni* and *Campylobacter coli* based on direct agglutination of heat-stable antigens. Journal of Clinical Microbiology 36:335-9.

Goossens, H., M. De Boeck, H. Coignau, L. Vlaes, C. Van den Borre, and J. P. Butzler. 1986. Modified selective medium for isolation of *Campylobacter* spp. from feces: comparison with Preston medium, a blood-free medium, and a filtration system. Journal of Clinical Microbiology **24:**840-3.

Grant, A. J., C. Coward, M.A. Jones, C.A. Woodall, P.A. Barrow and D.J. Maskell. 2005. Signature-tagged transposon mutagenesis studies demonstrate the dynamic nature of cecal colonization of 2-week-old chickens by *Campylobacter jejuni*. Applied and Environmental Microbiology 71(12): 8031-41.

Guerry, P. 1997. Nonlipopolysaccharide surface antigens of *Campylobacter* species. Journal of Infectious Diseases 176 Supplement 2:S122-4.

Gun-Munro, J., R. P. Rennie, J. H. Thornley, H. L. Richardson, D. Hodge, and J. Lynch. 1987. Laboratory and clinical evaluation of isolation media for *Campylobacter jejuni*. Journal of Clinical Microbiology **25:**2274-7.

Gupta, S., and M. C. J. Maiden. 2001. Exploring the evolution of diversity in pathogen populations. Trends in Microbiology 9:147-192.

Gupta, S., M. C. J. Maiden, I. M. Feavers, S. Nee, R. M. May and R. M. Anderson 1996. The maintenance of strain structure in populations of recombining infectious agents. Nature Medicine **2**(4): 437-442.

Hald, B., K. Knudsen, P. Lind, and M. Madsen. 2001. Study of the infectivity of saline-stored *Campylobacter jejuni* for day-old chicks. Applied and Environmental Microbiology 67:2388-92.

Hald, B., H. Skovgard, D. D. Bang, K. Pedersen, J. Dybdahl, J. B. Jespersen and M. Madsen (2004). Flies and *Campylobacter* infection of broiler flocks. Emerg Infect Dis **10**(8): 1490-2.

Hanel, I., J. Muller, W. Muller, and F. Schulze. 2004. Correlation between invasion of Caco-2 eukaryotic cells and colonization ability in the chick gut in *Campylobacter jejuni.* Veterinary Microbiology 101:75-82.

Hanninen, M. L., S. Pajarre, M. L. Klossner, and H. Rautelin. 1998. Typing of human *Campylobacter jejuni* isolates in Finland by pulsed- field gel electrophoresis. Journal of Clinical Microbiology 36:1787-9.

Hansson, I., M. Ederoth, L. Andersson, I. Vagsholm, and E. Olsson Engvall. 2005. Transmission of *Campylobacter* spp. to chickens during transport to slaughter. Journal of Applied Microbiology 99:1149-57.

Harrington, C. S., F. M. Thomson Carter, and P. E. Carter. 1997. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: implications for the flagellin gene typing scheme. Journal of Clinical Microbiology 35:2386-92.

Harrison, W. A., C. J. Griffith, D. Tennant, and A. C. Peters. 2001. Incidence of

Campylobacter and *Salmonella* isolated from retail chicken and associated packaging in South Wales. Letters in Applied Microbiology 33:450-4.

Hassane, D. C., R. B. Lee, and C. L. Pickett. 2003. *Campylobacter jejuni* cytolethal distending toxin promotes DNA repair responses in normal human cells. Infection and Immunity 71:541-5.

Hein, I., C. Schneck, M. Knogler, G. Feierl, P. Plesss, J. Kofer, R. Achmann, and M. Wagner. 2003. *Campylobacter jejuni* isolated from poultry and humans in Styria, Austria: epidemiology and ciprofloxacin resistance. Epidemiology and Infection 130:377-86.

Heuer, O. E., K. Pedersen, J. S. Andersen, and M. Madsen. 2001. Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. Letters in Applied Microbiology 33:269-74.

Hey, J. 1991. The structure of genealogies and the distribution of fixed differences between DNA sequence samples from natural populations. Genetics 128:831-40.

Hickey, T. E., A. L. McVeigh, D. A. Scott, R. E. Michielutti, A. Bixby, S. A. Carroll, A. L. Bourgeois, and P. Guerry. 2000. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infection and Immunity 68:6535-41.

Hiett, K. L., N. A. Cox, R. J. Buhr, and N. J. Stern. 2002. Genotype analyses of *Campylobacter* isolated from distinct segments of the reproductive tracts of broiler breeder hens. Current Microbiology 45:400-4.

Hirsch, R. P., and R. K. Riegelman. 1992. Statistical First Aid. Blackwell Science.

Hook, H., M. A. Fattah, H. Ericsson, I. Vagsholm, and M. L. Danielsson-Tham. 2005. Genotype dynamics of *Campylobacter jejuni* in a broiler flock. Veterinary Microbiology 106:109-17.

Hopkins, K. L., M. Desai, J. A. Frost, J. Stanley, and J. M. Logan. 2004. Fluorescent amplified fragment length polymorphism genotyping of *Campylobacter jejuni* and *Campylobacter coli* strains and its relationship with host specificity, serotyping, and phage typing. Journal of Clinical Microbiology 42:229-35.

HPA. 2006. Common gastrointestinal infections, England and Wales, laboratory reports: weeks 49-52/05. CDR Weekly 16:4.

Hudson, J. A., C. Nicol, J. Wright, R. Whyte, and S. K. Hasell. 1999. Seasonal variation of *Campylobacter* types from human cases, veterinary cases, raw chicken, milk and water. Journal of Medical Microbiology 87:115-24.

Hudson, R. R. 1993. Levels of DNA polymorphism and divergence yield important insights into evolutionary processes. Proceedings of the Nationall Academy of Sciences U S A 90:7425-6.

Hudson, R. R., M. Slatkin, and W. P. Maddison. 1992. Estimation of levels of gene

flow from DNA sequence data. Genetics 132:583-9.

Hughes, R. A., and D. R. Cornblath. 2005. Guillain-Barre syndrome. Lancet 366:1653-66.

Humphrey, T. J. 1989. An appraisal of the efficacy of pre-enrichment for the isolation of *Campylobacter jejuni* from water and food. Journal of Applied Bacteriology 66:119-26.

Humphrey, T. J., A. Henley, and D. G. Lanning. 1993. The colonization of broiler chickens with *Campylobacter jejuni*: some epidemiological investigations. Epidemiology and Infectection 110:601-7.

Hunter, P. R. 1990. Reproducibility and indices of discriminatory power of microbial typing methods. Journal of Clinical Microbiology 28:1903-5.

Huson, D. H. 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14:68-73.

Huson, D. H., and D. Bryant. 2006. Application of phylogenetic networks in evolutionary studies. Molecular Biology and Evolution 23:254-67.

Jacobs-Reitsma, W. F., N. M. Bolder, and R. W. A. W. Mulder. 1994. Caecal carriage of *Campylobacter* and *Salmonella* in Dutch broiler flocks at slaughter: a one-year study. Poultry Science 73:1260-1266.

Jin, S., A. Joe, J. Lynett, E. K. Hani, P. Sherman, and V. L. Chan. 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Molecular Microbiology 39:1225-36.

Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden. 2001. Sequence type analysis and recombinational tests (START). Bioinformatics 17:1230-1.

Jolley, K. A., and R. Urwin. 2001. Computational methods for meningococcal population studies. In A. J. Pollard and M. C. J. Maiden (ed.), Methods in Molecular Medicine, vol. 67: Meningococcal Disease: Methods and Protocols. Humana Press, Totowa, New Jersey.

Jones, D. R., K. E. Anderson, P. A. Curtis, and F. T. Jones. 2002. Microbial contamination in inoculated shell eggs: I. Effects of layer strain and hen age. Poultry Science 81:715-20.

Jones, K. 2001a. Campylobacters in water, sewage and the environment. Journal of Applied Microbiology 30:68S-79S.

Jones, K. 2001b. The *Campylobacter* conundrum. Trends in Microbiology 9:365-6.

Jones, K., S. Howard, and J. S. Wallace. 1999. Intermittent shedding of thermophilic Campylobacters by sheep at pasture. Journal of Applied Microbiology 86:531-536.

Kapperud, G., and O. Rosef. 1983. Avian wildlife reservoir of *Campylobacter fetus* subsp. *jejuni*, *Yersinia* spp., and *Salmonella* spp. in Norway. Applied and Environmental Microbiology 45:375-80.

Karenlampi, R., H. Rautelin, M. Hakkinen, and M. L. Hanninen. 2003. Temporal and geographical distribution and overlap of Penner heat-stable serotypes and pulsed-field gel electrophoresis genotypes of *Campylobacter jejuni* isolates collected from humans and chickens in Finland during a seasonal peak. Journal of Clinical Microbiology 41:4870-2.

Karlyshev, A. V., D. Linton, N. A. Gregson, A. J. Lastovica, and B. W. Wren. 2000. Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity. Molecular Microbiology **35:**529-41.

Kemp, R., A. J. Leatherbarrow, N. J. Williams, C. A. Hart, H. E. Clough, J. Turner, E. J. Wright, and N. P. French. 2005. Prevalence and genetic diversity of *Campylobacter* spp. in environmental water samples from a 100-square-kilometer predominantly dairy farming area. Applid and Environmental Microbiology 71:1876-82.

Kessel, A. S., I. A. Gillespie, S. J. O'Brien, G. K. Adak, T. J. Humphrey and L. R. Ward (2001). General outbreaks of infectious intestinal disease linked with poultry, England and Wales, 1992-1999. Commun Dis Public Health **4**(3): 171-7.

Ketley, J. M. 1997. Pathogenesis of enteric infection by *Campylobacter.* Microbiology 143:5-21.

Kirkwood, B. R., and J. A. C. Sterne. 2003. Medical Statistics, 2nd ed. Backwell Science.

