Diarrhoeal disease in children under the age of five in Ho Chi Minh City, Vietnam

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Abstract

The focus of the global diarrhoeal disease burden is in low and middle-income countries, where the disease epidemiology and aetiology is highly variable and not well characterised. The aim of this thesis was to challenge the knowledge gaps regarding diarrhoeal disease in children under the age of five in Ho Chi Minh City (HCMC), Vietnam. Firstly, a pilot surveillance in southern Vietnam demonstrated a preponderance of enteric viruses in hospitalised diarrhoeal children and reported the first rotavirus G12 in Vietnam; despite being geographically disproportional distributed, rotavirus (RoV) predominated followed by norovirus (NoV). On the basis of these data, a prospective multi-centre hospital-based surveillance was conducted in HCMC to study diarrhoeal disease in detail and investigate the extent and the epidemiology of the hypothesized NoV emergence. Faecal specimens from diarrhoea patients and diarrhoea-free children were screened for a panel of pathogens; RoV was again identified as the predominant agent, followed by NoV. Enteric bacteria were found at smaller proportions, and exhibited excessive antimicrobial resistance. As NoV was found to be highly endemic and a major cause of hospitalisation, a risk factor analysis for NoV infections was performed. Risk factors included young age, residential crowding and contact with symptomatic individuals. Additional analysis on the phylogenetic structure of NoV strains demonstrated diverse genotypes circulating, most commonly belonging to the GII.4 lineage. A spatiotemporal analysis of GII.4 variants, GII.4-2006b (Minerva) and the novel emergent GII.4-2010 (New Orleans), suggested a strain replacement phenomenon and detected a cluster of GII.4-2010 in the northeastern part of the city. These studies indicate prominent disease dynamics involved rapid evolution of viruses, necessitate studies on strain distribution and genomic analyses and potential source additionally contributing to genetic variations (animal reservoirs), and suggest considerable impact of RoV and NoV immunisation in Vietnam.

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Although words won't be enough to express my gratitude, I would like to thank and fully and whole-heartedly dedicate this thesis to my family, particularly my mom and my dear sister who have always been by my side and effortlessly supporting me for whoever I am, whatever I do and wherever I am!

Declaration

I would like to declare that the majority of the work presented in this thesis is my own and was conducted under the supervision of Dr Stephen Baker at the Oxford University Clinical Research Unit (OUCRU), Vietnam. Sample size in the multi-centre hospital-based surveillance (Chapter 4) was calculated with the help of Dr Marcel Wolbers (OUCRU Vietnam). Additionally, Ms Corinne Thompson (OUCRU Vietnam) has assisted in statistical analyses including risk factor analysis (Chapter 5) and SaTScan (Chapter 6). Moreover, the phylogenetic analyses of rotavirus (Chapter 3) and BEAST analysis of norovirus (Chapter 6) were constructed and interpreted with the instruction and advice from Dr Maia Rabaa and Prof. Edward C. Holmes (Pennsylvania State University, US), and from Dr Maciej Boni and Lam (OUCRU Vietnam), respectively. Other co-authors in my published papers related to my thesis are study clinicians and nurses at collaborating hospitals for patient enrolment, and microbiology lab staff at OUCRU for clinical sample receipt and initial processing. This thesis has not been submitted for a degree or other qualification to this or any other university.

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ABBREVIATIONS

95 % CI	95 % Confidence Interval
AdV	Enteric adenovirus
AMP	Ampicillin
AOR	Adjusted Odds Ratio
AstV	Astrovirus
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
AUG	Augmentin (Amoxicillin-Clavulanic acid)
AZT	Azithromycin
BA	Blood agar
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAZ	Ceftazidime
CAZ-CLA	Ceftazidime-Clavulanic acid
cDNA	Complementary DNA
CDS	coding sequences
CH1	Children’s Hospital 1
CH2	Children’s Hospital 2
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute

CN	Gentamicin
CRF	Case Report Form
CRO	Ceftriaxone
CTX	Cefotaxime
CTX-CLA	Cefotaxime-Clavulanic acid
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DT	Dong Thap province
DTCS	Dye terminator cycle sequencing
DTPH	Dong Thap Provincial Hospital
E. coli	*Escherichia coli*
ESBL	Extended-spectrum β-lactamase
GA	Gatifloxacin
GIS	Geographic Information System
GPS	Global Positioning System
HTD	The Hospital for Tropical Diseases
HCMC	Ho Chi Minh City
ICF	Informed Consent Form
ICTV	International Committee on Taxonomy of Viruses
IQR	Inter-quartile Range
KIA	Kliger iron agar
kp	kilobase pair

LB	Luria-Bertani
MC	MacConkey Agar
MIC	minimum inhibitory concentration
MDR	multi-drug resistance
MH	Mueller-Hinton
NA	Nalidixic acid
NCBI	National Centre for Biotechnology Information
NoV	Norovirus
NPV	Negative Predictive Value
NSP	Non-structural protein
OD	Optical Density
OFX	Ofloxacin
OR	Odds Ratio
ORF	Open reading frame
OUCRU	Oxford University Clinical Research Unit
OxTREC	Oxford Tropical Research Ethics Committee
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIS	Patient Information Form
PPV	Positive Predictive Value
RoV	Rotavirus
RT	Reverse Transcription

RT PCR	Reverse Transcription Polymerase Chain Reaction
Spp.	Species
SXT	Trimethoprim-sulfamethoxazole
Tm	melting temperature
UTR	Untranslated Region
VP	Viral proteins
WHO	World Health Organisation
XLD	Xylose-Lysine-Deoxycholate Agar

PUBLICATIONS DURING DPhil CANDIDATURE

1. **My PV**, Hoang NVM, Thompson C, Minh PV, Vinh NT, Thuy CT, Nga TTT, Chau NTS, Rabaa MA, Duy PT, Dung TTN, Phat VV, Phuc HL, Tuyet PTN, Hau NTT, Vinh H, Chinh NT, Thuong TC, Tuan HM, Campbell JI, Chau NVV, Hien TT, Farrar JJ, and Baker S. *A yearlong descriptive investigation of hospitalized pediatric diarrhea in Ho Chi Minh City, Vietnam*. Submitted for publication at PlosOne. 2013 Feb.
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1. INTRODUCTION

1.1. Diarrhoeal Disease

1.1.1. Global burden

Diarrhoeal disease presents a major challenge to global public health. Young children and infants in under-developed and developing countries are disproportionately affected, and diarrhoeal disease in such areas is often severe and frequently requires hospitalisation [1]. Severe cases may lead to dehydration, secondary complications, or death, without proper management and treatment. The global estimates of the annual deaths associated with diarrhoea in children under the age of five years reflect a declining trend, ranging between 1.76 to 1.87 million deaths in 2000-2004 [2-4], to approximately 1.34 million deaths in 2008 [5], and to the most recent estimate of around 800,000 deaths in 2010 [6]. In those estimated mortality figures, diarrhoea remains as the second leading cause of neonatal and child deaths, with a great proportion allocated in the deprived areas of Africa and Asia. In 2010, among 7.6 millions deaths due to all causes in children < 5 years of age, diarrhoea contributed to approximately 11 % of the overall childhood mortality (Figure 1-1) [6].

Globally, diarrhoeal disease is estimated to cause 2.5 billion cases annually in children under 5 years of age [7]; the disease burden in such age groups represents 90 % of the total burden of diarrhoeal infections [8]. These data suggest that every child in this key age group experiences three episodes of diarrhoea per year on average (range 2.8 - 6.3) [9]. A substantial proportion (> 80 %) of morbidity and mortality occurs in children in sub-Saharan Africa and Southeast Asia [4-7, 10]. Although the disease mortality has shown a decline over the past few decades, particularly in

developing countries, a concurrent decrease in overall disease morbidity has not occurred; thus, diarrhoeal disease remains one of the principal causes of childhood morbidity and mortality worldwide [5, 6, 9, 11].

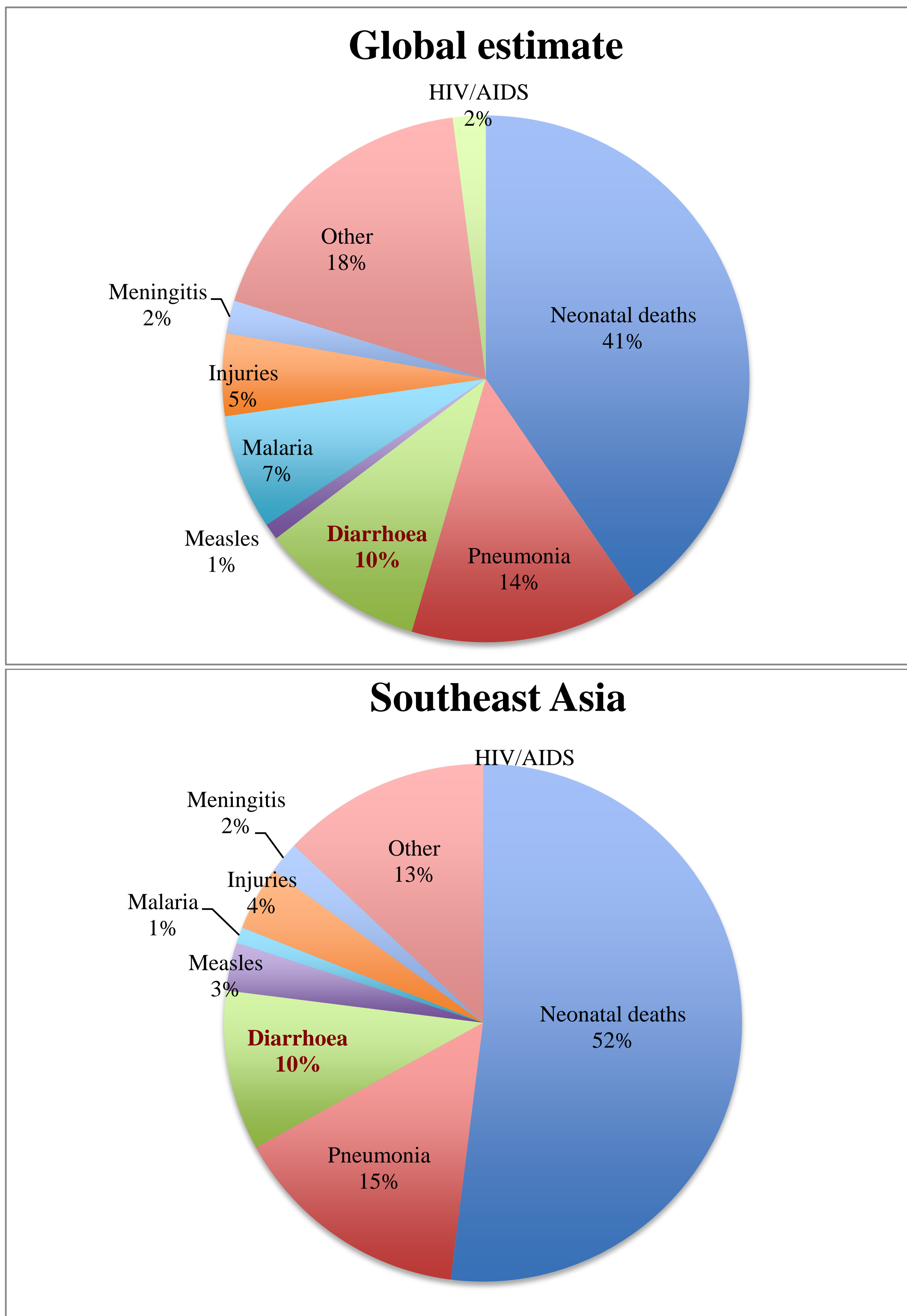


Figure 1-1. The major causes of death in children under the age of five years globally and in Southeast Asia in 2010

Source: Adapted from [6]. Among estimated neonatal deaths only attributed to all causes, diarrhoeal disease accounted for 1 % in each estimate for Southeast Asia and global. Major causes indicate postnatal causes while neonatal deaths refer to mortality in neonates.

1.1.2. Disease aetiology

The aetiological agents of diarrhoea are numerous and comprise several viral, bacterial and parasitic pathogens. The prevalence and relative importance of enteric pathogens differ geographically and temporally, and are influenced by socio-economic status, which is directly related to hygiene and sanitation. In developed countries, the majority of diarrhoeal infections are caused by viral pathogens, while in developing areas with poor hygiene and sanitation standards, enteric bacteria and parasites are more prevalent than enteric viruses as reviewed in [11].

Overall, viruses are responsible for the substantial burden of morbidity and mortality, comprising more than half of all global gastroenteritis cases and up to 40 % of all severe diarrhoea cases in developing countries [12]. The most prevalent viruses causing endemic childhood gastroenteritis are rotavirus (RoV), norovirus (NoV), enteric adenovirus group F type 40/41 (AdV), astrovirus (AstV), and sapovirus (SaV); among which RoV and NoV are the two most commonly identified agents [13-15].