Kopecko, D. J., L. Hu, and K. J. Zaal. 2001. *Campylobacter jejuni*-microtubule-dependent invasion. Trends in Microbiology 9:389-96.

Kramer, J. M., J. A. Frost, F. J. Bolton, and D. R. Wareing. 2000. *Campylobacter* contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. Journal of Food Protection 63:1654-9.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5:150-63.

Linton, D., A. J. Lawson, R. J. Owen, and J. Stanley. 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. Journal of Clinical Microbiology 35:2568-72.

Logue, C. M., J. S. Sherwood, L. M. Elijah, P. A. Olah, and M. R. Dockter. 2003. The incidence of *Campylobacter* spp. on processed turkey from processing plants in the midwestern United States. Journal of Applied Microbiology 95:234-41.

Louis, V. R., I. A. Gillespie, S. J. O'Brien, E. Russek-Cohen, A. D. Pearson, and R. R.

Colwell. 2005. Temperature-driven *Campylobacter* seasonality in England and Wales. Applied and Environmental Microbiology 71:85-92.

Lu, J., S. Sanchez, C. Hofacre, J. J. Maurer, B. G. Harmon, and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Applied and Environmental Microbiology 69:901-8.

Luechtefeld, N. A., M. J. Blaser, L. B. Reller, and W. L. Wang. 1980. Isolation of *Campylobacter fetus* subsp. *jejuni* from migratory waterfowl. Journal of Clinical Microbiology 12:406-8.

Madden, R. H., L. Moran, and P. Scates. 1998. Frequency of occurrence of *Campylobacter* spp. in red meats and poultry in Northern Ireland and their subsequent subtyping using polymerase chain reaction-restriction fragment length polymorphism and the random amplified polymorphic DNA method. Journal of Applied Microbiology 84:703-8.

Maiden, M. C. 2006. Multilocus Sequence Typing of Bacteria. The Annual Review of Microbiology.

Maiden, M. C. 2002. Population structure of *Neisseria meningitidis*, p. 151-170. In C. Ferreirós, M. T. Criado, and J. Vázquez (ed.), Emerging strategies in the fight against meningitis: molecular and cellular aspects. Horizon Scientific Press, Wymondham, Norfolk.

Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences USA 95:3140-3145.

Mamelli, L., J. M. Pages, M. E. Konkel, and J. M. Bolla. 2006. Expression and purification of native and truncated forms of CadF, an outer membrane protein of *Campylobacter*. International Journal of Biological Macromolecules **39:**135-40.

Mandrell, R. E., and M. R. Wachtel. 1999. Novel detection techniques for human pathogens that contaminate poultry. Current Opinion in Biotechnology **10:**273-8.

Manning, G., C. G. Dowson, M. C. Bagnall, I. H. Ahmed, M. West, and D. G. Newell. 2003. Multilocus sequence typing for comparison of veterinary and human isolates of *Campylobacter jejuni*. Applied and Environmental Microbiology 69:6370-9.

Manning, G., B. Duim, T. Wassenaar, J. A. Wagenaar, A. Ridley, and D. G. Newell. 2001. Evidence for a genetically stable strain of *Campylobacter jejuni*. Applied and Environmental Microbiology 67:1185-1189.

Mason, M. J., T. J. Humphrey, and K. W. Martin. 1999. Isolation of sublethally injured campylobacters from poultry and water sources. British Journal of Biomedical Science

56:2-5.

Maugeri, T. L., M. Carbone, M. T. Fera, G. P. Irrera, and C. Gugliandolo. 2004. Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. Journal of Applied Microbiology 97:354-61.

Mawdsley, J. L., R. D. Bardgett, R. J. Merry, B. F. Pain, and M. K. Theodorou. 1995. Pathogens in livestock waste, their potential for movement through soil and environmental pollution. Applied Soil Ecology 2:1-15.

Maynard Smith, J., N. H. Smith, M. O'Rourke, and B. G. Spratt. 1993. How clonal are bacteria? Proceedings of the National Academy of Sciences USA 90:4384-4388.

McCarthy, N. D., F. M. Colles, K. E. Dingle, M. C. Bagnall, M. C. J. Maiden, and D. Falush. 2006. Host specific gene pools in *Campylobacter jejuni.* Submitted.

Meerburg, B. G., W. F. Jacobs-Reitsma, J. A. Wagenaar, and A. Kijlstra. 2006. Presence of *Salmonella* and *Campylobacter* spp. in Wild Small Mammals on Organic Farms. Applied and Environmental Microbiology 72:960-2.

Meinersmann, R. J., L. O. Helsel, P. I. Fields, and K. L. Hiett. 1997. Discrimination of *Campylobacter jejuni* isolates by fla gene sequencing. Journal of Clinical Microbiology 35:2810-4.

Meinersmann, R. J., R. W. Phillips, K. L. Hiett, and P. Fedorka-Cray. 2005. Differentiation of *Campylobacter* populations as demonstrated by flagellin short variable region sequences. Applied and Environmental Microbiology 71:6368-74.

Miller, W. G., S. L. On, G. Wang, S. Fontanoz, A. J. Lastovica and R. E. Mandrell 2005. Extended multilocus sequence typing system for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. J Clin Microbiol **43**(5): 2315-29.

Miller, W. G., M. D. Englen, S. Kathariou, I. V. Wesley, G. Wang, L. Pittenger-Alley, R. M. Siletz, W. Muraoka, P. J. Fedorka-Cray, and R. E. Mandrell. 2006. Identification of host-associated alleles by multilocus sequence typing of *Campylobacter coli* strains from food animals. Microbiology 152:245-55.

Miwa, N., Y. Takegahara, K. Terai, H. Kato, and T. Takeuchi. 2003. *Campylobacter jejuni* contamination on broiler carcasses of *C. jejuni*-negative flocks during processing in a Japanese slaughterhouse. International Journal of Food Microbiology 84:105-9.

Monteville, M. R., and M. E. Konkel. 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infection and Immunity 70:6665-71.

Moore, J. E., T. S. Wilson, D. R. Wareing, T. J. Humphrey, and P. G. Murphy. 2002. Prevalence of thermophilic *Campylobacter* spp. in ready-to-eat foods and raw poultry in Northern Ireland. Journal of Food Protection 65:1326-8.

Morooka, T., A. Umeda, and K. Amako. 1985. Motility as an essential colonisation

factor for *Campylobacter jejuni*. Journal of General Microbiology 131:1973-1980.

Moulton, V. 2003. SplitsTree: A network-based tool for exploring evolutionary relationships in molecular data, p. 312-328. *In* M. Salemi and A.-M. Vandamme (ed.), The Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny. Cambridge University Press, Cambridge, UK

Muller, J., F. Schulze, W. Muller, and I. Hanel. 2005. PCR detection of virulence-associated genes in *Campylobacter jejuni* strains with differential ability to invade Caco-2 cells and to colonize the chick gut. Veterinary Microbiology.

Nachamkin, I., B. M. Allos, and T. Ho. 1998. *Campylobacter* species and Guillain-Barré syndrome. Clinical Microbiological Reviews 11:555-567.

Nachamkin, I., and M. B. Skirrow. 2000. Systematic Bacteriology. Arnold.

Newell, D. G., and C. Fearnley. 2003. Sources of *Campylobacter* colonization in broiler chickens. Applied and Environmental Microbiology 69:4343-51.

Newell, D. G., J. E. Shreeve, M. Toszeghy, G. Domingue, S. Bull, T. Humphrey, and G. Mead. 2001. Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. Applied and Environmental Microbiology 67:2636-40.

Nichols, G. L. 2005. Fly transmission of *Campylobacter*. Emerging Infectious Diseases 11:361-4.

Nielsen, E. M. 2002. Occurrence and strain diversity of thermophilic campylobacters in cattle of different age groups in dairy herds. Letters in Applied Microbiology 35:85-9.

Nielsen, E. M., J. Engberg, V. Fussing, L. Petersen, C. H. Brogren, and S. L. On. 2000. Evaluation of phenotypic and genotypic methods for subtyping *Campylobacter jejuni* isolates from humans, poultry, and cattle. Journal of Clinical Microbiology 38:3800-10.

Northcutt, J. K., M. E. Berrang, J. A. Dickens, D. L. Fletcher, and N. A. Cox. 2003. Effect of broiler age, feed withdrawal, and transportation on levels of coliforms, *Campylobacter*, *Escherichia coli* and *Salmonella* on carcasses before and after immersion chilling. Poultry Science 82:169-73.

Oberhelman, R. A., R. H. Gilman, P. Sheen, J. Cordova, D. N. Taylor, M. Zimic, R. Meza, J. Perez, C. LeBron, L. Cabrera, F. G. Rodgers, D. L. Woodward, and L. J. Price. 2003. *Campylobacter* transmission in a Peruvian shantytown: a longitudinal study using strain typing of campylobacter isolates from chickens and humans in household clusters. Journal of Infectious Disease 187:260-9.

Obiri-Danso, K., and K. Jones. 1999. Distribution and seasonality of microbial indicators and thermophilic campylobacters in two freshwater bathing sites on the River Lune in northwest England. Journal of Applied Microbiology 87:822-32.

Obiri-Danso, K., N. Paul, and K. Jones. 2001. The effects of UVB and temperature on

the survival of natural populations and pure cultures of *Campylobacter jejuni*, *Camp. coli*, *Camp. lari* and urease-positive thermophilic campylobacters (UPTC) in surface waters. Journal of Applied Microbiology 90:256-67.
On, S. L., and P. J. Jordan. 2003. Evaluation of 11 PCR assays for species-level identification of *Campylobacter jejuni* and *Campylobacter coli*. Journal of Clinical Microbiology 41:330-6.

Park, H., Y. C. Hung, and R. E. Brackett. 2002. Antimicrobial effect of electrolyzed water for inactivating *Campylobacter jejuni* during poultry washing. International Journal of Food Microbiology 72:77-83.

Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665-668.

Patrick, M. E., L. E. Christiansen, M. Waino, S. Ethelberg, H. Madsen and H.C. Wegener. 2004. Effects of climate on incidence of *Campylobacter* spp. in humans and prevalence in broiler flocks in Denmark. Applied and Environmental Microbiology 70(12): 7474-80.

Pattison, M. 2001. Practical intervention strategies for *Campylobacter*. Symposium Series Society of Applied Microbiology:121S-5S.

Pearson, A. D., M. H. Greenwood, R. K. Feltham, T. D. Healing, J. Donaldson, D. M. Jones, and R. R. Colwell. 1996. Microbial ecology of *Campylobacter jejuni* in a United Kingdom chicken supply chain: intermittent common source, vertical transmission, and amplification by flock propagation. Applied and Environmental Microbiology 62:4614-20.

Pei, Z., and M. J. Blaser. 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. The Journal of Biological Chemistry 268:18717-25.

Pei, Z. H., R. T. Ellison, 3rd, and M. J. Blaser. 1991. Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni*. The Journal of Biological Chemistry 266:16363-9.

Petersen, L., E. M. Nielsen, J. Engberg, S. L. On, and H. H. Dietz. 2001a. Comparison of genotypes and serotypes of *Campylobacter jejuni* isolated from Danish wild mammals and birds and from broiler flocks and humans. Applied and Environmental Microbiology 67:3115-21.

Petersen, L., E. M. Nielsen, and S. L. On. 2001b. Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks. Veterinary Microbiology 82:141-54.

Petersen, L., and A. Wedderkopp. 2001. Evidence that certain clones of *Campylobacter*

jejuni persist during successive broiler flock rotations. Applied and Environmental Microbiology 67:2739-45.

Petersen, S. T., J. Wiseman, and M. R. Bedford. 1999. Effects of age and diet on the viscosity of intestinal contents in broiler chicks. British Poultry Science 40:364-70.

Rautelin, H., J. Jusufovic, and M. L. Hanninen. 1999. Identification of hippurate-negative thermophilic campylobacters. Diagnostic Microbiology and Infectious Disease 35:9-12.

Refregier-Petton, J., N. Rose, M. Denis, and G. Salvat. 2001. Risk factors for *Campylobacter* spp. contamination in French broiler-chicken flocks at the end of the rearing period. Preventative Veterinary Medicine 50:89-100.

Richardson, J. F., J. A. Frost, J. M. Kramer, R. T. Thwaites, F. J. Bolton, D. R. Wareing, and J. A. Gordon. 2001. Coinfection with Campylobacter species: an epidemiological problem? Journal of Applied Microbiology 91:206-11.

Riordan, T., T. J. Humphrey, and A. Fowles. 1993. A point source outbreak of *Campylobacter* infection related to bird- pecked milk. Epidemiology and Infection 110:261-5.

Rivera-Amill, V., B. J. Kim, J. Seshu, and M. E. Konkel. 2001. Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. Journal of Infectectious Disese 183:1607-16.

Rivoal, K., M. Denis, G. Salvat, P. Colin, and G. Ermel. 1999. Molecular characterization of the diversity of *Campylobacter* spp. isolates collected from a poultry slaughterhouse: analysis of cross-contamination. Letters in Applied Microbiology 29:370-4.

Rivoal, K., C. Ragimbeau, G. Salvat, P. Colin, and G. Ermel. 2005. Genomic diversity of *Campylobacter coli* and *Campylobacter jejuni* isolates recovered from free-range broiler farms and comparison with isolates of various origins. Applied and Environmental Microbiology 71:6216-27.

Rosef, O., G. Kapperud, S. Lauwers, and B. Gondrosen. 1985. Serotyping of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter laridis* from domestic and wild animals. Applied and Environmental Microbiology 49:1507-10.

Rozas, J., and R. Rozas. 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15:174-5.

Sahin, O., N. Luo, S. Huang, and Q. Zhang. 2003. Effect of *Campylobacter*-specific maternal antibodies on *Campylobacter jejuni* colonization in young chickens. Applied and Environmental Microbiology 69:5372-9.

Sails, A. D., B. Swaminathan, and P. I. Fields. 2003a. Clonal complexes of *Campylobacter jejuni* identified by multilocus sequence typing correlate with strain associations identified by multilocus enzyme electrophoresis. Journal of Clinical

Microbiology 41:4058-67.

Sails, A. D., B. Swaminathan, and P. I. Fields. 2003b. Utility of Multilocus Sequence Typing as an Epidemiological Tool for Investigation of Outbreaks of Gastroenteritis Caused by *Campylobacter jejuni*. Journal of Clinical Microbiology 41:4733-9.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-25.

Sanger, F., S. Nicklen, and A. R. Coulson. 1992. DNA sequencing with chain-terminating inhibitors. 1977. Biotechnology 24:104-8.

Savill, M. G., J. A. Hudson, A. Ball, J. D. Klena, P. Scholes, R. J. Whyte, R. E. McCormick, and D. Jankovic. 2001. Enumeration of *Campylobacter* in New Zealand recreational and drinking waters. Journal of Applied Microbiology 91:38-46.

Schlesselman, J. J. 1982. Case-control studies; design, conduct, analysis. Oxford University Press.

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin version 2.000:a software for population genetic data analysis. University of Geneva, Geneva.

Schouls, L. M., S. Reulen, B. Duim, J. A. Wagenaar, R. J. Willems, K. E. Dingle, F. M. Colles, and J. D. Van Embden. 2003. Comparative Genotyping of *Campylobacter jejuni* by Amplified Fragment Length Polymorphism, Multilocus Sequence Typing, and Short Repeat Sequencing: Strain Diversity, Host Range, and Recombination. Journal of Clinical Microbiology 41:15-26.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Applied and Environmental Microbiology 51:837-884.

Shreeve, J. E., M. Toszeghy, M. Pattison, and D. G. Newell. 2000. Sequential spread of *Campylobacter* infection in a multipen broiler house. Avian Disease 44:983-8.

Shreeve, J. E., M. Toszeghy, A. Ridley, and D. G. Newell. 2002. The carry-over of *Campylobacter* isolates between sequential poultry flocks. Avian Disease 46:378-85.

Siemer, B. L., E. M. Nielsen, and S. L. On. 2005. Identification and molecular epidemiology of *Campylobacter coli* isolates from human gastroenteritis, food, and animal sources by amplified fragment length polymorphism analysis and Penner serotyping. Applied Environmental Microbiology 71:1953-8.

Skirrow, M. B. 1994. Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. Journal of Comparative Pathology 111:113-149.

Slader, J., G. Domingue, F. Jorgensen, K. McAlpine, R. J. Owen, F. J. Bolton, and T. J. Humphrey. 2002. Impact of transport crate reuse and of catching and processing on *Campylobacter* and *Salmonella* contamination of broiler chickens. Applied and Environmental Microbiology 68:713-9.

Snelling, W. J., J. P. McKenna, C. J. Hack, J. E. Moore, and J. S. Dooley. 2006. An examination of the diversity of a novel *Campylobacter* reservoir. Archives of Microbiology 186:31-40.

Sopwith, W., M. Ashton, J. A. Frost, K. Tocque, S. J. O'Brien, M. Regan, and Q. Syed. 2003. Enhanced surveillance of *Campylobacter* infection in the North West of England. Journal of Infection 46:35-45.

Sopwith, W., A. Birtles, M. Matthews, A. Fox, S. Gee, M. Painter, M. Regan, Q. Syed, and E. Bolton. 2006. *Campylobacter jejuni* Multilocus Sequence Types in Humans, Northwest England, 2003-2004. Emerging Infectious Disease 12:In press.

Spratt, B. G., and M. C. J. Maiden. 1999. Bacterial population genetics, evolution and epidemiology. Proceedings of the Royal Society of London. Series B: Biological Sciences 354:701-10.

Staden, R. 1996. The Staden sequence analysis package. Molecular Biotechnology 5:233-241.

Stanley, K. N., J. S. Wallace, J. E. Currie, P. J. Diggle, and K. Jones. 1998a. The seasonal variation of thermophilic Campylobacters in beef cattle, dairy cattle and calves. Journal of Applied Microbiology 85:472-80.

Stanley, K. N., J. S. Wallace, J. E. Currie, P. J. Diggle, and K. Jones. 1998b. Seasonal variation of thermophilic campylobacters in lambs at slaughter. Journal of Applied Microbiology 84:1111-6.

Stanley, K. N., J. S. Wallace, and K. Jones. 1998c. Note: Thermophilic campylobacters in dairy slurries on Lancashire farms: seasonal effects of storage and land application. Journal of Applied Microbiology 85:405-409.

Stanley, K. N., and K. Jones. 1998d. High frequency of metronidazole resistance among strains of *Campylobacter jejuni* isolated from birds. Letters in Applied Microbiology 27:247-50.

Stern, N. J., M. A. Myszewski, H. M. Barnhart, and D. W. Dreesen. 1997. Flagellin A gene restriction fragment length polymorphism patterns of *Campylobacter* spp. isolates from broiler production sources. Avian Disease 41:899-905.

Suerbaum, S., M. Lohrengel, A. Sonneveld, F. Ruberg, and M. Kist. 2001. Allelic diversity and recombination in *Campylobacter jejuni*. Journal of Bacteriology 183:2553-2559.

Suzuki, S., and T. Gojobori. 2003. Analysis of coding sequences, p. 283-311. *In* M. Salemi and A.-M. Vandamme (ed.), The Phylogenetic handbook: A Practical Approach to DNA and Protein Phylogeny. Cambridge University Press.

Tam, C. C., S. J. O'Brien, G. K. Adak, S. M. Meakins, and J. A. Frost. 2003. *Campylobacter coli* - an important foodborne pathogen. Journal of Infection 47:28-32.

Thakur, S., and W. A. Gebreyes. 2005. *Campylobacter coli* in swine production: antimicrobial resistance mechanisms and molecular epidemiology. Journal of Clin Microbiology 43:5705-14.