Children in developing countries are often exposed to pathogenic enteric bacteria at a very young age, and the predominant bacterial pathogens vary by the age of the child, by time, and by geography [11]. Commonly identified diarrhoea-causing bacteria in young children include *Shigella* spp., non-typhoidal *Salmonella* spp., *Campylobacter* spp., diarrhoeagenic *Escherichia coli* (*E.coli*), and to a lesser extent *Yersinia enterocolitica*, *Aeromonas hydrophila*, and *Plesiomonas shigelloides* [11, 16].

Parasitic pathogens are generally less prevalent than viral and bacterial pathogens in diarrhoeal infections, and the prevalence of parasitic infections is high

in economically deprived areas, including many tropical countries [12]. Common diarrhoeal parasites include *Giardia lamblia*, *Entamoeba histolytica,* and *Cryptosporidium* spp. [11, 12]. *Giardia lamblia* is the most common intestinal parasite causing diarrhoeal disease worldwide [17], while *Cryptosporidium* is more prevalent in HIV/AIDS or immunocompromised patients [18] and immunocompetent malnourished children in developing countries [12].

Data from previous surveillance of the prevalence and epidemiologic importance of diarrhoeal pathogens have been challenged by Levine *et al.* [19], mostly concerning limitations in the study settings and diagnostic methods used. In that light, a prospective multi-national, age-stratified, case-control surveillance has currently been being performed to determine the population-based incidence, epidemiologic and aetiological profiles, and clinical characteristics and adverse outcomes of acute moderate-to-severe diarrhoea in children under five in 7 countries in sub-Saharan Africa and South Asia [19-21]. Using the state-of-art microbiological diagnostic methods, this largest case-control study, when completed, would generate the most comprehensive landscape of diarrhoeal disease and provide accurately defined enteric pathogens associated with childhood diarrhoea in developing countries.

1.1.3. Clinical manifestations

Acute diarrhoea manifests as an increase in the frequency and/or volume of stools, and typically lasts for less than two weeks while persistent diarrhoea lasts for more than 14 days [22]. Bloody stools may occur in infections caused by invasive pathogens. Fever is a common clinical presentation and is usually associated with

invasive enteric bacterial pathogens including *Shigella* spp., non-typhoidal *Salmonella* spp., and *Campylobacter* spp. Vomiting occurs more frequently in viral gastroenteritis than in infections caused by enteric bacteria and parasites. The clinical characteristics of selected viral, bacterial and parasitic infections are summarised in Table 1-1. Diagnosis of enteric pathogens based solely on clinical manifestations is generally insufficient to rapidly distinguish between viral, bacterial and parasitic pathogens, or to predict the clinical outcome.

Table 1-1. Typical clinical characteristics of selected enteric pathogens causing acute diarrhoea in young children

Pathogen	Type	Clinical characteristics					Reference
		Fever	Abdominal pain	Bloody stool	Vomiting/ nausea	Durations	
RoV	dsRNA virus	Common	Common	NC	Common	3-8 days	[17, 23]
NoV	(+)ssRNA virus	Variable	Common	NC	Common	1-3 days	[17, 23]
Vibrio cholera	Gram(-) bacteria	Variable	Variable	Variable	Variable	3-4 days	[17, 23]
Shigella spp.	Gram(-) bacteria	Common	Common	Occurs	Common	5-7 days	[17, 23]
Salmonella spp.	Gram(-) bacteria	Common	Common	Occurs	Occurs	4-7 days	[17, 23, 24]
Campylobacter spp.	Gram(-) bacteria	Common	Common	Occurs	Occurs	2-10 days	[17, 23, 25]
Giardia lamblia	Protozoan	NC	Common	NC	Occurs	2-6 weeks	[17]
Entamoeba histolytica	Protozoan	Occurs	Occurs	Variable	Variable	Weeks to months	[17]
Cryptosporidium	Protozoan	Variable	Variable	NC	Occurs	Weeks	[23, 26, 27]

NC: Not Characteristic

1.1.4. Disease management and prevention

There are two main approaches for the clinical management of diarrhoeal disease. For acute watery diarrhoea (no blood in stools), case management involves (1) prevention/treatment of dehydration by fluid replacement (oral/intravenous rehydration therapy), (2) reduction of disease duration and severity by zinc supplementation and (3) prevention of nutritional damage by continuing feeding [22]. A timely and accurate assessment of dehydration status is, therefore, essential for effective management of diarrhoeal morbidity. The use of antimicrobials is controversial, having potential benefits associated with reducing the effect of bacterial pathogens. However, antimicrobials should not be routinely administered to treat acute diarrhoeal disease as most infections are self-limiting and inappropriate use may promote antimicrobial resistance in commensal organisms [22, 28]. Yet, for acute bloody diarrhoea or severe cases of diarrhoea caused by invasive pathogens, in addition to the aforementioned three-point management, a course of antimicrobial treatment should be prescribed, preferentially with fluoroquinolones, such as ciprofloxacin [22].

A large portion of diarrhoeal disease is preventable through improvements of sanitation and hygiene, provision of safe water and food, and micronutrient supplementation. Specific vaccines for diarrhoeal pathogens are currently limited to RoV as despite on-going research and development, there are, currently, no effective vaccines for pathogens such as NoV, *Shigella*, *Campylobacter* and non-typhoidal *Salmonella* species. The commercially available RoV vaccines have been shown to be safe and efficacious against RoV gastroenteritis and are now implemented into the national immunisation programs in a number of countries [29]. Cost adjustment for a

wider implementation of the vaccines in developing areas (Africa and Asia) is essential to decrease the global RoV burden [29].

1.2. Viral diarrhoea

1.2.1. Rotavirus

1.2.1.1. Biology

RoV was first identified as a cause of diarrhoea by electron microscopy (EM) in 1973 from an intestinal biopsy of a child with diarrhoea [30]. A year later, 'rotavirus' was suggested as a name for the virus, indicative of its wheel-like shape under the EM, and this was officially approved in 1978 by the International Committee on Taxonomy of Viruses (ICTV) [31, 32].

RoV forms a genus *Rotavirus* belonging to the *Reoviridae* family of viruses. The virion possesses an icosahedral structure of 65 to 75 nm (as determined by conventional EM) in size. The non-enveloped triple-layered virus shelters a genome of 11 independent double-stranded RNA segments (Table 1-2). Each RNA segment contains a single open reading frame (ORF) encoding for one protein, expressing six structural viral proteins (VPs) and five non-structural proteins (NSPs) (Figure 1-2), with an exception of segment 11 in some group A viruses coding for another protein (NSP6) in addition to NSP5 protein [31]. The 5' and 3' terminals of each RNA segment consist of a short conserved untranslated region (UTR). The sequence diversity found among these conserved UTRs in group A, B and C viruses is likely to restrict genetic reassortment between viruses of different groups [31].

Each of the structural proteins (designated as VP1-4, VP6 and VP7) and non-structural proteins (designated as NSP1-6) plays an essential role in virus replication,

propagation and infection [31]. VP1, VP2 and VP3 play essential roles in viral RNA replication, forming the innermost layer of the triple layer virion. VP4 forms a spike protein on the surface of the virion, determining viral growth, virulence and protein attachment. VP4 cleaves into VP8* and VP5 (Table 1-2) in the presence of trypsin, enhancing viral infectivity. VP6, the major structural protein, forms the inner viral capsid (middle layer of the virion). This protein is highly immunogenic and contains group- and subgroup-specific epitopes. VP7 is a glycosylated protein that forms the outermost layer of the virion together with the VP4 protein (Figure 1-2). Both VP7 and VP4 are the targets for neutralising antibodies and are used for the identification of genotypes (see section 1.2.1.2 below) [31].

Non-structural NSP1 protein has a role in host-specific interferon suppression that may have an effect on host range restriction [33-35], and has the greatest sequence diversity of all RoV proteins. NSP2 functions to catalyse the packaging of viral mRNA into core-like replication intermediates and binds to NSP5 and VP1 proteins. NSP3 is involved in translational regulation. NSP4 is the RoV enterotoxin and was the first putative viral enterotoxin identified [36], yet its role in inducing diarrhoea in humans remain unclear [31]. NSP5 is suggested to play a role in viral replication. In some RoV strains, NSP6 is the second protein product of segment 11, which interacts with NSP5 protein and hence may play a role in viral replication [31].

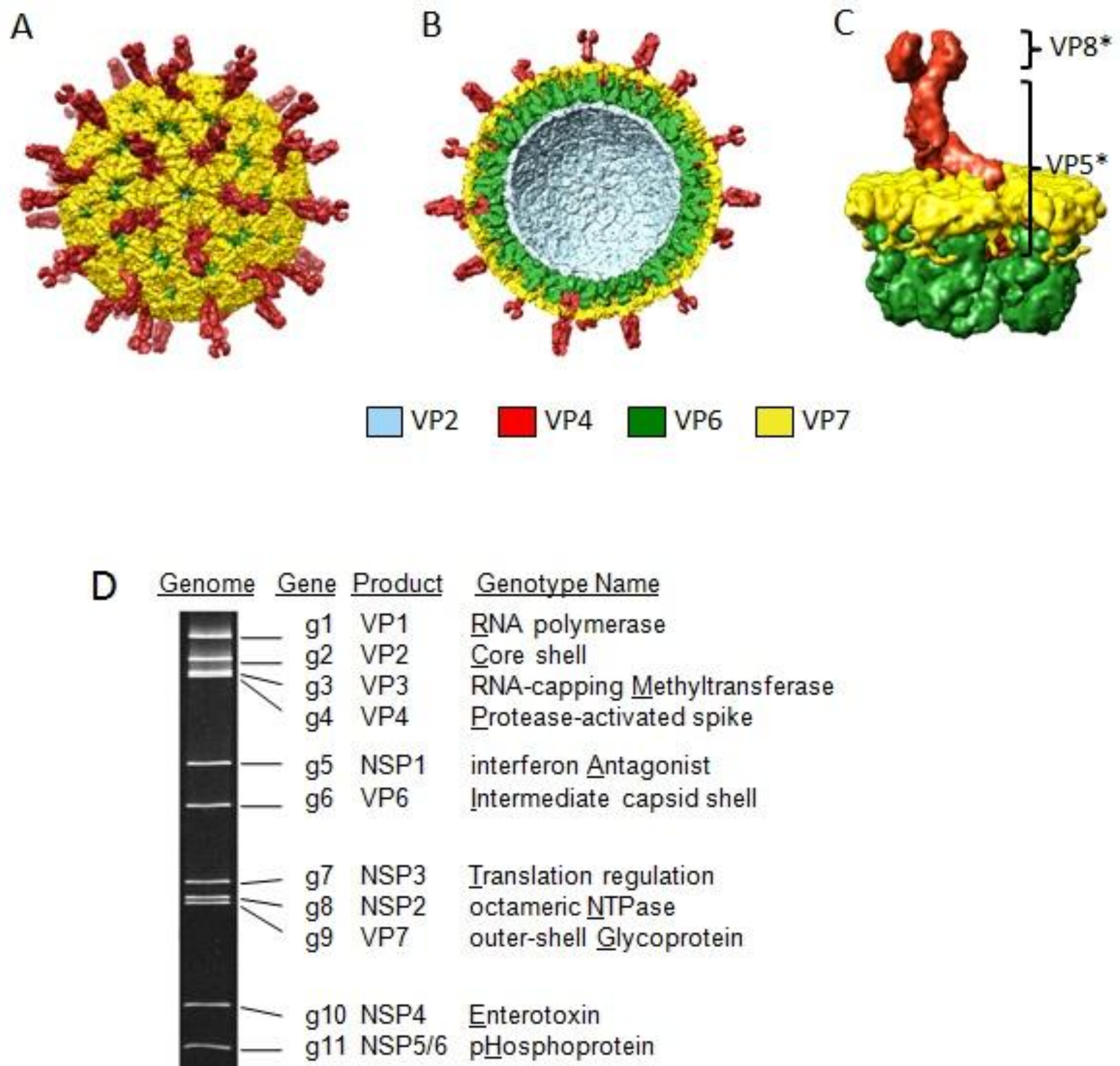


Figure 1-2. The structural organization of RoV virion

Source: [37]

(A) Exterior view of the triple-layered virion with VP4 (red) spikes extruding from the VP7 (yellow) outer capsid shell.

(B) The cross-sectional view of the virion showing three shell layers: outer capsid (VP4 and VP7), inner capsid (VP6) and core layer (VP2).

(C) Two components of VP4 spike: protruding VP8* and elongated VP5.

(D) Double-stranded RNA segments of the genome, separated by size on a polyacrylamide gel. Segment numbers are labelled as g1-g11, and corresponding proteins with associated functions.

1.2.1.2. Classification

Viruses within the genus *Rotavirus* are classified into eight groups (serogroups) based on their antigenic and genetic characteristics of the internal structural protein VP6 [38]; groups are indicated by letters from A to G [32] as well as the recently identified group H [38]. To date, viruses in groups A, B, C (and H) are known to cause disease in both humans and a variety of mammalian and avian species while viruses in other groups have been found only in non-human mammals and avian species. Genetic exchange can occur among viruses within one group (of the same or different genotypes), but not among viruses belonging to different groups [39].