Thomas, L. M., Long, K.A., Good, R.T. Panaccio, M. and Widders P.R. 1997. Genotypic diversity amongst *Campylobacter jejuni* isolates in a commercial brioler flock. Applied and Environmental Microbiology 63:1874-1877.

Urwin, R., and M. C. Maiden. 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends in Microbiology 11:479-487.

Usuki, S., S. A. Thompson, M. H. Rivner, K. Taguchi, K. Shibata, T. Ariga, and R. K. Yu. 2005. Molecular mimicry: Sensitization of Lewis rats with *Campylobacter jejuni* lipopolysaccharides induces formation of antibody toward GD3 ganglioside. Journal of Neuroscience Research 83:274-284.

Vandamme, A.-M. 2003. Basic concepts of molecular evolution., p. 1-21. *In* M. Salemi and A.-M. Vandamme (ed.), The Phylogenetic handbook; a practical approach to DNA and protein phylogeny. Cambridge University Press.

Van de Peer, Y. 2003. Phylogeny inference based on distance methods, p. 101-119. *In* M. Salemi and A.-M. Vandamme (ed.), The Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny. Cambridge University Press, Cambridge, UK.

Vuckovic, D., M. Abram, M. Bubonja, B. Wraber, and M. Doric. 2006. Host resistance to primary and secondary *Campylobacter jejuni* infections in C57Bl/6 mice. Microbial Pathogenesis 40:35-9.

Waldenstrom, J., T. Broman, I. Carlsson, D. Hasselquist, R. P. Achterberg, J. A. Wagenaar, and B. Olsen. 2002. Prevalence of *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter coli* in different ecological guilds and taxa of migrating birds. Applied and Environmental Microbiology 68:5911-7.

Waldenstrom, J., F. Colles, N. McCarthy, O. Hellgren, B. Olsen, D. Hasselquist, and M. C. Maiden. 2005a. Epidemiology and population structure of *Campylobacter jejuni* from wild birds. Manuscript.

Waldenstrom, J., S. L. On, R. Ottvall, D. Hasselquist, and B. Olsen. 2005b. Species diversity fo campylobacteria in a wild bird community in Sweden. Submitted.

Wallace, J. S., K. N. Stanley, J. E. Currie, P. J. Diggle, and K. Jones. 1997. Seasonality of thermophilic *Campylobacter* populations in chickens. Journal of Applied Microbiology 82:219-224.

Wallace, J. S., K. N. Stanley, and K. Jones. 1998. The colonisation of turkeys by thermophilic Campylobacters. Journal of Applied Microbiology 85:224-230.

Wang, G., C. G. Clark, T. M. Taylor, C. Pucknell, C. Barton, L. Price, D. L.

Woodward, and F. G. Rodgers. 2002. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. Journal of Clinical Microbiology 40:4744-7.

Wareing, D. R. A., F. J. Bolton, A. J. Fox, P. A. Wright, and D. L. A. Greenway. 2002. Phenotypic diversity of *Campylobacter* isolates from sporadic cases of human enteritis in the UK. Journal of Applied Microbiology 92:502-509.

Wassenaar, T. M., and D. G. Newell. 2000. Genotyping of *Campylobacter* species. Applied and Environmental Microbiology 66:1-9.

Wassenaar, T. M., B. N. Fry and B. A. M. van der Zeijst (1995). Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. Microbiology_141: 95-101.

Weijtens, M. J., R. D. Reinders, H. A. Urlings, and J. Van der Plas. 1999. *Campylobacter* infections in fattening pigs; excretion pattern and genetic diversity. Journal of Applied Microbiology 86:63-70.

Wilkinson-Herbots, H. M., and R. Ettridge. 2004. The effect of unequal migration rates on FST. Theoretical Population Biology 66:185-97.

Willis, W. L., C. Murray, and C. Talbott. 2002. *Campylobacter* isolation trends of cage versus floor broiler chickens: a one-year study. Poultry Science 81:629-31.

Wittwer, M., J. Keller, T. M. Wassenaar, R. Stephan, D. Howald, G. Regula, and B. Bissig-Choisat. 2005. Genetic diversity and antibiotic resistance patterns in a *Campylobacter* population isolated from poultry farms in Switzerland. Applied and Environmental Microbiology 71:2840-7.

Wokke, J. H., and L. H. van den Berg. 2005. A way out of the maze: *Campylobacter jejuni* gene polymorphisms define Guillain-Barre syndrome. Neurology 65:1350-1.

Workman, S. N., G. E. Mathison, and M. C. Lavoie. 2005. Pet dogs and chicken meat as reservoirs of *Campylobacter* spp. in Barbados. Journal of Clinical Microbiology 43:2642-50.

Wright, S. (1951). The genetical structure of populations. Annals of Eugenics_**15**: 323-354.

Zhang, Q., J. C. Meitzler, S. Huang, and T. Morishita. 2000. Sequence polymorphism, predicted secondary structures, and surface-exposed conformational epitopes of *Campylobacter* major outer membrane protein. Infection and Immunity 68:5679-89.

Zia, S., D. Wareing, C. Sutton, E. Bolton, D. Mitchell, and J. A. Goodacre. 2003. Health problems following *Campylobacter jejuni* enteritis in a Lancashire population. Rheumatology (Oxford) 42:1083-8.

Zilbauer, M., N. Dorrell, P. K. Boughan, A. Harris, B. W. Wren, N. J. Klein, and M. Bajaj-Elliott. 2005. Intestinal innate immunity to *Campylobacter jejuni* results in

induction of bactericidal human beta-defensins 2 and 3. Infection and Immunity 73:7281-9.

Zimmer, M., H. Barnhart, U. Idris, and M. D. Lee. 2003. Detection of *Campylobacter jejuni* strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens. Avian Disease 47:101-7.

Ziprin, R. L., M. E. Hume, C. R. Young, and R. B. Harvey. 2002. Cecal colonization of chicks by porcine strains of *Campylobacter coli.* Avian Disease 46:473-7.

Appendices

Appendix 1. *Campylobacter* genotypes from pigs, cattle, human disease and retail meat.

Table 1. The *C. jejuni* genotypes isolated from 91 human diseases in Lancashire 1999.

Clonal complex	Number of isolates	ST	Complex variant[a]	Frequency	Clonal complex	Number of isolates	ST	Complex variant[a]	Frequency
257	22	257	C	11	353	4	5	SLV	1
		163	SLV	1			356	DLV	1
		316	SLV	1			363	DLV	1
		361	SLV	1			82	TLV	1
		366	SLV	1	574	4	305	SLV	1
		367	SLV	5			571	SLV	3
		372	SLV	1	48	3	48	C	3
		421	SLV	1	443	3	443	C	1
21	13	21	C	3			440	SLV	1
		50	SLV	5			84	DLV	1
		104	SLV	2	49	2	570	SLV	2
		53	SLV	2	354	2	354	C	2
		24	DLV	1	607	1	170	TLV	1
45	12	45	C	8	Unassigned	13	85		1
		25	SLV	1			384		1
		137	SLV	1			449		1
		233	SLV	1			34		1
		546	TLV	1			319		1
52	6	52	C	2			350		3
		172	SLV	1			364		1
		413	SLV	1			407		1
		161	TLV	1			441		2
		379	TLV	1			448		1
61	6	61	C	5					
		417	SLV	1					

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant.
[a]The relationship to the central genotype.

Table 2. The *Campylobacter* genotypes isolated from Oxfordshire cattle in 2004.

Clonal complex	Number of isolates	ST	Complex variant[a]	Frequency
21	6	21	C	1
		19	SLV	1
		104	SLV	1
		262	SLV	3
42	5	42	C	3
		1013	SLV	1
		1085	SLV	1
45	1	45	C	1
49	2	49	C	2
61	2	61	C	1
		955	DLV	1
828	15	827	SLV	12
		1084	TLV	1
Unassigned		436		2

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant.
[a]The relationship to the central genotype.

Table 3. The *C. coli* genotypes isolated from Oxfordshire pigs in 2004.

Clonal complex	Number of isolates	ST	Complex variant[a]	Frequency
828	8	886	TLV	4
		887	TLV	2
		888	TLV	2

C, central genotype; SLV, single locus variant; DLV, double locus variant; TLV, triple locus variant.
[a]The relationship to the central genotype.

Table 4. The *Campylobacter* genotypes isolated from 722 cases of human disease in the UK between 1977 and 2004; data from the *Campylobacter* MLST database.

Clonal complex	Number of STs	Frequency (%)
21	70	189 (26.2)
45	30	63 (8.7)
48	24	59 (8.2)
257	20	55 (7.6)
61	15	44 (6.1)
206	18	37 (5.1)
353	17	21 (2.9)
828	19	21 (2.9)
52	8	13 (1.8)
42	7	12 (1.7)
22	9	11 (1.5)
403	8	10 (1.4)
49	4	9 (1.3)
446	6	8 (1.1)
574	4	6 (0.8)
607	6	6 (0.8)
433	20	5 (0.7)
460	4	4 (0.6)
177	1	3 (0.4)
362	3	3 (0.4)
573	3	3 (0.4)
661	3	3 (0.4)
1034	2	2 (0.3)
508	2	2 (0.3)
677	1	1 (0.1)
Unassigned	59	65

Table 5. The *Campylobacter* genotypes isolated from 1358 cases of human disease worldwide between 1977 and 2005; data from the *Campylobacter* MLST database.