Group A RoV is a well-recognized cause of diarrhoeal disease in young children, responsible for more than 90 % of RoV infections worldwide [33]. Group B RoV has been found to cause outbreaks of severe diarrhoea in adults [40] and more recently in sporadic acute childhood diarrhoea [41]. Viruses in group C have been sporadically found in children [42] and adults [43] with diarrhoea. The epidemiological distribution of these group B and group C viruses are poorly defined [33]. Group A RoV bears the most important epidemiologic role in childhood diarrhoea globally and is, inarguably, the most important group of the genus *Rotavirus* in human infections; hence this group of viruses is the focus for the subsequent sections and is interchangeably referred hereafter as RoV unless otherwise noted.

Viruses in group A RoV are categorized using the following schemes [31, 33]:

i. serotypes: subgroups, G-serotypes, P-serotypes based on the antigenic properties of VP6, VP7 and VP4, respectively;
ii. long, short, supershort or atypical electropherotypes based on the migration pattern of RNA segments by polyacrylamide gel electrophoresis (segregation on segment size and weight);
iii. genogroups based on whole-genome RNA hybridization patterns; for example, 3 common human genogroups: Wa, DS-1, AU-1;
iv. genotypes based on nucleotide sequence analysis of two outer capsid proteins, VP7 (G genotypes) and VP4 (P genotypes), hence forming a dual classification system.

The current RoV classification system is recommended by the Rotavirus Classification Working Group (RCWG) performed through the online tool RotaC [44]. This system is based on the sequence homology of the ORF of each gene segment, in addition to the common dual genotyping system for VP7 and VP4, to entail the complete genome constellation of each RoV strain [45]. Particularly, the nomenclature Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx represents the genotypes of segments encoding VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5, respectively (Table 1-2). A total of 27 G-types and 35 P-types (Table 1-2) have been defined for human and animal RoV [45], and at least 73 GP combinations have been reported in human RoV infections [46].

Table 1-2. The genome constellation of RoV

Gene encoding	Function	Segment number	Length bp	% Nu cut-off	No. identified
VP7	**G**lycosylated	9	1062	80	27**G**
VP4	**P**rotease-sensitive	4	2362	80	35**P**
VP6	**I**nner capsid	6	1356	85	16**I**
VP1	**R**dRp	1	3302	83	9**R**
VP2	**C**ore protein	2	2690	84	9**C**
VP3	**M**ethyltransferase	3	2591	81	8**M**
NSP1	Interferon **A**ntagonist	5	1611	79	16**A**
NSP2	**N**TPase	8	1059	85	9**N**
NSP3	**T**ranslation enhancer	7	1049	85	12**T**
NSP4	**E**nterotoxin	10	751	85	14**E**
NSP5	p**H**osphoprotein	11	667	91	11**H**

% Nu cut-off: Nucleotide percentage identity cut-off values for genotype assignation for each gene segment.

Source: [45, 47, 48].

1.2.1.3. RoV evolution

Genetic diversity of the RoV genome is derived from multiple mechanisms: genome rearrangement, point mutations (drift), genetic reassortments (shift), and interspecies transmission (as reviewed in [33, 49]). Rearrangement of the RoV genome indicates partial insertions or duplications or deletions of genome segments or their ORFs; this phenomenon rarely occurs in nature, and is mostly reported within the non-structural proteins and VP6 [33, 49-51].

One of the natural properties of RNA viruses is low replication fidelity of the viral polymerase; data from *in vitro* study of RoV suggested a mean mutation rate of gene segment 11 of under $5x10^{-5}$ mutations per replicated nucleotide, comparable to other RNA viruses [52, 53]. Given biological nature of high mutation rate and size (~ 18kb genome) of RoV genome, every virus progeny genome is estimated to possess at least one mutation compared to its parental genome [33, 49]. Each gene segment may evolve at different rates due to different levels of selection pressures. Among 11 segments, genes encoding for the outer capsid proteins, VP7 and VP4, are expected to evolve at much greater rates than those encoding internal structural proteins, mostly due to host immune selection pressures [31, 33]. Data from a large-scale comparative genomic analysis demonstrated higher evolutionary rates observed in VP7 and VP4 genes, and presence of changes in the neutralising antibody binding regions potentially leading to new antigenic variants [54]. Overall, accumulation of point mutations over time may facilitate the emergence of immune escape viruses through antigenic drift [31, 33, 54].

Reassortment of gene segments can occur in a host cell experiencing mixed infection with two or more RoV strains of the same genogroup (see section 1.2.1.1 and 1.2.1.2), giving rise to a novel strain (genetic shift) [55]. The genetic

reassortment phenomenon in addition to the error-prone nature of RNA viruses via accumulation of point mutations results in increasing viral diversity and facilitate rapid genetic shift and antigenic drift [54]. The frequency of natural RoV reassortment appears to be higher in developing countries compared to developed countries [56, 57]. A higher frequency of mixed RoV infections and close contact among human, livestock and other animals may play a key role in creating greater diversity in co-circulating strains of common and uncommon genotypes or GP combinations, potentially accelerating genetic exchange and the emergence of novel strains in less developed regions [33, 56, 57].

In general, RoV from one species has been observed to be incapable of efficiently spreading and maintaining itself in another species, forming the basis for developing Jennerian RoV vaccines (RotaTeq™, RV5) [31]. Interspecies primary transmission from animals to human, albeit uncommon, have been reported in diarrhoeal children from Belgium (G9P[6] from pig to human, and G3P[14] from rabbit to human) and Israel (G6P[1] from calf to human) [50, 58, 59]. Interspecies transmission and subsequent genetic reassortment between animal and human RoV genes (among group A viruses) can increase the diversity of circulating strains, and allow reassorted virus to infect multiple host species, thus extending the host range [31, 33]. Human RoV containing gene segments originated from animal RoV have been identified in children with diarrhoea, such as bovine-related G10P[11] (India) [60], porcine-related G5 (Brazil, Cameroon and Vietnam) [61-63], and ruminant-related G8P[4] (India) [64].

1.2.1.4. Pathogenesis and clinical presentation

RoV infections in young children generally manifest as watery diarrhoea (mild to severe), low-grade fever and vomiting potentially leading to dehydration; disease is usually self-limiting and resolves within 4 to 8 days [33, 65]. The incubation period is short, generally less than 48 hours [33]. Asymptomatic RoV infection in children is common and thought to be modulated by host and viral genetic factors [66, 67].

RoV infection can be symptomatic or asymptomatic, suggesting that both the viral and host factors play a role in modulating the clinical outcome of the infection. With respect to viral factors, asymptomatic disease may be resulted from infection by some VP4 alleles that are inherently attenuated in virulence (nursery strains) [68], or by RoV strains transmitted from different host species leading to naturally attenuated viruses (host range restriction) [31, 33]. Among potential host factors, age of infected individual is probably the most important element that affects the clinical disease outcome; RoV infection in neonates is rarely symptomatic, which may be explained by the transplacental transfer of maternal antibodies [69, 70]. Malnutrition is another host factor that could lead to an increased severity in symptomatic RoV infections [71-73].

Much of our understanding of RoV disease pathogenesis is derived primarily from studies of animal models. RoV-induced diarrhoea is multifactorial and involves malabsorption and secretion pathways in addition to other components that are related to villus ischemia and intestinal motility. Malabsorptive diarrhoea is induced by a number of changes in the villus epithelium mediated by virus infection and action of RoV enterotoxin (NSP4); changes include destruction of villus enterocytes, reduced expression of digestive enzymes (lactase, sucrose, alkaline phosphatase, and

maltase), and paracellular leakage due to functional modifications in tight junctions between enterocytes [31, 36, 74-80]. The enteric nervous system (ENS) and NSP4 appear to be the key modulators of the secretory diarrhoea, through activation of cellular Cl^- channels in crypt cells leading to increased efflux/secretion of Cl^- and consequently water [36, 79-85]. Stimulation of ENS has been suggested to be triggered by NSP4 enterotoxin or the chemokines and other factors secreted from infected epithelial cells (such as 5-hydroxytryptamine) [84]. Villus ischemia and changes in intestinal motility have been described in some animal model studies, yet the significance of experimental observation and their clear role in disease in children are subjective to define in future studies [80, 86, 87]. RoV spreading to extraintestinal tissues and viraemia, albeit rare, have been reported; however, the clinical consequences of systemic infections and causation underlying extraintestinal spread of viruses remain undetermined [88-92].

1.2.1.5. RoV immunity

Understanding on natural immune mechanisms against RoV illnesses is incomplete. Despite a paucity of information from human studies, it is generally surmised that the two outer capsid proteins VP7 and VP4 independently stimulate the production of neutralising antibodies, consequently conferring host protection and resistance against RoV diarrhoea [31]. Homotypic (serotype-specific) and heretotypic (cross-reactive) immunity following RoV natural infection and vaccination has been observed; however, the mechanism and specific roles of VP7 and VP4 in inducing such complex immune response are poorly characterised [93-99].

Overall, RoV infections confer a variable level of natural immunity against subsequent infections; the first infection after neonatal period is symptomatic and provides protective immunity against reinfections [96, 98-101]. Cohort studies showed that the first RoV infection provided 43 %, 70 % and 77 % protection against subsequent RoV disease in children from Vellore, Guinea-Bissau and Mexico, respectively [98, 99, 102]. Furthermore, the protective efficacy against moderate to severe RoV-induced gastroenteritis was 100 % in Mexican children after two infections, compared with 79 % in Indian children after three infections [98, 99, 103].

RoV disease is most severe when the first RoV infection occurs in children of 3 to 24 months of age, when the circulating maternal transplacental RoV-specific antibodies (IgG) are on the wane, indicating protective effects of transplacental acquired IgG antibodies [70, 104]. A year-round RoV exposure to high environmental viral loads and diverse genotypes circulating would potentially lead to very early RoV infections, soon after the waning of acquired maternal antibodies, in the poorest to low-income countries in Africa and Asia [56, 96, 105]. If the mode of producing neutralising antibodies to RoV is age-dependent (the younger the child, the lower the antibody response [106]), such an early infection in infants may result in an inefficient protective immunity, which would in part contribute to reduced efficacy of protection induced by natural infection and vaccination in poor areas of Africa and Asia [96, 106].

1.2.1.6. Epidemiology

RoV is the dominant agent of viral diarrhoea and is the suspected aetiological agent in 39 % and 45 % of all hospital admissions related to diarrhoea globally and

in Asia, respectively [107, 108]. RoV infection is ubiquitous in young children, infecting nearly all children before they reach five years of age [1, 65, 104, 109-111]. On a global scale, RoV is responsible for approximately 111 million diarrhoeal episodes, 25 million clinic visits and 2 million hospitalisations every year in children under five [111]. This translates to an estimated risk of 1 in 5 children being infected with RoV requiring outpatient care and 1 in 65 requiring hospitalisation [111].

More recent global estimate has suggested that approximately 453,000 deaths in children under the age of five years were attributed to RoV in 2008, with an annual mortality rate of 1 in every 260 children [112]. This RoV-related mortality represents 37 % of all diarrhoeal deaths and 5 % of all-cause deaths in this key age group [112]. A major burden of global RoV-associated mortality occurs in developing regions, particularly economically deprived countries in sub-Saharan Africa and Asia [112]. Six countries from Africa and Asia contributed to nearly 60 % of global RoV-associated mortality, including India, Nigeria, Pakistan, Democratic Republic of Congo, Ethiopia, and Afghanistan, in which India alone accounts for ~ 22 % (99,000 deaths) of the global estimated RoV-related deaths [112]. The highest RoV-related mortality was observed in children under five years of age in Afghanistan (518 deaths per 100,000 children < 5 years old), compared to the estimate of < 1 death per 100,000 children of the same age group in other 63 countries [112].

RoV infections are associated with a wide range of strains circulation, among which RoV genotypes G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and recently to a lesser extent G12P[8] are currently the most commonly identified genotypes, accounting for about 90 % of human RoV infections [33, 46, 56, 113, 114]. The distribution of RoV genotypes has been shown to differ by time and by geography,

with the greatest diversity reported in developing countries [56, 57, 107]. Disparity in age distribution of RoV disease has also been reported between low and high-income countries. In particular, RoV-related hospitalisation tended to shift toward younger age children in developing countries than in developed countries [105]. The peak incidence of RoV infections in children in developing countries is estimated from 6 to 11 months of age, while higher incidence is observed in children in their second year of life in developed countries [33].

The main transmission route of RoV is faecal-oral; the virus can be transmitted readily through person-to-person contact as the virus has a low infectious dose (as low as 10 viral particles) [33]. The infections peak in the cooler months in temperate zones, while showing less clear seasonality in tropical countries [115]. Studies of RoV infection patterns showed contradictory findings; higher birth rates were found to associate with earlier timing of RoV epidemics in the United States (US) [116, 117], yet the same pattern was not observed in countries across Europe [118]. Variations in RoV infections, transmission and disease patterns are, suggestively, affected by complex interactions of multiple factors including population demographics (such as birth rates, malnutrition, low birth weight, preterm birth, socio-economic status), environmental factors and strains circulating [33, 116, 118-120].