Clonal complex	Number of STs	Frequency (%)
21	98	190 (21.4)
45	46	115 (8.5)
48	36	112 (8.2)
828	64	69 (5.1)
257	24	61 (4.5)
403	11	58 (4.3)
353	36	56 (4.1)
61	19	52 (3.8)
22	16	50 (3.7)
508	6	49 (3.6)
206	21	47 (3.5)
42	10	36 (2.7)
354	20	26 (1.9)
658	12	26 (1.9)
443	16	24 (1.8)
52	13	22 (1.6)
49	7	20 (1.5)
41	1	18 (1.3)
283	10	15 (1.1)
362	3	12 (0.9)
607	10	11 (0.8)
574	6	10 (0.7)
460	9	9 (0.7)
446	6	8 (0.6)
433	4	6 (0.4)
1150	5	5 (0.4)
177	1	3 (0.2)
573	3	3 (0.2)
661	3	3 (0.2)
1034	2	2 (0.1)
179	1	1 (0.1)
677	1	1 (0.1)
Unassigned	127	138 (10.2)

Table 6. The genotypes identified amongst 130 *Campylobacter* isolates from retail chicken meat in the UK between 1982 and 2005; data from the *Campylobacter* MLST database.

Clonal complex	Number of STs	Frequency (%)
21	24	34 (29.2)
45	23	24 (18.5)
257	5	10 (7.7)
354	7	8 (6.2)
573	4	5 (3.8)
48	4	4 (3.1)
283	3	3 (2.3)
433	4	3 (2.3)
828	3	3 (2.3)
206	2	2 (1.5)
574	2	2 (1.5)
661	2	2 (1.5)
22	1	1 (0.8)
353	1	1 (0.8)
443	4	1 (0.8)
460	1	1 (0.8)
49	1	1 (0.8)
52	1	1 (0.8)
61	1	1 (0.8)
Unassigned	19	19 (14.6)

Table 7. The genotypes identified amongst 144 *Campylobacter* isolates from retail chicken meat worldwide between 1982 and 2005; data from the *Campylobacter* MLST database.

Clonal complex	Number of STs	Frequency (%)
21	98	190 (21.4)
45	46	115 (8.5)
48	36	112 (8.2)
828	64	69 (5.1)
257	24	61 (4.5)
403	11	58 (4.3)
353	36	56 (4.1)
61	19	52 (3.8)
22	16	50 (3.7)
508	6	49 (3.6)
206	21	47 (3.5)
42	10	36 (2.7)
354	20	26 (1.9)
658	12	26 (1.9)
443	16	24 (1.8)
52	13	22 (1.6)
49	7	20 (1.5)
41	1	18 (1.3)
283	10	15 (1.1)
362	3	12 (0.9)
607	10	11 (0.8)
574	6	10 (0.7)
460	9	9 (0.7)
446	6	8 (0.6)
433	4	6 (0.4)
1150	5	5 (0.4)
177	1	3 (0.2)
573	3	3 (0.2)
661	3	3 (0.2)
1034	2	2 (0.1)
179	1	1 (0.1)
677	1	1 (0.1)
Unassigned	127	138 (10.2)

Appendix 2 *Campylobacter* STs from all isolates.

Table 1. The distribution of *Campylobacter* genotypes amongst animal sources, human disease and retail chicken meat, shown as STs.

Clonal Complex	ST	Geese^{O} n=166	Starlings^{O} n=293	Poultry^{O} n=868	Cattle^{O} n=31	Sheep^{O} n=4	Pigs^{O} n=8	Poultry^{L} n=28	Cattle^{L} n=33	Sheep^{L} n=42	Starlings^{L} n=9	Humans^{DB} n=1358	Chicken^{DB} n=144
21	19			9.68	0.37					0.90		1.50	2.80
	21				0.37					0.90		5.20	0.70
	53			0.23				0.90	2.70	0.90	1.80	1.50	
	104				0.37							1.30	
	141			0.12								0.10	
	193	0.60										0.10	
	262			3.11	1.12				8.10	3.60		0.50	0.70
	264			0.12									
	376									0.90		0.10	
	518									0.90			
	519									0.90			
	520									0.90			
	1383		0.34										
No. additional STs												*90*	*21*
22	22								1.8	0.90		2.60	
	512								0.9				
No. additional STs												*15*	
42	42		0.34		1.12				0.90	8.00		1.20	
	517									0.90			
	1013				0.37								
	1085				0.37								
No. additional STs												9	
45	45		1.71	0.58	0.37			22.30	0.90			3.30	1.40
	100							0.90					
	137	4.22				25.00					0.90	1.00	
	334		0.34									0.10	
	514								0.90				
	515								0.90				
	706	0.60											
	998		0.34										

Clonal Complex	ST	Geese^{O} n=166	Starlings^{O} n=293	Poultry^{O} n=868	Cattle^{O} n=31	Sheep^{O} n=4	Pigs^{O} n=8	Poultry^{L} n=28	Cattle^{L} n=33	Sheep^{L} n=42	Starlings^{L} n=9	Humans^{DB} n=1358	Chicken^{DB} n=144
45 cont'd	1025		0.34										
No. additional STs												*43*	*23*
48	38		0.68						1.80	0.90		0.40	
	48								1.80	1.80		2.60	
	205								0.90			0.60	
	475			2.42								1.60	
	813			0.23									
	516								0.90				
No. additional STs												*33*	*4*
49	49				0.75							1.00	0.70
No. additional STs												*7*	*1*
52	52									1.80		0.50	
No. additional STs												*12*	*2*
61	61				0.37				0.90	9.90		2.10	0.70
	81									1.80			
	955				0.37								
No. additional STs												*18*	*1*
177	144		1.02										
	177		15.70										
	563		0.68									0.20	
	521								2.70				
	685		0.34										
	1004		1.02										
	1014		0.34										
	1381		0.68										
	1382		0.68										
	1388		0.68										
	1394		1.02										
	1482		0.34										
	1485		0.34										
	1500		0.34										

Clonal Complex	ST	Geese^{O} n=166	Starlings^{O} n=293	Poultry^{O} n=868	Cattle^{O} n=31	Sheep^{O} n=4	Pigs^{O} n=8	Poultry^{L} n=28	Cattle^{L} n=33	Sheep^{L} n=42	Starlings^{L} n=9	Humans^{DB} n=1358	Chicken^{DB} n=144
177	1506		0.34										
continued	1533		0.34										
	1535		0.34										
179	220		1.02										
	1207											0.10	
No. additional STs												*1*	
206	206									1.80		1.20	
No. additional STs												*20*	*2*
257	257		0.68	1.38				0.90			2.70		
	513								0.90				
No. additional STs												*24*	*5*
283	267		0.34									0.40	0.70
No. additional STs												*9*	*3*
460	741												0.70
	1538			0.12									
No. additional STs												*9*	*1*
573	573			12.21									
	817			0.12									
No. additional STs												*3*	*4*
574	574		0.34	3.57									
No. additional STs												*6*	*2*
661	814			12.90									
	958			1.38									
No. additional STs												*3*	*2*
677	677		0.68										
	1024		0.34										
	1534		0.34										
No. additional STs												*1*	
692	690								0.90				
	692	2.41											
	699	4.22											

Clonal Complex	ST	Geese^{O} n=166	Starlings^{O} n=293	Poultry^{O} n=868	Cattle^{O} n=31	Sheep^{O} n=4	Pigs^{O} n=8	Poultry^{L} n=28	Cattle^{L} n=33	Sheep^{L} n=42	Starlings^{L} n=9	Humans^{DB} n=1358	Chicken^{DB} n=144
692 cont'd	707	3.61											
828coli	827				4.49							0.10	
	828			3.46								0.10	
	829			0.35								0.10	
	830			0.12								0.10	
	854			11.98									
	855			15.21									
	860			1.15								0.10	
	868						50.00						
	871			1.50									
	887						25.00						
	888						25.00						
	902			3.23									
	1084				0.37								
	1088			0.12									
	1089			2.53									
	1614			0.12									
	1615			0.12									
No. additional STs												*59*	*11*
1034	694	0.44											
	697	0.22											
	698	0.22											
	711	0.44											
	788	0.22											
	977	0.22										0.10	
	1029	0.22											
	1033	3.50											
	1608	0.22											
No. additional STs												*1*	
1150coli	1487			1.61									
No. additional STs												*5*	

Clonal Complex	ST	Geese[O] n=166	Starlings[O] n=293	Poultry[O] n=868	Cattle[O] n=31	Sheep[O] n=4	Pigs[O] n=8	Poultry[L] n=28	Cattle[L] n=33	Sheep[L] n=42	Starlings[L] n=9	Humans[DB] n=1358	Chicken[DB] n=144
1332	696	5.42				25.00							
Unassigned	436		0.68		0.75							0.10	
	683		4.78										
	684		0.34										
	688								0.90				
	691	3.01											
	693	5.42											
	695	3.01											
	700	4.82											
	701	4.82											
	704	1.81											
	708	0.60											
	709	3.61											
	710	7.83											
	785	0.60											
	784					25.00							
	786	0.60											
	787					25.00							
	789	0.60											
	993	0.60											
	997		0.68										
	999		0.34										
	1000		0.34										
	1002		0.34										
	1023		3.07	0.12									
	1026		0.34										
	1028	1.81											
	1030	2.41											
	1031	6.02											
	1032	1.20											
	1090			10.14									

Clonal Complex	ST	Geese^{O} n=166	Starlings^{O} n=293	Poultry^{O} n=868	Cattle^{O} n=31	Sheep^{O} n=4	Pigs^{O} n=8	Poultry^{L} n=28	Cattle^{L} n=33	Sheep^{L} n=42	Starlings^{L} n=9	Humans^{DB} n=1358	Chicken^{DB} n=144
Unassigned	1357			0.12									
continued	1384		0.34										
	1389		0.34										
	1481		0.34										
	1483		0.34										
	1484		0.34										
	1486		0.34										
	1501		0.34										
	1502		0.34										
	1504		0.34										
	1508		0.34										
	1536		0.34										
	1537		0.34										
	1539		0.34										
	1606	1.81											
	1607	0.60											
	1609		0.34										
	1612		0.34										
	1613		0.34										
No. additional STs												*126*	*23*

^{O}=Oxfordshire, ^{L}= Lancashire ^{DB}=Isolates from *Campylobacter* MLST database. a=all isolates listed on the database,with a worldwide distribution.

Shaded boxes = STs isolated from more than one source.

Values are percentage of isolates.