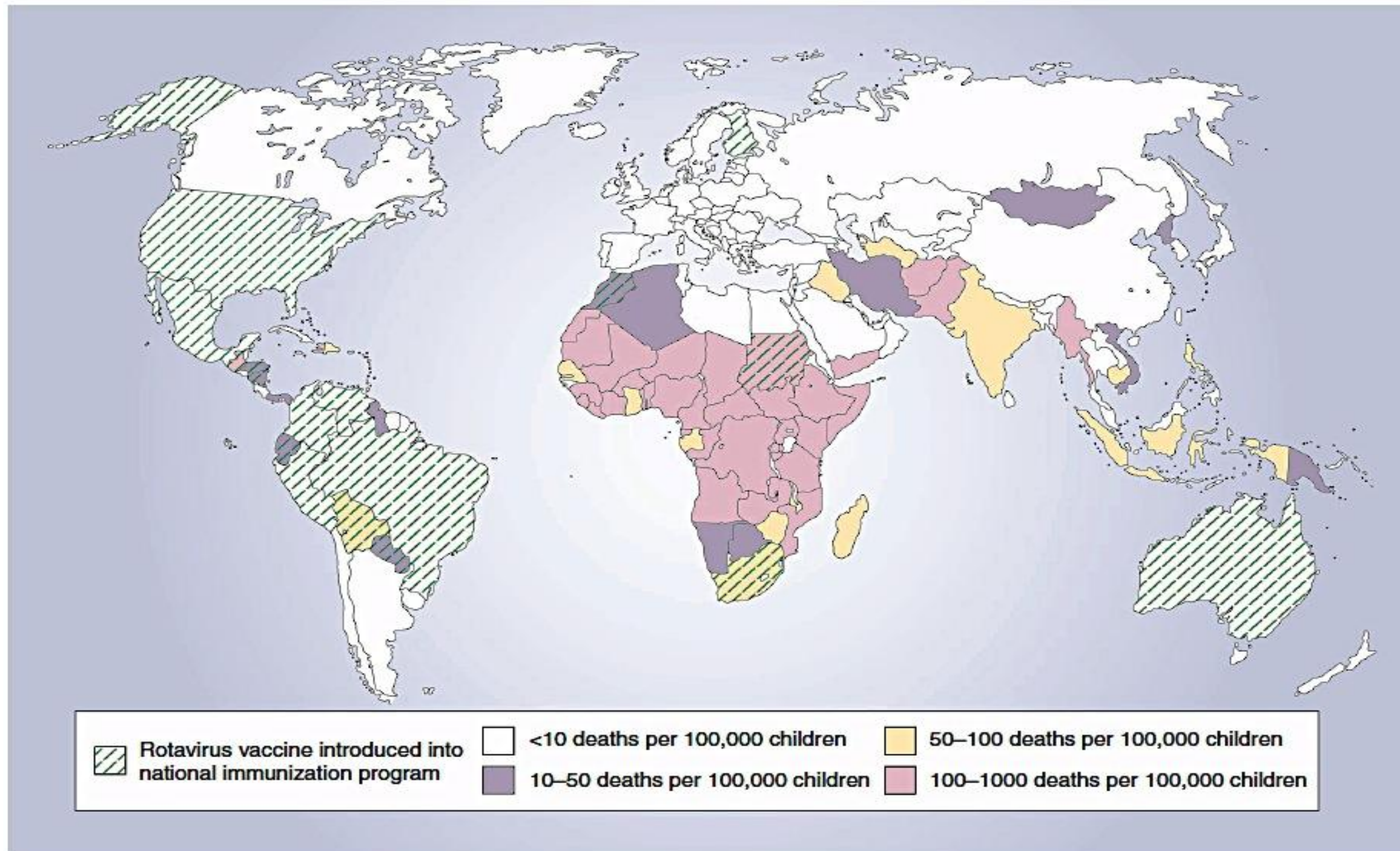


Figure 1-3. RoV mortality rates among children < 5 years of age and status of RoV vaccination program.
Source: [121]. The majority burden of RoV-related mortality occurs in developing countries in the sub-Saharan Africa and Asia regions, where RoV vaccination is not yet integrated into national childhood immunisation program.

The development of a RoV vaccine has been a protracted example of the vaccine development industry. The first RoV vaccine was RotaShield, a live tetravalent reassortant between rhesus-human strains that was licensed by the US FDA in 1998 [122]. The vaccine was withdrawn from the market after one year of launching due to observed cases of intussusception among RoV vaccinated children [123]. The licensure of two live attenuated vaccines, RotaTeq™ (RV5, Merck) and Rotarix™ (RV1, GSK), followed [124, 125], and these are the two major vaccines currently commercially available to protect against RoV. RotaTeq™ is a live pentavalent bovine-human reassortant, based on the Jennerian approach, containing strains of human G1, G2, G3, G4 and P[8] types with a G6P[5] bovine backbone, hence representing a naturally attenuated virus. This bovine-human reassortant component of RotaTeq™ virus can replicate in a human host, to a lower extent than that in cows, yet is capable of inducing a protective immune response [33]. Rotarix™ is a live-attenuated monovalent vaccine of the G1P[8] type.

Clinical trials of these vaccines demonstrate that both vaccines are safe and efficacious against RoV acute gastroenteritis (> 70 %) and severe RoV acute gastroenteritis (90 %) as well as in reducing the incidence of diarrhoea-associated hospitalisations (> 40 %) [124-126]. Both vaccines have been reported to have a lower efficacy against RoV gastroenteritis in less developed countries [127-131], suggestively attributable to multiple factors including vaccine interference by maternal antibodies [70, 132, 133], co-administration with other oral vaccines such as OPV [29], RoV infection at an earlier age [96, 106], and the pattern of RoV year-round infections with multiple genotypes circulating at high viral challenge loads [56]. Nevertheless, the vaccines may still prevent a substantial number of RoV-related deaths in economically deprived areas, i.e. cost-effective strategy, albeit with

lower overall vaccine efficacy [29, 121, 127, 134-141]. RoV vaccination has been implemented as part of the National Immunisation Program in many countries, yet is largely voluntary in many developing countries where it may be needed the most (Figure 1-3). Recent identification of RoV either with or without vaccine-derived segments in vaccinated children, albeit at very low frequency, has raised a concern toward the use of the current licensed vaccine [142, 143]. Several alternative vaccine candidates are currently being evaluated, such as the neonatal RoV strains 116E (G9P[11]) in India and RV3 (G3P[6]) in Australia [144].

1.2.2. Norovirus

1.2.2.1. Biology

NoV, previously known as human Norwalk-like virus, forms a genus *Norovirus* belonging to the *Caliciviridae* family of viruses [145]. Viruses within this genus *Norovirus* are known to cause diseases in both humans and mammalian animals [145].

NoV is a small non-enveloped RNA virus of ~ 35 nm [145]. The genome contains a polyadenylated positive-sense single-stranded RNA segment of approximately 7.5 – 7.7 kb in length [145]. The RNA genome is organised in three ORFs encoding several structural and non-structural proteins; the ORF1 and ORF2 overlap by a short region (14 – 20 bp), and ORF2 and ORF3 overlap by one (or a few) nucleotide (Figure 1-4) [145]. ORF1 is the longest ORF in the NoV genome, encoding a large polyprotein that, subsequently, is proteolytically cleaved into six non-structural proteins, including an N-terminal protein (NS1-2), an NTPase protein (NS3), a 3A-like protein (NS4), a viral protein-genome linked protein (NS5), a 3C-

like protease (3CL^{pro}, mediator of the proteolysis process) (NS6) and an RNA-dependent RNA polymerase (NS7) (Figure 1-4) [145].

ORF2 encodes a major structural protein, VP1, that functions in the processes of virus self-assembly and capsid formation, host interactions and immunogenicity [145]. The monomer VP1 protein consists of two distinct structural domains, the shell (S) and the protruding (P) domains. The N-terminal arm at 5' end of ORF2 together with the conserved S domain form the structurally interior core of the intact viral capsid. The P domain, extending from the virion surface, comprises of two sub-regions, the protruding stem P1 connecting with the S domain via a flexible hinge, and the P2 with the highest sequence diversity that forms the outermost external surface of the viral particle (Figure 1-4) [146]. This P2 domain contains motifs that may involve in interaction with the potential neutralising antibody recognition sites [147, 148] and putative host binding sites [148-151]. Although specific cellular receptor(s) facilitating NoV entry is largely undetermined, it has been identified that the P2 binds to the histo-blood group antigens (HBGAs), which are presumably preliminary binding site in initiating human infection [149-152].

The last open reading frame (ORF3) is located at 3' end of the NoV genome, encoding a minor capsid protein, VP2 (Figure 1-4). The specific role of VP2 is unclear, although its functions are thought to include assisting in viral capsid assembly and stability [153] and participating in recruiting genomic RNA into the virion [154]. Recently, an alternative ORF (ORF4) has been identified in murine NoV (MNV, see classification section 1.2.2.2 below), an alternate reading frame located within the ORF2 overlapping with the VP1-coding region, which encodes a novel virulence factor protein (VF1) [155].

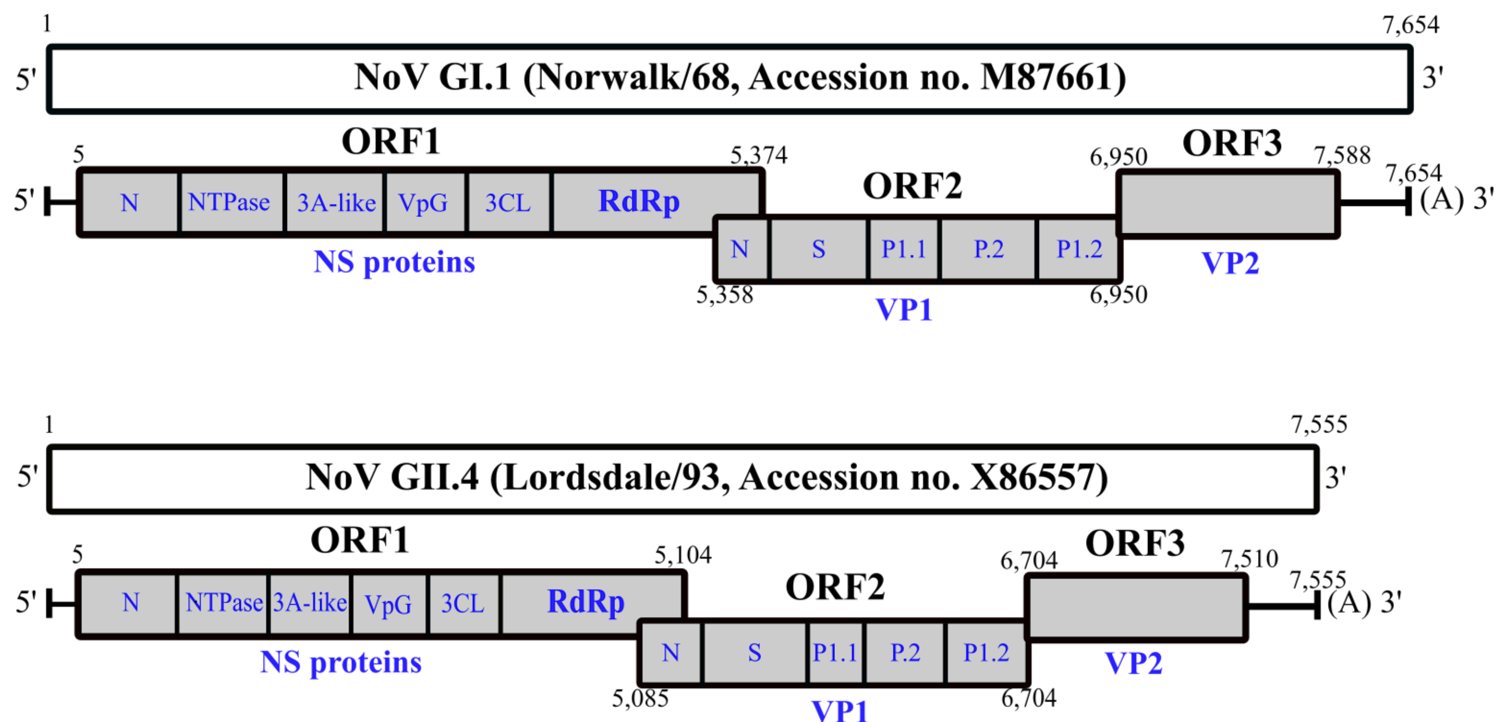


Figure 1-4. The genomic organization of human NoV strains, GI (GI.1, Norwalk/68) and GII (GII.4, Lordsdale/93)

Schematic representation showing the genome organisations of NoV GI.1 Norwalk/68 (upper) and NoV GII.4 Lordsdale/93 (lower). Each viral genome comprises of three ORFs with 3' end of ORF1 overlapping the 5' end of ORF2 by 17 bp (Norwalk/68) or 20 bp (Lordsdale/93), and 3' end of ORF2 overlapping 5' end of ORF3 by 1 bp. ORF1 encodes a large polyprotein, which is proteolytically cleaved into 6 non-structural (NS) proteins, including N-terminal (N) protein, NTPase, 3A-like protein, viral protein-genome (VpG), 3C-like protease (3CL), and RdRp. ORF2 encodes the major structural protein VP1, which self-assembles into the viral capsid protein. Distinct structural domains within ORF2 are found to organise into the N-terminal (N) arm, the shell (S) domain, the P1 (divided into P1.1 and P1.2 subdomains) and P2 domains. ORF3 encodes a minor capsid protein VP2. The genome consists of a polyadenylated tail (A) at the 3' end.