Appendix 3.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2003, p. 7409–7413
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.12.7409–7413.2003

Vol. 69, No. 12

Genetic Diversity of *Campylobacter jejuni* Isolates from Farm Animals and the Farm Environment

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Received 10 April 2003/Accepted 8 September 2003

The genetic diversity of *Campylobacter jejuni* isolates from farm animals and their environment was investigated by multilocus sequence typing (MLST). A total of 30 genotypes, defined by allelic profiles (assigned to sequence types [STs]), were found in 112 *C. jejuni* isolates originating in poultry, cattle, sheep, starlings, and slurry. All but two of these genotypes belonged to one of nine *C. jejuni* clonal complexes previously identified in isolates from human disease and retail food samples and one clonal complex previously associated with an environmental source. There was some evidence for the association of certain clonal complexes with particular farm animals: isolates belonging to the ST-45 complex predominated among poultry isolates but were absent among sheep isolates, while isolates belonging to the ST-61 and ST-42 complexes were predominant among sheep isolates but were absent from the poultry isolates. In contrast, ST-21 complex isolates were distributed among the different isolation sources. Comparison with MLST data from 91 human disease isolates showed small but significant genetic differentiation between the farm and human isolates; however, representatives of six clonal complexes were found in both samples. These data demonstrate that MLST and the clonal complex model can be used to identify and compare the genotypes of *C. jejuni* isolates from farm animals and the environment with those from retail food and human disease.

Campylobacter jejuni continues to be the most common etiological agent of bacterial gastroenteritis in the developed world, with an estimated 2.5 million cases occurring per year in the United States (22) and over 54,000 cases reported to the United Kingdom Public Health Laboratory Service during 2001 (1). It is also a major problem in developing countries, particularly among young children (7). In contrast to many other food-borne bacterial pathogens, the majority of cases of campylobacteriosis in humans are considered to be sporadic, with few cases of disease traceable to point sources. It is thought that the consumption of inadequately cooked contaminated meat, particularly that from poultry, is a major source of human infection, but this has not been established unequivocally (8). Investigations into the epidemiology of human infection by this bacterium have been complicated by a number of factors, including its high genetic and antigenic diversity (2) and its wide, perhaps ubiquitous, distribution. In addition, serological typing methods have lacked reproducibility among laboratories and discriminatory power, with a large number of isolates reported as untypeable (12, 28, 41).

C. jejuni is an apparently harmless commensal of the gastrointestinal tract of many domestic and wild animals, especially birds (5). It is also readily isolated from a range of environmental locations, including soil, surface water, and the sand of bathing beaches, probably as a consequence of contact with various contamination sources, including animal feces (3, 5, 20, 26, 30). Environmental reservoirs of *C. jejuni* can act as sources of infection for humans; for example, wild birds pecking milk bottle tops have been shown to cause outbreaks of human campylobacteriosis (24). However, the contribution of such sources to the overall burden of human disease and to the colonization of farm animals has not been established. Whether only some of the *C. jejuni* present in environmental reservoirs are pathogenic to humans (20) and whether all animals and microenvironments harbor the same strains are important questions that remain incompletely addressed. Population genetic approaches that examine differentiation between populations provide a novel approach to resolving this problem.

Multilocus sequence typing (MLST) is a molecular isolate characterization technique that exploits recent advances in high-throughput molecular technology to generate accurate isolate characterization data efficiently and inexpensively. Data are highly reproducible among laboratories and can be shared electronically via the Internet (10). It has sufficient resolution to accommodate the high levels of diversity present in many bacterial pathogen populations but can also rationalize this diversity into groups of isolates with related genotypes. MLST has been successfully employed to characterize a variety of bacteria, including *C. jejuni* (11, 35), but to date studies of *C. jejuni* have focused on isolates obtained from human disease and retail food products, with relatively few environmental isolates having been investigated.

In the present work, MLST was used to characterize 112 isolates from a variety of farm and environmental sources with the aim of establishing the genetic diversity of *C. jejuni* in the farm setting and comparing this diversity with that found among collections of human disease isolates. There was small but significant differentiation between the two populations, although many of the genotypes found among isolates derived from human disease were present in the environmental sam-

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TABLE 1. Allelic diversity among 112 *C. jejuni* isolates from farm animal and environmental sources

Locus	Fragment size (bp)	No. of alleles	No. of variable sites[b]	% Variable sites[b]	d_N/d_S ratio[b]	Pairwise F_{ST}[a]	P values[c]
asp	477	5	13	2.7	0.045	0.044	0.005
gln	477	8	17	3.6	0.093	0.028	0.000
glt	402	12	16	4	0.048	0.094	0.000
gly	507	11	28	5.5	0.045	0.053	0.000
pgm	498	11	34	6.8	0.026	0.081	0.000
tkt	459	9	26	5.7	0.008	0.051	0.000
unc	489	6	71 (4)	14.5 (0.8)	0.143 (0.000)	0.015	0.135
All loci	3,309	30	207 (140)	6.3 (4.2)	0.030 (0.037)	0.005	0.000

[a] Comparison with the 91 human disease isolates from stool cultures in the United Kingdom during 1999.
[b] Figures in parentheses exclude *uncA* allele 17, which may have come from a different *Campylobacter* species (11).
[c] F_{ST} P values calculated at a 0.05 significance level.

ples. The results indicated the potential for combining MLST data with population genetic analyses in elucidating the relative importance of the various possible sources of human infection by *C. jejuni*.

MATERIALS AND METHODS

Bacterial isolates. A total of 112 *C. jejuni* isolates obtained from poultry, cattle, sheep, and environmental sources from northwestern England during the 1990s were investigated. These comprised 16 isolates from the whole intestine of broiler birds from a poultry processing plant supplied by 35 farms located within a 150-mile radius (38); 12 isolates from fresh feces of turkey chicks kept on a dairy farm (39); 14 isolates from the small intestines of adult beef cattle at an abattoir receiving cattle and sheep from northwestern England, northern Wales, and southwestern Scotland; 9 isolates from fresh feces of newborn calves (32); 9 isolates from the small intestines of lambs at the same abattoir as the beef cattle (33); 14 isolates from intestinal contents of sheep at slaughter; 10 isolates from fresh feces of sheep grazing on salt marsh; 9 isolates from fresh feces of sheep grazing on upland fell (21); 10 isolates from slurry storage tanks (34); and 9 isolates from starling feces collected on the same dairy farm as the isolates from turkey chicks (31).

Microbiological isolate characterization. The isolates had been presumptively identified as thermophilic *Campylobacter* species on the basis of microscopy and catalase and oxidase reactions. Isolates from the sheep and poultry were biotyped by using the modified scheme of Bolton et al. (4). Some isolates from sheep grazing on salt marsh and fell pastures were biotyped by using the MAST ID Camp Identification System (Mast Diagnostics, Bootle, United Kingdom). Where necessary, identification of some of the isolates to species level was confirmed by nucleotide sequencing of an 812-bp segment of 23S RNA, using the 43a and 69ar primers designed by van Camp et al. (36).

DNA preparation. The isolates, which had been stored at −80°C in a cryopreservative medium, were revived on Columbia agar (CM 0331; Oxoid Ltd., Basingstoke, United Kingdom) with 5% horse blood in a microaerophilic atmosphere produced by using gas-generating sachets (CN0025A; Oxoid Ltd.; and 96125; bioMerieux, Basingstoke, United Kingdom) at 42°C for 48 h. A suspension of approximately 10^6 cells per ml was prepared in sample buffer, and chromosomal DNA was extracted by using IsoQuick nucleic acid extraction kits (ISC Bioexpress, Kaysville, Utah) and the rapid DNA extraction protocol according to the manufacturer's instructions.

MLST. The previously published protocol for *C. jejuni* MLST was used (11). Briefly, fragments of seven housekeeping genes (aspartase A, *aspA*; glutamine synthetase, *glnA*; citrate synthase, *gltA*; serine hydroxymethyl transferase, *glyA*; phosphoglucomutase, *pgm*; transketolase, *tkt*; and ATP synthase subunit, *uncA*) were amplified by PCR, and the nucleotide sequence of the amplicons was determined with the published oligonucleotide primers and reaction conditions. Nucleotide sequence extension reaction products were separated and detected on an ABI Prism 3700 or an ABI Prism 377 automated DNA analyzer. Nucleotide sequences were determined at least once on each DNA strand and were assembled with the STADEN software package (29). Allele numbers and sequence types (ST) were assigned by using the *Campylobacter* MLST database (http://campylobacter.mlst.net/).

Data from human disease isolates. Data from 91 human disease isolates of *C. jejuni*, isolated from cases of gastroenteritis from stool cultures in northwestern England during 1999, were obtained from the PubMLST isolate database (http://campylobacter.mlst.net) by using the "Search database—advanced queries" feature with the following criteria: id, <1316; country, United Kingdom; year, 1999; source, human stool. These data included sequence types for 40 previously published (10) and 51 unpublished isolates.

Data analysis. The STs were assigned to clonal complexes as described previously (10). Central genotypes were defined by using UPGMA cluster analysis and the BURST algorithm (13), both implemented with the computer program START (18), and split decomposition analysis was implemented with the program SPLITSTREE (17). Isolates were defined as belonging to a clonal complex if they shared at least four alleles with the central ST. The ratio of nonsynonymous to synonymous substitutions (d_N/d_S), and numbers of fixed differences and shared polymorphisms were calculated by using the DNASP software package version 3.53 (25). The pairwise F_{ST} and test of significance calculations were performed by using the Arlequin software package, version 2.000 (27). For some of the analyses the gene sequences for each of the MLST loci were concatenated to give a single continuous nucleotide sequence of 3,309 bp for each isolate.