1.2.2.2. Classification

The current classification divides NoV into five genogroups (GI – GV) on the basis of sequence divergence within the major capsid protein (VP1) (Figure 1-5) [156-159]. Strains in each genogroup can be further classified into genotypes and variants (sub-genotypes) [156, 158, 160, 161]. Based on the uncorrected pairwise distance of the complete VP1 amino acid sequences, the cut-off values of genetic differences for genogroups, genotypes and sub-genotypes (variants) are 45-61.4 %, 14.3-43.8 %, and < 14.3 %, respectively [156].

Initially, the genotype determined based on one region of the genome (either the RdRp or the capsid) was presumably the genotype defined based on the whole genome due to the unsegmented nature of NoV genome [157, 158, 161-164]. However, recent evidence of recombination occurring at the ORF1/ORF2 junction has signified the importance to determine both the polymerase and capsid genotypes for a better genotypic characterisation of strains, leading to the current nomenclature considering both polymerase and capsid genotypes [165, 166]. The web-based dual genotyping tool, from RIVM (the Dutch National Institute for Public Health and the Environment)-NoroNet, has so far differentiated GI and GII viruses into 14 GI and 29 GII polymerase genotypes, and 8 GI and 23 GII capsid genotypes [160]. Due to the challenges of obtaining complete capsid sequences or the RdRp gene particularly in resource-limited settings, molecular characterisation of partial coding sequences within ORF1 [162, 163, 167-169] and ORF2 [158, 161, 170, 171] is targeted for routine NoV detection, genogrouping and genotyping.

Human NoV have been found within genogroup I, II and IV; GI and GII are responsible for most human infections [156], with viruses of the GII.4 cluster being

responsible for the majority of global NoV-associated outbreaks and sporadic settings [172, 173] and more than 80 % of all NoV outbreaks in the US [133]. NoV are also found to infect a number of mammals, including pigs (GII.11, GII.18, and GII.19), sheep and cows (ovine/bovine GIII), lions and dogs (GIV.2), and mice (MNV, forming a distinct GV) (Figure 1-5) [159, 169, 174-181]. Although animal NoV, such as porcine and canine strains, can be found in two human genogroups GII and GIV, the ability of these animal NoV to infect and transmit between human hosts (zoonosis) has not been confirmed. No human NoV strain has yet been found to contain genetic information derived from a non-human NoV lineage origin [145, 172]. For the purposes of the work presented here, human NoV is the focus of the subsequent sections and referred hereafter as NoV unless otherwise noted.

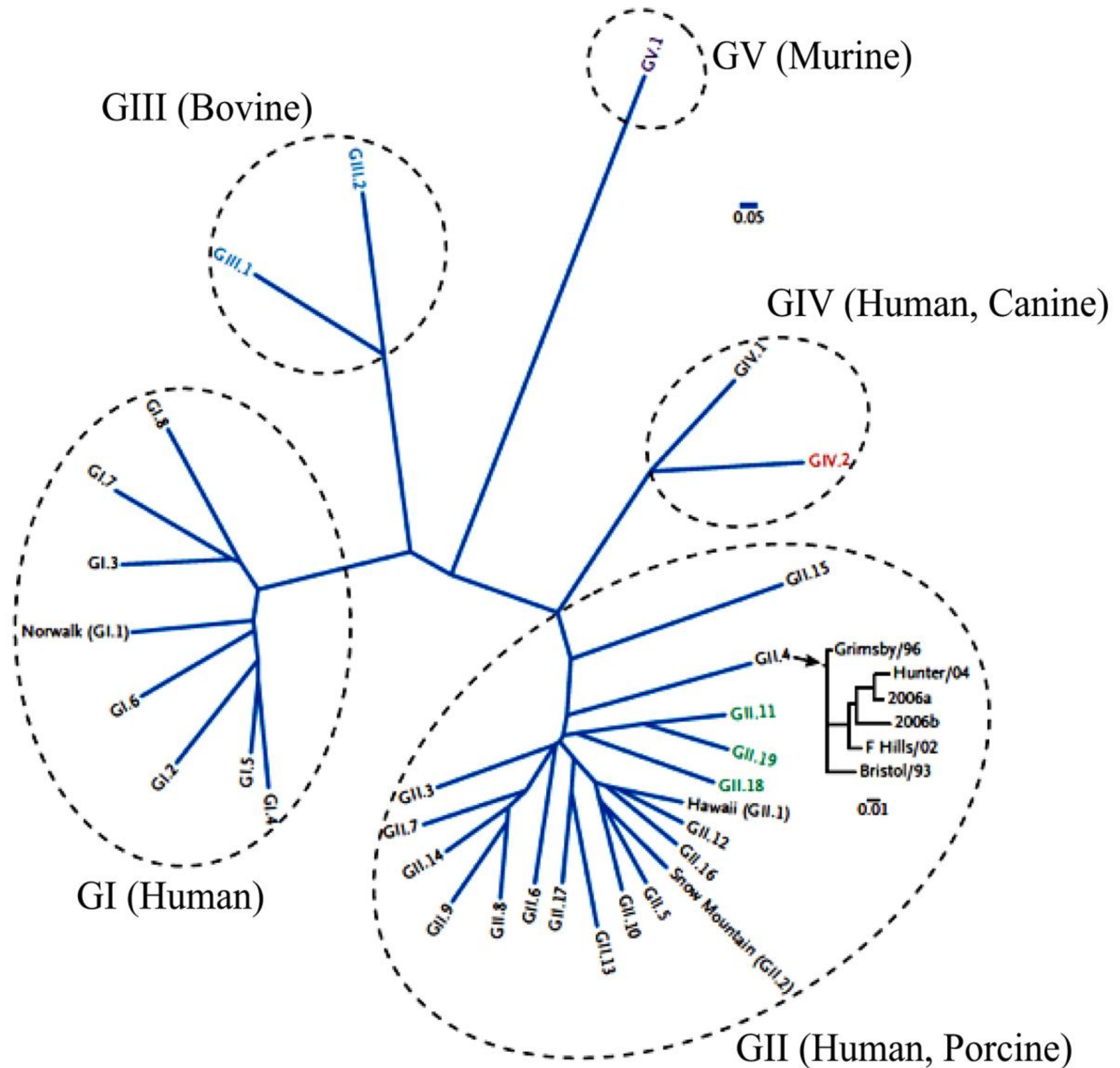


Figure 1-5. The phylogenetic tree of five major NoV genogroups

Source: Adapted from [133]. The tree was constructed based on 52 complete capsid amino acid sequences, and the scale bar of 0.05 represented the number of amino acid substitutions per site. Each dotted circle indicated a genogroup of viruses, from GI to GV with corresponding infecting species indicated in brackets. Human prototype viruses were shown in black; porcine viruses GII.11, GII.18 and GII.19 were shown in green; bovine viruses were shown in blue (GIII); murine viruses were shown in purple (GV); and a lion virus (GIV.2) was shown in red. Sub-phylogenetic tree of variants belonging the NoV GII.4 was also shown, with the scale bar representing the expected number of substitutions per site. Viruses in GI, GIII and GV are found to exclusively infect human, bovine and murine, respectively. Strains infecting both human and a few mammals can be in GII and GIV.

1.2.2.3. Mechanisms of NoV evolution

The two primary mechanisms of NoV evolution are random point mutation and homologous recombination [182]. Strains within a single genotype, GII.4, have been implicated in global epidemics since 1995 (Figure 1-6) [182]. Recent investigations suggest that the accumulation of mutations of the capsid P2 domain over time has led to significant variation, resulting in antigenic drift which influences the ability of pre-existing herd immunity to neutralise circulating strains and subsequently permits the emergence of new epidemic NoV variants [148, 182-189]. Due to the error-prone nature of RNA polymerases, RNA viruses show high evolutionary rates, ranging from 10^{-3} to 10^{-5} substitutions per nucleotide site per year [53, 190]. Evolutionary rates for NoV have been estimated to lie at the higher range, ranging between $1.9x10^{-3}$ and $5.6x10^{-3}$ substitutions per site per year based on complete capsid sequences [184, 191, 192]. It has been suggested that the higher potential epidemiological fitness of GII.4 viruses can be attributed to higher rates of evolution of the capsid proteins [184]. In particular, the lower fidelity of replication in combination with higher diversity in the P2 domain indicates a higher rate of mutations among GII.4 viruses at the antigenic sites conferring to a fitness benefit and hence allowing the viruses to persist in the human population [182-184, 193].

As in many RNA viruses, recombination is an important mechanism for NoV evolution [194]. NoV recombines frequently, thus generating genetic diversity in the NoV genome [165, 195-197]. Two proposed mechanisms of RNA recombination include reassortment and copy-choice recombination [198], of which the latter is the primary mechanism of recombination in unsegmented viruses but also occurs in segmented viruses [199]. In the copy-choice model, a recombinant RNA is formed during a mixed infection when the viral RdRp complex switches from one RNA

template to another during transcription/replication, resulting in homologous recombination [194]. Homologous recombination has been described in NoV and is proposed to occur via the copy-choice model [151, 165, 166, 195, 196]. The proposed sites of NoV recombination have been identified within the polymerase gene (ORF1), and immediately upstream or downstream of the conserved 20-bp ORF1/ORF2 overlap in GII strains [165, 166]. In addition to recombination observed at the ORF2/ORF3 junction [200, 201], intragenic recombination within the ORF2 has also been reported [202, 203]. Such recombination in ORF2 region may potentially alter the orientation of the capsid domains and protein conformation, blocking the neutralisation by the extant antibodies [182]. Intergenic recombination between different genotypes has also been reported, which likewise drives the NoV evolution by accelerating the mutation rate and increasing genetic diversity and overall viral fitness [165, 195-197, 204, 205].

Several factors have been suggested to influence the rate of evolution in NoV such as the duration of herd immunity and host genetic factors that determine susceptibility or resistance to NoV binding (such as HBGAs) as discussed in section 1.2.2.5. Evidence shows that mutations in the NoV GII.4-1996 (US95/96) variant have extended the ability of this variant to bind more HBGA types, consequently expanding the susceptible population size and hence allowing for the global spread of this lineage [183]. Additionally, evidence suggests that heterogeneity among strains of the GII.4 lineage and the presence of some herd immunity results in antigenic drift and persistence of the GII.4 lineage, and act as the driving force in NoV molecular evolution [173, 183, 185-188, 206-208].