RESULTS

Diversity of MLST allele sequences. It was possible to determine the nucleotide sequences at each of the MLST loci from all of the farm and environmental isolates with previously published methods and reagents. The number of unique sequences at each locus varied from 5 for the *aspA* locus to 12 for the *gltA* locus, with the percentage of variable sites in this data set ranging from 2.7% for the *aspA* locus to 14.5% at the *uncA* locus. The apparent high diversity of the *uncA* locus was caused by a single allele, allele 17, which occurred 18 times in the data set. Exclusion of this allele gave diversity for this locus of 0.8%. The ratio of nonsynonymous to synonymous substitutions (d_N/d_S) observed ranged from 0.000 to 0.143 (Table 1). All except two of the MLST allele sequences found in this isolate collection had been described previously.

Diversity of sequence types. The 112 isolates contained a total of 30 different STs (Table 2). The most common ST was ST-45, which was represented by 26 isolates, with several STs occurring only once in the data set (Table 2). The four most predominant STs (ST-45, ST-42, ST-61, and ST-262) represented approximately half of the isolates (61 out of 112 isolates, or 54%). Virtually all of the isolates (110 out of 112 isolates, or 98%) were assigned to 1 of 10 previously described clonal complexes, with the remaining two isolates unassigned as they did not share four alleles in common with any of the previously recognized central genotypes. The clonal complexes were represented by between 2 isolates (ST-52 complex) and 30 isolates (ST-45 complex). The number of STs within each

TABLE 2. Distribution of STs among 112 *C. jejuni* isolates from farm animals and their environment and their resolution into clonal complexes

Clonal complex	ST no.	No. of isolates[a]
21	21	1
	19	1
	53	7
	262	13
	376	1
	518	1
	519	1
	520	1
22	22	3
	512	1
42	42	10
	517	1
45	45	26
	100	1
	137	1
	514	1
	515	1
48	48	4
	38	3
	205	1
	216	1
52	52	2
61	61	12
	81	2
177	521	3
206	206	6
257	257	4
	513	1
Unassigned	688	1
	690	1

[a] The total numbers of isolates for each clonal complex are as follows: complex 21, 26; complex 22, 4; complex 42, 11; complex 45, 30; complex 48, 9; complex 52, 2; complex 61, 14; complex 177, 3; complex 206, 6; complex 257, 5; and the unassigned complex, 2.

clonal complex ranged from one (ST-52, ST-177, and ST-206 complexes) to eight (ST-21 complex). With the exception of the ST-21 complex, the previously assigned central genotype was the most predominant ST present in each of the clonal complexes identified in this sample. A total of 12 STs were novel to this study.

Distribution of genotypes among isolation sources. The most common clonal complexes among the farm isolates were ST-45 complex (30 isolates), ST-21 complex (26 isolates), ST-61 complex (14 isolates), and ST-42 complex (11 isolates) (Table 2). The remaining clonal complexes were represented by nine or fewer isolates, with the ST-52 complex, represented by two isolates, being the smallest. The number of clonal complexes identified in each isolation source varied from two for turkey chicks, broiler chicks, and slurry to six for adult beef cattle at slaughter and sheep at slaughter (Table 3). The ST-21 complex was the most widely distributed clonal complex, present in 8 of the 10 different isolation sources. The ST-52 and ST-177 complexes were the least widely distributed and were each identified in one isolation source. The ST-45 complex was predominant among turkey and broiler chick isolation sources and was absent from ovine isolation sources. Conversely, ST-42 and ST-61 complexes were predominant among ovine and bovine sources but were absent from avian sources. Two clonal complexes were identified in both turkey and broiler chicks, although only the ST-45 complex was common to both isolation sources in this study. Adult cattle and calves shared the ST-21, ST-45, and ST-48 complexes, but each had further complexes. Sheep at slaughter, sheep grazing on salt marsh, and sheep grazing on fell (hill) land shared three clonal complexes, ST-21 complex, ST-45 complex, and ST-61 complex. Adult sheep and lambs shared the ST-61 and ST-48 complexes, but only the ST-61 complex was found in all ovine groups. Isolates belonging to the ST-21 complex, ST-45 complex, and ST-257 complex were present in starlings, which were the only source from which the ST-177 complex was recovered. Both of the unassigned STs (ST-690 and ST-688) were from bovine isolation sources.

Comparison of genotypes with human disease isolates. The gene flow analysis (pairwise F_{ST}) between the 112 isolates in this study and the 91 isolates from human disease gave values (0.015 to 0.094) that ranged from significantly different to zero

TABLE 3. Frequency and distribution of clonal complexes among farm and environmental sources of *C. jejuni*

Source (*n*)	Clonal complex													
	21	22	42	45	48	52	61	177	206	257	353	443	354	U[a]
Turkey chicks (12)	—[b]	—	—	11	—	—	—	—	—	1	—	—	—	—
Broiler chicks (16)	1	—	—	15	—	—	—	—	—	—	—	—	—	—
Calves (9)	3	—	—	2	2	—	—	—	—	1	—	—	—	1
Adult beef cattle at slaughter (14)	2	—	1	1	4	—	1	—	4	—	—	—	—	1
Slurry (10)	7	3	—	—	—	—	—	—	—	—	—	—	—	—
Lambs at slaughter (9)	—	1	—	—	2	—	6	—	—	—	—	—	—	—
Sheep at slaughter (14)	5	—	3	—	1	2	1	—	2	—	—	—	—	—
Sheep grazing in salt marsh (10)	5	—	3	—	—	—	2	—	—	—	—	—	—	—
Sheep grazing in fell (9)	1	—	4	—	—	—	4	—	—	—	—	—	—	—
Starlings (9)	2	—	—	1	—	—	—	3	—	3	—	—	—	—
Humans (91)	13	—	—	13	3	6	6	—	—	22	4	4	4	16

[a] U, unassigned isolates.
[b] —, zero.

for all of the alleles, except for *uncA* (Table 1) and for the concatenated sequences. A total of 183 polymorphisms were shared between the two data sets, and there were no fixed nucleotide differences between them.

DISCUSSION

The contributions of the various possible sources of infection to the total disease burden of human campylobacteriosis have not been definitively established. In addition to the problems inherent in analyzing a sporadic zoonotic infection, this has been to a large extent due to the lack of a reliable and portable scheme for isolate characterization that enables comparisons among different studies. The *Campylobacter* MLST scheme was devised to address this problem and has been shown to be effective for the analysis of isolates from human disease and retail food (11). The analyses described here demonstrate that this scheme can be applied to isolates from the farm environment without modification, allowing direct and unambiguous comparison among *C. jejuni* isolates from a variety of sources (10, 11).

The nucleotide diversity present in the farm-derived isolates was similar to that described for human disease and retail food isolates (10) in terms of the number of alleles, nucleotide variability, and d_N/d_S ratio at each locus (Table 1). The largest degree of variability was seen at the *uncA* locus with the inclusion of allele 17, which may have originated in a different *Campylobacter* species, such as *Campylobacter coli* (11). All but two of the alleles, 16 of the 30 STs, and all of the clonal complexes identified in the farm and environment isolates had been previously described among the human disease and retail food isolates (10, 11), with the majority of alleles and STs identified in this study being commonly found among isolates on the *Campylobacter* PubMLST database.

The data were consistent with the idea that particular genotypes, indicated by clonal complex, are associated with given host sources, as suggested previously by using MLST, serotyping, and pulsed-field gel electrophoresis (10, 14, 28, 40). Isolates belonging to the ST-45 clonal complex were dominant among turkey and broiler chick samples and were absent from sheep and lamb samples. In contrast, ST-61 and ST-42 complexes were dominant among sheep isolates but were absent from poultry isolates: similar trends have been reported for retail food sources (10). The ST-21 complex appeared to have a wide distribution; this clonal complex probably corresponds to the large stable cluster of isolates capable of colonizing a wide range of hosts identified with other techniques (14, 28).

Several other observations concerning the distribution of clonal complexes among isolation source, while based on smaller numbers of isolates, warrant further investigation. Wild birds have been implicated in spreading infection in the farm environment on numerous occasions, although the extent of their contribution is as yet unknown (6, 9, 19, 20, 23, 37). The results from this investigation suggest that a range of *C. jejuni* genotypes may be prevalent in wild birds. Starlings were found to carry *C. jejuni* belonging to STs from complexes associated with poultry and environment sources as well as the ST-21 complex; however, none of the complexes potentially associated with cattle or sheep was present. This could be a reflection of host adaptation, behavioral patterns of the birds, or small sample size. A larger number of clonal complexes were represented in animals at slaughter than in the other animal groups, perhaps reflecting different sampling sites or contamination within the slaughterhouse. Differences in clonal complex distribution between both adult cattle and calves and adult sheep and lambs could reflect the different conditions and farming practices in which the animal age groups are kept or could reflect host immunological maturity (20, 21, 32, 33).

Of the 30 STs, 8 accounted for 73% (82 out of 112) of the isolates, implying that these genotypes may be particularly stable. Possible explanations of such stability remain to be determined but could reflect stabilizing selection imposed by, for example, niche adaptation. Members of the ST-21 complex in particular might be well adapted for long-term survival given their apparently ubiquitous distribution. This idea was further supported by the observation that they were one of only two genotypes isolated from slurry to which a potentially large number of different genotypes were added on a regular basis (34). With the exception of ST-21 complex, the most common genotype in each of the major clonal complexes was the central ST, which is usually the most common ST among the clonal complexes in the *Campylobacter* PubMLST database. In contrast to isolate data available in the database, ST-262 was the most common ST-21 complex genotype in this study, found among slurry, sheep, and cattle isolates, while ST-53 isolates, the second most common type in the complex, were present among calves, sheep, chicks, and starlings. There were 12 (10% of the total number of isolates) STs unique to this investigation, which all differed from the central genotype of the clonal complex at one or two loci. These could be specific host-adapted STs but are more likely to represent recent variants from the persistent central genotypes.