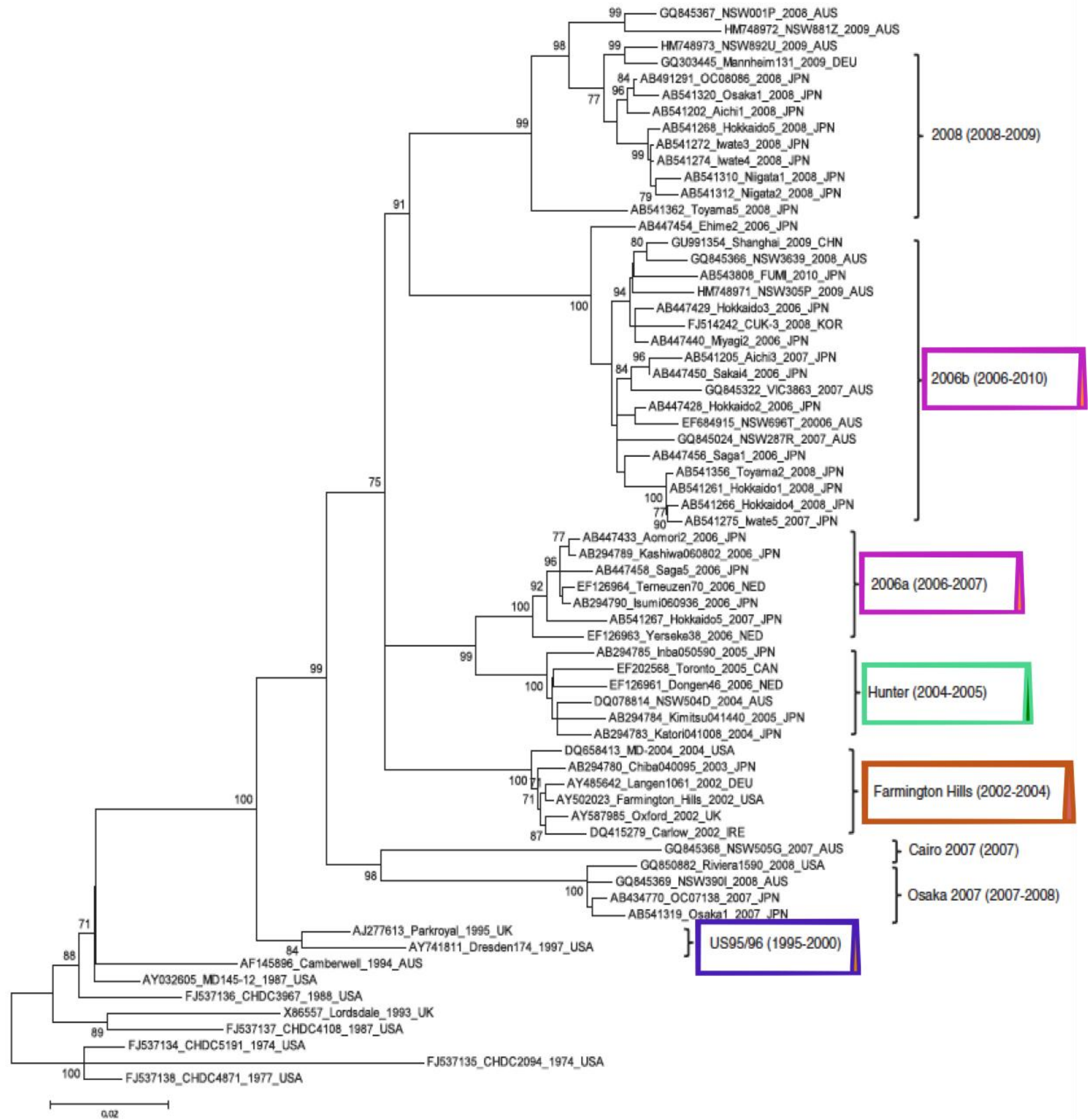


Figure 1-6. The phylogenetic tree of NoV GII.4 variants

Source: Adapted from [182]. The figure shows the maximum likelihood tree of the viral capsid gene (VP1) of GII.4 viruses identified from 1976 to 2010 [182]. The tree is drawn to scale with branch lengths proportional to the number of nucleotide substitutions per site. Replicates were performed 1,000 times and only bootstrap values > 70 are shown. Coloured boxes highlight the strains that have been implicated in global epidemics. Strains are named according to GenBank accession ID_strain name_Year of isolation_Country of isolation. Countries are abbreviated using three letter codes.

1.2.2.4. Pathogenesis and clinical presentation

NoV infection is characterised by an acute onset of vomiting and diarrhoea, accompanied by other symptoms including nausea, malaise and abdominal pain [145]. Diarrhoea is usually loose watery, non-bloody, without visible mucous or leucocytes and a low-grade fever may occur in some patients [145]. The disease is usually mild and self-limiting, with a mean incubation period of 24 to 48 hours. Symptoms resolve within 12 to 60 hours on average [209]. However, infections in the very young (under 1 year of age), elderly and immunosuppressed or immunocompromised patients are often more severe such that symptoms may persist for ≥ 4 days [209]. Weeks of prolonged viral shedding (≥ 3 weeks) can also occur in asymptomatic infections [145, 209]. NoV infections in transplant or severely immunosuppressed patients can be protracted to chronic diarrhoea and virus shedding can last for months to years [209].

Much of understanding on NoV pathogenesis stems from experimental human volunteers inoculated with NoV GI.1 virus [210]. Examination of biopsies at the duodeno-jejunal junction from infected individuals showed histopathological lesions in the jejunum, correlating with a broadening and blunting of intestinal villi and crypt cell hyperplasia of the small intestine, leading to speculation that NoV replication occurs in the small intestine [210, 211]. Although the mechanisms inducing symptomatic infections are unknown, it has been suggested that the decrease in small intestinal brush border enzymatic activities (sucrose, trehalase, alkaline phosphatase) results in transient carbohydrate malabsorption [212]. The secretory function of the stomach (pepsin, HCl, intrinsic factor) is not affected but gastric emptying is markedly delayed, indicating that reduced gastric motility is associated with symptoms such as nausea and vomiting in NoV gastroenteritis [213].

1.2.2.5. NoV immunity

The mechanisms of host immune responses and protective immunity against NoV infections are complex and poorly characterised. Due to a lack of efficient tissue culture and animal model systems for human NoV, much of knowledge on NoV immunity is acquired from human volunteer challenge studies. It is generally known that people of all ages can be infected with NoV, suggesting that the immunity against natural NoV infection may be short-term and incomplete. The short-term immunity following NoV GI.1 infection has been shown in early human challenge studies, which lasted for 6 to 14 weeks [214-217]. It has also been found that all symptomatic volunteers following the initial challenge developed gastroenteritis when re-challenged 27 to 42 months later with the homologous virus confirming that the immunity is short-lived irrespective of increased serum antibodies against NoV GI.1 [215, 217]. Recently, a Phase 1-2 vaccine trial demonstrated that intranasal GI.1 VLPs (virus-like particles) vaccine provided protection against NoV disease three weeks following vaccination [218]. Nevertheless, the topic of long-term immunity is debatable since a few studies showed protection against GI.1-induced gastroenteritis after 6 months post-challenge in some but not all volunteers [219-221].

Existence of natural resistance to NoV infection has been suggested as 50 % of the challenged individuals (N = 6; total volunteers: 12) did not develop clinical illness or an immune response upon the initial and subsequent challenges [215]. Susceptibility and resistance to NoV infection have been shown to link with a host genetic factor, the HBGA expression, in particular a linkage between NoV binding with three major HBGAs, including ABO, secretor [H antigen] and the Lewis factors [209, 222-224]. HBGAs are antigens present on the surfaces of mucosal epithelial

cells and red blood cells, or free antigens circulating in biological fluids (saliva and milk) [209, 222-224]. The Fucosyltransferase (FUT) 2 enzyme, which mediates the biosynthesis of these antigens, is suggested to play a key role; the genetic differences within this particular enzyme within the human population result in varied levels of susceptibility/resistance to NoV disease [224]. In particular, individuals possessing a G248A mutation in the FUT2 gene do not express the H type 1 HBGA due to failure to synthesise/modify the blood group H antigen from precursor disaccharides [224]. Such individuals with non-functional FUT2 enzyme are considered "non-secretors" (Se^-), and are resistant to NoV GI.1 as GI.1 uses H type 1 as an attachment factor, leading to resistance against clinical infection with NoV GI.1 in 20 % of European people (who are generally non-secretor type) [222, 225]. Studies on association between ABO blood phenotypes and NoV GI.1 clinical infections found that individuals with an O phenotype were more susceptible to infections, while those with a type B tended to be more resistant to NoV infections [226]. In Vietnam, the relative proportions of blood groups A, B, AB and O are 21.2 %, 30.1 %, 6.6 % and 42.1 %, respectively (unpublished data).

Different NoV strains may exhibit differential HBGA binding patterns [226-228]. In contrast to GI viruses, binding of NoV GII strains to HBGAs has been demonstrated to be independent of the secretor status of the individual [226, 227, 229-231]. Particularly, strains within the NoV GII.4 cluster are capable of binding to all secretor (Se^+) individuals regardless of their ABO phenotype (about 80 % of the populations) [226, 230]. The different HBGA binding patterns leading to a greater susceptible population for GII infections may partially explain the overwhelming prevalence and spread of NoV GII viruses, in particular GII.4 strains, over GI viruses in NoV-associated gastroenteritis.

1.2.2.6. Epidemiology

NoV infects people of all ages and have been detected in both developed and developing regions; NoV infections can be epidemic (such as foodborne gastroenteritis and health-care associated outbreaks) or sporadic (such as acute sporadic gastroenteritis in children and in adults) [232-235].

NoV is suggested to be the leading cause of foodborne illnesses in the US [234-237] in addition to being the principal agent of global non-bacterial outbreaks of gastroenteritis in all age groups (~ 50 % of all gastroenteritis outbreaks) [238]. Foodborne NoV gastroenteritis outbreaks are commonly associated with consumption of faecally contaminated food (such as oysters harvested from sewage contaminated water) or food from infectious food-handlers [236, 239-243]. Outbreaks of health-care associated NoV gastroenteritis occur in closed or semi-closed settings such as hospital wards, nursing homes, cruise ships, military institutions, schools and restaurants [145, 209, 244]. Elderly, transplant or immunosuppressed patients are more likely to be affected from acquired NoV infections; such infections often result in prolonged disease duration, delayed discharge or even hospital-ward closures to prevent transmission [145, 209, 244-247].

NoV is the second most common cause of acute sporadic viral gastroenteritis in young children after RoV and among the leading causes of severe childhood diarrhoea worldwide [133, 148, 209, 214]. In developed countries, NoV is responsible for an estimated 64,200 diarrhoeal episodes requiring hospitalisation and 906,000 diarrhoea-related clinic visits in children under five years of age every year [232]. Yet it has been estimated that the burden of NoV-associated illness is greater among children younger than 5 years of age in developing areas; in particular, the

virus has been calculated to cause 1.1 million childhood diarrhoea episodes that require hospitalisation, an annual incidence of 197 NoV-related hospitalisations per 100,000 children with 218,000 annual deaths in children < 5 years old [232].

Overall, viruses in GI and GII genogroups are responsible for the vast majority of human NoV infections, with strains in the GII.4 genotype playing a prominent role in both outbreak and sporadic settings [133, 148, 156, 248]. Similar to data from outbreak-based surveillance showing the predomination of GII.4 strains, studies of sporadic acute NoV gastroenteritis showed a concurrent emergence and dominance of GII.4 viruses alongside with those associated with outbreaks, such as globally epidemic variants GII.4-1996 (US95/96), GII.4-2002 (Farmington Hills), GII.4-2004 (Hunter), GII.4-2006a (Laurens) and GII.4-2006b (Minerva) [133, 148, 173, 183, 249]. The mechanisms underlying NoV GII.4 evolution, persistence and dominance have been discussed in section 1.2.2.3. Notably, GII.4-2006b is an example of strain replacement phenomenon within the GII.4 lineage, in which the variant emerged in 2005 and subsequently displaced previously circulating strains to dominate in most human NoV infections in many countries including Vietnam [250-252]. In addition to the aforementioned pandemic GII.4 strains, other variants in the GII.4 lineage have been identified including GII.4-2002CN (Henry), GII.4-2003 (Asia) and GII.4-2007 (Osaka), which were likely to associate with local epidemics [253-255]. Other genotypes in GII are also accused of a number of human NoV infections, such as GII.3, GII.2, GII.6, and GII.13 [250-252, 256-259].

NoV infections occur year-round but disease incidence may increase in the colder months [260]. NoV is highly infectious and has a very low infectious dose (ID_{50} = 18 infectious particles) [261]. The virus is mainly transmitted through person-to-person contact, through the faecal-oral route (such as contaminated food

and water) or through airborne or fomite transmission (such as vomitus) [145, 238, 262, 263]. Hence, proximity to infected people with vomiting presents a risk factor for NoV infections [145]. Vaccine research and development for NoV has been impeded by the unavailability of efficient cell cultures and small animal model systems. Although as yet there is no licensed vaccine against NoV, a number of vaccine candidates are currently in development or under evaluation [218, 264-267].

1.2.3. Other common enteric viruses

Astroviruses (AstV) are non-enveloped, positive-sense, single-stranded RNA viruses within the genus *Astrovirus*, family *Astroviridae* [268]. AstV distributes globally, and eight serotypes have been found to cause human disease [269]. The estimated mean prevalence of AstV in hospitalised diarrhoeal patients in developing countries is 3.7 % (range 0 – 13.9 %) [14]. Transmission occurs mainly via direct person-to-person contact through the faecal-oral route. AstV infections commonly occur in children younger than four years of age and peak during the winter months [269]. Clinical infection is characterised by diarrhoea, fever, vomiting, nausea, abdominal pain and malaise [268]. The disease is often self-limiting and last for an average of 5 to 6 days; asymptomatic infections are common [270]. Viral shedding can occur for up to 2 weeks or may be further prolonged in immunodeficient individuals [271].

Adenoviruses (AdV) are non-enveloped, double-stranded DNA viruses of the family *Adenoviridae* [272]. AdV causes a range of human infections including gastroenteritis, acute respiratory infections and urinary tract infections. There are 51 AdV serotypes, which are further divided into six subgenera (A-F), among which AdV subgroup F (serotype 40/41) has been associated with acute gastroenteritis

[272]. AdV is responsible for approximately 6.4 % of childhood diarrhoeal episodes (range 1.4 – 19.9 %) in developing areas [14]. Enteric AdV illnesses occur year-round and show a peak in children under the age of four [15]. The virus is highly transmissible during the first few days of acute illness; persistent infections and prolonged shedding are common, as are asymptomatic infections and re-infections [270]. The clinical presentation of AdV infections is similar to that of RoV infections and can be more severe in immunocompromised individuals. Fever and respiratory symptoms are uncommon in diseases caused by enteric AdV.

Sapovirus (SaV) belongs to the *Caliciviridae* family [151]. Sapoviruses are genetically diverse and can be divided into five genogroups (GI-GV), of which viruses in GI, GII, GIV and GV are found to cause human diseases (GIII causes disease in pigs) [273]. Infection caused by SaV presents similarly to NoV gastroenteritis but is often milder with a lower frequency of vomiting [274]. The disease is acute, self-limiting and does not usually require hospitalisation; high rates of asymptomatic infection are thought to occur [274]. The incubation period of SaV infection is approximately 24 to 48 hours and illness usually lasts for 3 to 4 days but may be prolonged in immunocompromised patients. Transmission occurs via the faecal-oral route [274]. SaV infections tend to occur predominantly among infants and young children under five years of age, from 0.8 % to 9.3 % [275].

1.3. Bacterial diarrhoea

1.3.1. Shigella

1.3.1.1. Microbiology

Shigellae are a genus of invasive Gram-negative bacilli within the *Enterobacteriacae* family. Four *Shigella* species (or groups) are recognized, including *Shigella dysenteriae* (group A, with 15 serotypes), *Shigella flexneri* (group B, with 14 serotypes and subserotypes), *Shigella boydii* (group C, with 20 serotypes) and *Shigella sonnei* (group D, with a single serotype) [276].

Initially, *S.sonnei* was thought to be the predominant *Shigella* species in developed countries, whereas *S.flexneri* was considered the dominant species in developing countries. However, recent evidence demonstrated a shift in the prevalence of these two species, in which *S.sonnei* has become predominant in rapidly developing or transitional countries such as Vietnam [277]. *S.dysenteriae* serotype 1 causes epidemic severe dysentery in the poorest areas of the world [278]. *S.dysenteriae* serotypes non-serotype 1 and *S.boydii* are cumulatively responsible for 3 % diarrhoeal cases in developed countries, and 12 % of the overall shigellosis burden in developing countries [279].

1.3.1.2. Characteristics of infections

Shigellosis is generally characterised by diarrhoea with presence of visible blood in stools, with or without mucus, abdominal cramps and tenesmus, fever and anorexia [276]. Yet, some patients may present solely with acute watery diarrhoea and none of the aforementioned symptoms. Dehydration is not common and is rarely severe if present. Typically, patients are clinically symptomatic after an incubation

period of 1 to 4 days, and recovered within 7 to 10 days without any antimicrobial intervention [276]. Shigellosis is generally self-limiting, but complications or death may occur in severe cases without proper and timely management [211, 280, 281]. *Shigella* spp. induce a spectrum of clinical manifestations, among which *S.sonnei* causes the mildest infections and low mortality risk, and infections caused by *S.dysenteriae* serotype 1 lie at the most severe end with higher mortality risk [276]. However, it was observed in southern Vietnam that *S.sonnei* induced more severe clinical infections than by *S.flexneri* [277].

Shigella spp. is the most common aetiology of bacillary dysentery and a prominent problem in developing countries. Based on a review of studies published from 1966 to 1997, the majority of shigellosis burden occurred in developing regions, with an annual estimate of 163.2 million endemic shigellosis episodes resulting in 1.1 million deaths every year in these countries [279]. In contrast, 1.5 million shigellosis episodes were estimated to occur every year in developed countries [279]. The majority of shigellosis-related morbidity (69 %) and mortality (61 %) occur in children younger than five years of age [279]. The peak incidence of *Shigella* infections occurs in children of 12 to 47 months of age [279, 282, 283]. Currently, there is no vaccine available to prevent shigellosis.

Routes of transmission of *Shigella* spp. include the faecal-oral route, contaminated food and water or person-to-person contact provided its low infectious dose (10 – 200 viable organisms) [284]. Peaks of *Shigella* infections exhibit distinct geographical and temporal patterns, i.e. summer time in temperate areas [285] versus wet season in tropical countries [277]. Shigellosis in tropical areas may be associated with poor hygiene and water quality [286-288].

1.3.2. Non-typhoidal *Salmonella*

1.3.2.1. Microbiology

Salmonella are rod-shaped, motile, Gram-negative, oxidase-negative, non-spore forming bacteria of the *Enterobacteriaceae* family, which can be subdivided into *Salmonella enterica* and *Salmonella bongori* species [289]. Non-typhoidal *Salmonella* (NTS) belong to the species *Salmonella enterica* and are broadly referred to as "non-typhoidal" to distinguish them from *Salmonella* Typhi and *Salmonella* Paratyphi which cause enteric fever [289].

Bacteria within the NTS can be serologically differentiated based on the antigenic characteristics of the two surface antigens: the flagellar "H" and the oligosaccharide "O". Among these, *Salmonella enteritidis* serogroup D and *Salmonella* Typhimurium serogroup B are the two most common serotypes causing NTS infections worldwide [289, 290].

1.3.2.2. Characteristics of infections

Enteric infections caused by NTS have similar clinical manifestations to those induced by other enteric bacteria, and thus can rarely be distinguished based on presenting clinical characteristics. NTS-induced gastroenteritis is characterised by abdominal pain, fever, watery diarrhoea, and occasionally mucoid or bloody diarrhoea [290]. Vomiting and/or nausea occur frequently but are not severe or protracted. The incubation period is varied, depending on the host and bacterial serotype, but typically ranges from 6 to 72 hours [290]. The disease is usually self-limiting but can be more severe in the very young and elderly or immunocompromised, with a typical duration of illness of 4 to 7 days [24]. About 5

% of all cases of NTS gastroenteritis may progress into bacteraemia and require medical attention [290, 291].

NTS gastroenteritis imposes a substantial burden in both developed and developing regions [290, 292, 293]. Globally, *Salmonella*-associated gastroenteritis is estimated to be responsible for an annual number of 93.8 million cases and 155 thousands deaths; 86 % of all cases are thought to be foodborne infections [292]. In the US, FoodNet surveillance has estimated that nearly 1.4 million persons are infected with NTS annually, resulting in 168,000 clinic visits, 15,000 hospitalisations and 400 deaths (incidence: 12.3 cases per 100,000 population) [293]. Outbreaks of NTS gastroenteritis are common and often of zoonotic origin [293]. Severe NTS infections are more frequently observed in resource-limited countries, with mortality rates ranging from 18 to 24 % [294]. In these severe illnesses, antimicrobials should be prescribed [22, 290]. There is no effective vaccine against NTS; hence, disease prevention and control programs mostly rely on improving personal hygiene and sanitation with particular attention paid to food safety.

NTS can be transmitted through multiple routes, including consumption of food of animal origin (eggs, poultry, undercooked meat and dairy products), fresh products that have been contaminated with animal waste, consumption of contaminated water, or direct contact with infective humans or animals [290].

1.3.3. Campylobacter **spp.**

1.3.3.1. Microbiology

Campylobacter are small Gram-negative curved or rod-shaped bacilli, historically misidentified as *Vibrio* spp. This non-fastidous group of bacteria can be

cultured effectively at 42 °C. Seventeen species of *Campylobacter* have been identified thus far, among which *Campylobacter jejuni* and *Campylobacter coli* are the most commonly isolated species causing disease in humans [295]. In particular, over 99 % of reported *Campylobacter* infections are attributed to *C.jejuni* [296].

1.3.3.2. Characteristics of infections

Campylobacter enteritis is clinically indistinguishable from acute bacterial diarrhoea, typically acute, self-limiting with diarrhoea, fever and abdominal cramps after an incubation period of 2 to 5 days [296]. Disease induced by *Campylobacter* spp. usually involves loose/watery or bloody diarrhoea, 8 to 10 episodes per day on the peak day of illness, lasting for a median of 6 days [25, 297-299]. Illness usually resolves without antimicrobial regimes, but may occasionally cause relapsing diarrhoea lasting for weeks or involve post-infectious complication such as Guillain-Barre´ syndrome (GBS) [296, 300, 301]. The presence of while blood cells (WBC) and red blood cells (RBC) is frequently found in the patients' stool samples [299].

Campylobacter spp. is one of the leading bacterial causes of acute gastroenteritis worldwide, and particularly prominent among childhood gastroenteritis cases in developing countries [25, 302]. Studies on *Campylobacter* infections in developed countries reveal unique distributions by age and sex compared to other bacterial enteric pathogens; most infections occur before children reach 1 year of age and markedly more males are infected than females [296]. In developing countries, *Campylobacter* infections are hyperendemic, particularly in children under 2 years of age with low mortality risk [295, 296]. Asymptomatic *Campylobacter* infections appear to be common in developing countries [303], but

relatively uncommon in developed countries [304]. No clear seasonal pattern of *Campylobacter* gastroenteritis has been observed in tropical areas [302]. The primary transmission route of *Campylobacter* is believed to be foodborne contamination via consumption and handling of chicken [296, 305, 306]. A high infectious dose (800 – 10^6 bacilli) is required, suggesting that transmission via person-to-person contact is rare [307].

1.3.4. Antimicrobial resistance

Timely and accurate use of antimicrobial regimens may be beneficial for patients infected with certain bacteria and protozoans; nevertheless, excessive or inappropriate prescription of antimicrobials has many drawbacks, particularly increasing resistance among enteric bacteria [308]. Although antimicrobials are not routinely prescribed for treatment of acute diarrhoea [22], a substantial proportion of diarrhoeal patients have been treated with at least one antimicrobial. This inappropriate prescription practice is particularly relevant in resource-limited countries where (1) antimicrobials are largely available over the counter, (2) patients may have intuitive expectations of medical treatment and lack a general awareness of the dangers of antimicrobial overuse [22], (3) routine identification of diarrhoeal aetiology is not routinely performed and (4) policies that determine antimicrobial regimens are not stringent. Reasons for the increase in resistance over the last several decades include mutations occurring in resistance genes, genetic exchange among enteric microorganisms, resistant bacterial clonal spread and expansion, and selective pressure in hospitals and communities facilitating the emergence and proliferation of resistance [309].

1.4. Diarrhoeal disease in Vietnam

Vietnam is a rapidly developing country in Southeast Asia with a gross national income (GNI) per capita of USD 1,260 as estimated in 2011 [310, 311]. More than 87 million people live in Vietnam, over 7 million of which are under the age of five years. The mortality rate among children under five years of age is 23/1,000 live births [310]. Diarrhoea is the fifth most common cause of deaths in children younger than 5 years of age in Vietnam, accounting for an annual 10 % of deaths in this age group (Figure 1-7) [310]. Routine identification of the aetiological agent of diarrhoeal disease is seldom performed in hospitals in Vietnam as in other resource-limited regions, leaving a significant gap in the general understanding of this disease.

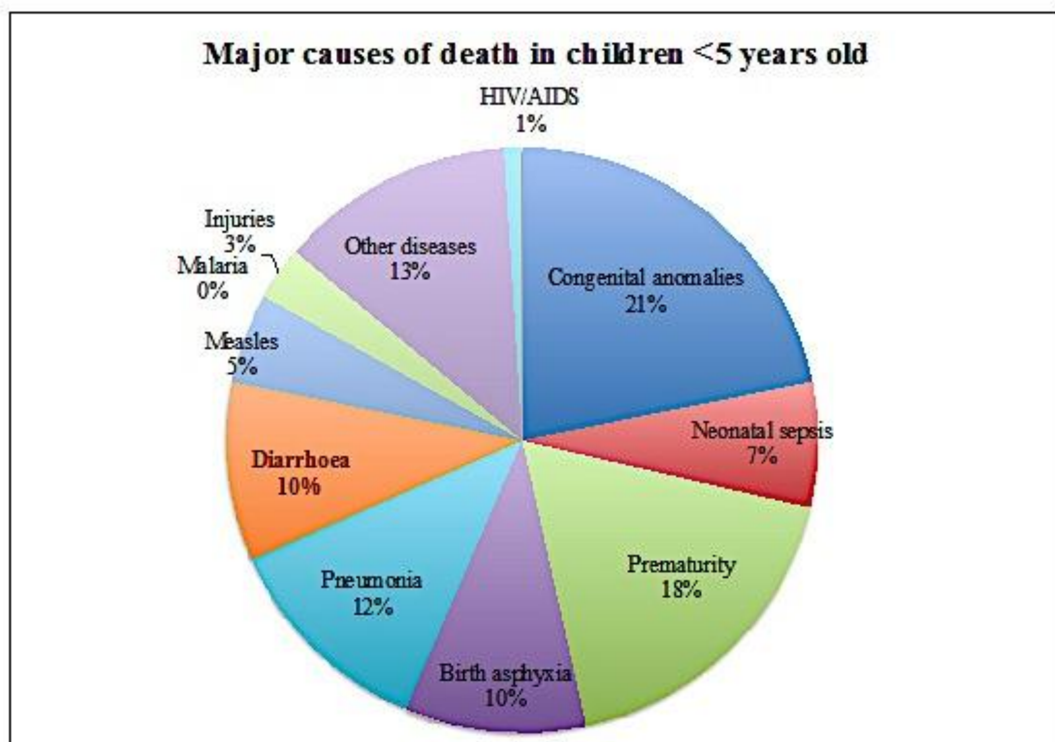


Figure 1-7. Major causes of death in children <5 years old in 2010 in Vietnam.