A number of studies have indicated that isolates from human disease and farm animals are very similar (12, 14–16, 19). In the largest MLST study of human disease published to date, six clonal complexes accounted for more than 60% of human disease isolates, namely, the ST-21, ST-45, ST-206, ST-61, ST-48, and ST-257 complexes (10). The same clonal complexes were also predominant among retail meat isolates in the *Campylobacter* PubMLST database, although the frequency of each varies according to isolation source. These clonal complexes were all present among the farm and environmental isolates. Further, while the two isolate collections shared many polymorphisms, there were no fixed differences between them. These observations are consistent with the farm populations of *C. jejuni* being a source for food contamination and human infection. The slightly different clonal complex composition of the human disease and farm isolate collections was reflected by the nonzero values of pairwise F_{ST} values for most loci and for the concatenated sequences. This is consistent with other populations of *C. jejuni* contributing to human disease. The high degree of discrimination possible with such analyses presents the prospect of employing MLST data to improve estimates of the contribution of different *Campylobacter* populations to the human disease burden.

ACKNOWLEDGMENTS

This work was supported by contract number OZ0604 from the United Kingdom Department of Environment, Food, and Rural Affairs in collaboration with Eric Bolton, David Wareing, Andrew Fox,

and Roisin Ure of the Public Health Laboratory Service, North West group.

This work made use of the *Campylobacter* PubMLST database, located at http://campylobacter.mlst.net/, developed by Man-Suen Chan and Keith Jolley, University of Oxford.

REFERENCES

1. **Anonymous.** 2001. Trends in selected gastrointestinal infections—2001. CDR Wkly. **11:**1–2.
2. **Boer, P., J. A. Wagenaar, R. P. Achterberg, J. P. Putten, L. M. Schouls, and B. Duim.** 2002. Generation of *Campylobacter jejuni* genetic diversity in vivo. Mol. Microbiol. **44:**351–359.
3. **Bolton, F. J., S. B. Surman, K. Martin, D. R. A. Wareing, and T. J. Humphrey.** 1999. Presence of *Campylobacter* and *Salmonellae* in sand from bathing beaches. Epidemiol. Infect. **122:**7–13.
4. **Bolton, F. J., D. R. A. Wareing, M. B. Skirrow, and D. N. Hutchinson.** 1992. Identification and biotyping of campylobacters, p. 151–161. *In* R. G. Board, D. Jones, and F. A. Skinner (ed.), Identification methods in applied and environmental microbiology. Blackwell Scientific Publications Ltd., London, United Kingdom.
5. **Broman, T., H. Palmgren, S. Bergstrom, M. Sellin, J. Waldenstrom, M. L. Danielsson-Tham, and B. Olsen.** 2002. *Campylobacter jejuni* in black-headed gulls (*Larus ridibundus*): prevalence, genotypes, and influence on *C. jejuni* epidemiology. J. Clin. Microbiol. **40:**4594–4602.
6. **Chuma, T., S. Hashimoto, and K. Okamoto.** 2000. Detection of thermophilic *Campylobacter* from sparrows by multiplex PCR: the role of sparrows as a source of contamination of broilers with *Campylobacter*. J. Vet. Med. Sci. **62:**1291–1295.
7. **Coker, A. O., R. D. Isokpehi, B. N. Thomas, K. O. Amisu, and C. L. Obi.** 2002. Human campylobacteriosis in developing countries. Emerg. Infect. Dis. **8:**237–244.
8. **Corry, J. E., and H. I. Atabay.** 2001. Poultry as a source of *Campylobacter* and related organisms. J. Appl. Microbiol. **90:**96S–114S.
9. **Craven, S. E., N. J. Stern, E. Line, J. S. Bailey, N. A. Cox, and P. Fedorka-Cray.** 2000. Determination of the incidence of *Salmonella spp.*, *Campylobacter jejuni*, and *Clostridium perfringens* in wild birds near broiler chicken houses by sampling intestinal droppings. Avian Dis. **44:**715–720.
10. **Dingle, K. E., F. M. Colles, R. Ure, J. Wagenaar, B. Duim, F. J. Bolton, A. J. Fox, D. R. A. Wareing, and M. C. J. Maiden.** 2002. Molecular characterisation of *Campylobacter jejuni* clones: a rational basis for epidemiological investigations. Emerg. Infect. Dis. **8:**949–955.
11. **Dingle, K. E., F. M. Colles, D. R. A. Wareing, R. Ure, A. J. Fox, F. J. Bolton, H. J. Bootsma, R. J. L. Willems, R. Urwin, and M. C. J. Maiden.** 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J. Clin. Microbiol. **39:**14–23.
12. **Duim, B., T. M. Wassenaar, A. Rigter, and J. Wagenaar.** 1999. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl. Environ. Microbiol. **65:**2369–2375.
13. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. USA **99:**7687–7692.
14. **Fitzgerald, C., K. Stanley, S. Andrew, and K. Jones.** 2001. Use of pulsed-field gel electrophoresis and flagellin gene typing in identifying clonal groups of *Campylobacter jejuni* and *Campylobacter coli* in farm and clinical environments. Appl. Environ. Microbiol. **67:**1429–1436.
15. **Hanninen, M. L., P. Perko-Makela, A. Pitkala, and H. Rautelin.** 2000. A three-year study of *Campylobacter jejuni* genotypes in humans with domestically acquired infections and in chicken samples from the Helsinki area. J. Clin. Microbiol. **38:**1998–2000.
16. **Hudson, J. A., C. Nicol, J. Wright, R. Whyte, and S. K. Hasell.** 1999. Seasonal variation of *Campylobacter* types from human cases, veterinary cases, raw chicken, milk and water. J. Med. Microbiol. **87:**115–124.
17. **Huson, D. H.** 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics **14:**68–73.
18. **Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden.** 2001. Sequence type analysis and recombinational tests (START). Bioinformatics **17:**1230–1231.
19. **Jones, K.** 2001. The *Campylobacter* conundrum. Trends Microbiol. **9:**365–366.
20. **Jones, K.** 2001. Campylobacters in water, sewage and the environment. J. Appl. Microbiol. **30:**68S–79S.
21. **Jones, K., S. Howard, and J. S. Wallace.** 1999. Intermittent shedding of thermophilic *Campylobacters* by sheep at pasture. J. Appl. Microbiol. **86:**531–536.
22. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
23. **Petersen, L., E. M. Nielsen, J. Engberg, S. L. On, and H. H. Dietz.** 2001. Comparison of genotypes and serotypes of *Campylobacter jejuni* isolated from Danish wild mammals and birds and from broiler flocks and humans. Appl. Environ. Microbiol. **67:**3115–3121.
24. **Riordan, T., T. J. Humphrey, and A. Fowles.** 1993. A point source outbreak of *Campylobacter* infection related to bird-pecked milk. Epidemiol. Infect. **110:**261–265.
25. **Rozas, J., and R. Rozas.** 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics **15:**174–175.
26. **Santamaria, J., and G. A. Toranzos.** 2003. Enteric pathogens and soil: a short review. Int. Microbiol. **6:**5–9.
27. **Schneider, S., D. Roessli, and L. Excoffer.** 2000. Arlequin version 2.000: a software for population genetic data analysis. University of Geneva, Geneva, Switzerland.
28. **Schouls, L. M., S. Reulen, B. Duim, J. A. Wagenaar, R. J. Willems, K. E. Dingle, F. M. Colles, and J. D. Van Embden.** 2003. Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: strain diversity, host range, and recombination. J. Clin. Microbiol. **41:**15–26.
29. **Staden, R.** 1996. The Staden sequence analysis package. Mol. Bio/Technol. **5:**233–241.
30. **Stanley, K., R. Cunningham, and K. Jones.** 1998. Isolation of *Campylobacter jejuni* from groundwater. J. Appl. Microbiol. **85:**187–191.
31. **Stanley, K. N., and K. Jones.** 1998. High frequency of metronidazole resistance among strains of *Campylobacter jejuni* isolated from birds. Lett. Appl. Microbiology. **27:**247–250.
32. **Stanley, K. N., J. S. Wallace, J. E. Currie, P. J. Diggle, and K. Jones.** 1998. The seasonal variation of thermophilic campylobacters in beef cattle, dairy cattle and calves. J. Appl. Microbiol. **85:**472–480.
33. **Stanley, K. N., J. S. Wallace, J. E. Currie, P. J. Diggle, and K. Jones.** 1998. Seasonal variation of thermophilic campylobacters in lambs at slaughter. J. Appl. Microbiol. **84:**1111–1116.
34. **Stanley, K. N., J. S. Wallace, and K. Jones.** 1998. Thermophilic campylobacters in dairy slurries on Lancashire farms: seasonal effects of storage and land application. J. Appl. Microbiol. **85:**405–409.
35. **Urwin, R., and M. C. Maiden.** Multi-locus sequence typing—a tool for global epidemiology. Trends Microbiol., in press.
36. **Van Camp, G., S. Chapelle, and R. De Wachter.** 1993. Amplification and sequencing of variable regions in bacterial 23S ribosomal RNA genes with conserved primer sequences. Curr. Microbiol. **27:**147–151.
37. **Waldenstrom, J., T. Broman, I. Carlsson, D. Hasselquist, R. P. Achterberg, J. A. Wagenaar, and B. Olsen.** 2002. Prevalence of *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter coli* in different ecological guilds and taxa of migrating birds. Appl. Environ. Microbiol. **68:**5911–5917.
38. **Wallace, J. S., K. N. Stanley, J. E. Currie, P. J. Diggle, and K. Jones.** 1997. Seasonality of thermophilic *Campylobacter* populations in chickens. J. Appl. Microbiol. **82:**219–224.
39. **Wallace, J. S., K. N. Stanley, and K. Jones.** 1998. The colonisation of turkeys by thermophilic campylobacters. J. Appl. Microbiol. **85:**224–230.
40. **Wareing, D. R. A., F. J. Bolton, A. J. Fox, P. A. Wright, and D. L. A. Greenway.** 2002. Phenotypic diversity of *Campylobacter* isolates from sporadic cases of human enteritis in the UK. J. Appl. Microbiol. **92:**502–509.
41. **Wassenaar, T. M., and D. G. Newell.** 2000. Genotyping of *Campylobacter* species. Appl. Environ. Microbiol. **66:**1–9.