Source: WHO health profile for Vietnam [310]. Among non-neonate children, diarrhoea is the second leading cause of mortality, following pneumonia.

1.4.1. Viral diarrhoea in Vietnam

1.4.1.1. RoV in Vietnam

RoV has been estimated to be responsible for between 44 % and 67.4 % of all childhood diarrhoeal infections requiring hospitalisation (Table 1-3) [252, 312, 313]. A national burden of RoV gastroenteritis has been estimated from a sentinel RoV surveillance program from 1998-2003, which enrolled patients at six hospitals, three from northern Vietnam (Vietnamese-Swedish Hospital, St. Paul Hospital and Hai Phong Hospital for children) and three from southern Vietnam (Khanh Hoa Provincial Hospital, Children's Hospitals 1 and 2) [312]. The study estimated that 5,300 – 6,800 children under the age of five years die of RoV infection each year in Vietnam, representing 8 to 11 % of all deaths in this age group (cumulative risk per child by age 5 years, 1 in 200 to 1 in 285) [312]. Since then, investigations on RoV prevalence and genotype distribution were sparse (Table 1-3), showing varied frequencies of detection in different locations and during different years. In Ho Chi Minh City (HCMC), RoV detection ranged from as low as 39 % [314] to as high as 67.4 % [252]. Among all RoV studies (Table 1-3), G1P[8] is the most common genotype, circulating in the presence of other common RoV types such as G2, G3 and G4; however, G3P[8] was found to be predominant in Nha Trang in 2005-2006 (Tamura, Nishikawa et al. 2010) and in Haiphong in 2006 – 2007 [315]. Clinical trials of RotaTeq™ vaccine in Vietnam showed 63.9 % efficacy against severe RoV acute gastroenteritis [131]. A Vietnamese RoV vaccine, Rotavin-M1 (live-attenuated G1P1A[8]), is being currently evaluated [316, 317].

Table 1-3. Prevalence of RoV and NoV in hospitalised paediatric diarrhoeal patients in Vietnam.

Year of study	Location	Hospital	No. of Samples	% RoV	% NoV	Reference
1994-1996	Rural HCMC	N/A	158	50 % (G1)	N/A	[318]
Sep-Dec 2000	HCMC	HTD	123	39 % (G1)	N/A	[314]
1999-2000	HCMC	CH1	1339	N/A	5.4 % (GII)	[319]
1999-2000	HCMC	CH1	1355	65.6 % (G1)	N/A	[320]
2002-2003	HCMC	CH1	1010	67.4 % (G1)	5.5 % (GII)	[252]
2005-2006	HCMC	CH1	502	N/A	6.4 % (GII)	[250]
1998-2003	Nha Trang	N/A	>10,000	55 %	N/A	[321]
2005-2006	Nha Trang	N/A	183	47.5 % (G3)	6.6 % (GII)	[322]
2001-2002	Hanoi	N/A	587	46.7 %	N/A	[323]
2006-2007	Haiphong	HPCH*	978	52 % (G3)	N/A	[315]
2007-2008	Hanoi	NPH*	501	42 %	36 % (GII)	[251]

* HPCH: Hai Phong Children's Hospital; NPH: National Paediatric Hospital. N/A: Not available.

Types in bracket indicate the predominant genotypes and genogroups for RoV and NoV, respectively.

1.4.1.2. NoV in Vietnam

The reported prevalence of acute NoV disease among Vietnamese children varies in different locations and between different study periods (Table 1-3). Rates of NoV disease range from 5.4 % to 6.4 % [250, 252, 319] in HCMC, 6.6 % in Nha Trang [322] and up to 36 % in Hanoi [251]. Among all listed studies performed in Vietnam, NoV GI viruses were detected in only one study in HCMC in 1999-2000 [319], while all studies demonstrated the predominance of GII viruses, particularly GII.4 viruses [250-252] or GII.12 [322].

1.4.2. Bacterial diarrhoea in Vietnam

While most of our understanding of enteric bacteria in Vietnam come from studies performed in the north, there is limited data on bacterial diarrhoea in the south apart from one extensive study of shigellosis [277]. This has led to a gap in knowledge of the burden of diarrhoea caused by enteric bacteria in southern Vietnam. Available data show an array of circulating enteric bacteria that are capable of causing human diseases, with high levels of antimicrobial resistance to Ampicillin, Chloramphenicol, Trimethoprim-sulfamethoxazole and to a lesser extent Ciprofloxacin [324-327].

1.5. Hypotheses, objectives, and aims

The need remains for a comprehensive investigation on various aspects of diarrhoea among young children in HCMC to understand the epidemiology and aetiology of this disease. The aims of this thesis were to describe and further investigate the aetiology, clinical and socio-economic features of diarrhoeal disease in children under the age of five years in HCMC, Vietnam. It was hypothesized that NoV plays an important, but largely overlooked, role in acute childhood diarrhoea in this location; therefore, further analyses on NoV were also performed.

The specific questions and aims of this study were as follows:

1. What are the frequencies of four major enteric viruses causing diarrhoeal disease in hospitalised children in HCMC (urban setting) in comparison to Dong Thap province (rural setting) (Chapter 3)?

   The aim was to identify the proportion of paediatric diarrhoea cases attributed to four major viruses, including group A RoV, NoV (GI and GII), enteric AdV and AstV. Differences in the geographical distribution of four viruses between two locations and relative importance of viruses were noted. Additionally, RoV, which was hypothesized to be the predominant agent following a literature review, was further characterised by sequencing and genotyping all identified strains.

2. What are the epidemiological characteristics of diarrhoeal disease in children under five years of age residing in HCMC (Chapter 4)?
   This chapter narrowed down the investigation to one location (HCMC) in order to gain a more detailed perspective of disease characteristics. The

objectives of this chapter were to obtain a general picture of diarrhoea aetiology, its clinical and demographic characteristics, and antimicrobial resistance profiles in diarrhoeal patients in comparison with diarrhoea-free children living within HCMC. A prospective multi-centre hospital-based surveillance formed the basis of this chapter.

3. What are the clinical, socio-demographic characteristics and risk factors of symptomatic NoV infections in young children in HCMC (Chapter 5)?

   This chapter details epidemiological perspectives on NoV infections, including demographic and clinical features, treatments and behavioural factors, from which risk factors of symptomatic NoV infections were investigated.

4. What are the epidemiological and evolutionary dynamics of NoV GII.4 variants circulating in HCMC (Chapter 6)?

   This chapter provides insights into the genetic aspects of NoV strains identified from Chapter 4. The aim of this chapter was to elucidate the dynamic interactions between genetic, spatial and temporal factors associated with NoV strains, with a particular focus on NoV GII.4 variants.

2. STUDY SETTINGS AND MATERIALS AND METHODS

2.1. Case definition

Diarrhoea was defined as three or more passages of loose or watery stool (a loose stool being one that would take the shape of the container) or at least one bloody stool within a 24-hour period [22].

Definition of vomiting and fever (axillary temperature) and dehydration were taken from WHO recommendations and Integrated Management of Childhood Illness (IMCI) [22, 328] and was assessed by study clinicians. Vomiting was defined as the forceful expulsion of gastric contents at least once in a 24-hour period. Fever was characterised as an axillary temperature of > 37.2 ^{o}C. Thresholds of 37.2 to 39 ^{o}C and > 39 ^{o}C were considered moderate and severe fevers, respectively.

Levels of dehydration were assessed according to the recommendations of the WHO Program for Control of Diarrhoeal Diseases and IMCI [22, 328]. The IMCI guidelines have recommended four indicators used to classify a child with dehydration: the sensorium; the presence or absence of sunken eyes; whether a child drinks poorly or eagerly; and how slowly the skin pinch goes back (slowly versus very slowly).

A child with severe dehydration would have at least two of the following four signs: sensorium is abnormally sleepy or lethargic, sunken eyes, drinking poorly or not at all, and a very slow skin pinch. A child with moderate or some signs of dehydration would have two of the following: restlessness or irritability, sunken eyes, drinking eagerly or slow skin pinch. A child with either one or none of these signs was classified as having no signs of dehydration [22, 328].

2.2. Study settings

2.2.1. For the enteric viruses study (Chapter 3)

2.2.1.1. Study sites and ethics

For the study of prevalence of enteric viruses in Dong Thap (DT) province and HCMC, verbal informed consent was obtained from the parents or legal guardians of minors enrolled. This work was approved by the institutional ethical review boards of the Hospital for Tropical Diseases (HTD) in HCMC and Dong Thap Provincial Hospital (DPTH) in DT. DTPH is 154 km away from HCMC and located within the Mekong Delta region of southern Vietnam; it is a rural location, with a lower population density than HCMC.

Patient recruitment was performed over one calendar month, from November 1, 2008 to November 30, 2008 at two hospitals, paediatric ward B at HTD in HCMC and the paediatric infections ward at DTPH in DT.

2.2.1.2. Sample collection

We enrolled all paediatric (under the age of 15 years) patients who had been hospitalised at HTD or DTPH because of acute watery diarrhoea without any additional underlying complications, such as febrile convulsions, extensive dehydration or stools containing visible blood or mucus (based on clinicians' judgment). The age of each patient was recorded and a stool specimen from each patient was collected in a sterile container on the day of admission and was stored at -20 ^{o}C. Samples collected at DTPH were transported in batch in cold chain to the microbiology laboratory at the Oxford University Clinical Research Unit (OUCRU) based at HTD in HCMC for analysis.

2.2.2. For the prospective multi-site hospital-based study (Chapter 4)

2.2.2.1. Study sites and ethics

The study was conducted at three tertiary care hospitals in urban HCMC; Children's Hospital 1 (CH1), Children's Hospital 2 (CH2), and Hospital for Tropical Diseases. Ethical approval was granted by the Oxford Tropical Research Ethics Committee (OxTREC No. 0109) and the Local Scientific and Ethical Committees of the participating hospitals. A Patient Information Sheet (PIS) was sent to potential patients (Appendix 9.1.1) and controls (Appendix 9.1.2) to provide details on the study before seeking consent from parents or legal guardians. Obtaining written Informed Consent (ICF) (Appendix 9.2.1 and 9.2.2 for patient and for control enrolment, respectively) from parents or legal guardians was mandatory for participation in the study.

2.2.2.2. Enrolment

2.2.2.2.1. Inclusion criteria for cases

Children from 0 to 60 months of age who were admitted to study sites with acute diarrhoeal disease from May 3, 2009 to April 29, 2010 were considered for inclusion in this study as diarrhoeal cases. These patients had to be residing in HCMC, and should not have had antimicrobials for at least three days prior to hospital admission. Obtaining a written informed consent from a parent or legal guardian was mandatory for participation in the study. If the patient's guardian wished to withdraw consent to the study, they were removed from the study, but any available data was still analysed.

2.2.2.2.2. Inclusion criteria for diarrhoea-free controls

Children of the same age range (0 to 60 months) without diarrhoea attending CH1 or CH2 for nutritional health check or for other gastrointestinal issues unrelated to diarrhoea or gastroenteritis were considered to enrol in the control group between March and December 2010. These children must have been living within HCMC without any history of diarrhoea, respiratory illness or antimicrobial regimens for seven days prior to study enrolment. Obtaining a written informed consent from a parent or legal guardian was mandatory for participation in the study. If the children' guardians wished to withdraw consent to the study, they were removed from the study, but any available data was still analysed.

2.2.2.3. Sample size

As the multi-centre hospital-based study was a descriptive study, the sample size was partially arbitrary; however, in order to calculate differences between spatial patterns and identification of pathogens we aimed to collect approximately 1,500 stool samples over the course of the study. Additionally due to limitations in resources, 1,500 feacal specimens were an estimation of how many samples could be handled over the period of a year. A sample size of 1,500 would statistically guarantee sufficient precision for the estimate of the proportion of patients with the viral and bacterial infection. For example, we hypothetically estimated that viral infection accounts for 40 % of the sample size and bacterial infection accounts for 20 %, the 95 % confidence interval is approximately +/- 1.75 % and +/-1.43 % respectively. If we aimed to compare a feature of viral and bacterial infection, such as duration of stay in hospital, using a two-sided t-test at 5 % significance level, a

sample size of 1,500 entrants would enable us with at least 90 % power to detect differences of 0.25 days or higher between groups of patients with different causative pathogens.

2.2.2.4. Sampling and sample collection

The study was aimed to avoid any bias originating in sample collection, i.e. disease severity or specific symptoms, and any excessive stresses on staff working in these units. Therefore, the first five patients meeting the inclusion criteria from each of the three study sites were included in the study. Due to feasibility of sample collection, culture and identification samples were only be collected and cultured on Monday through Friday. Whilst this may introduce some sample bias (those that attend hospitals at the weekend only), the study duration and sample size would diminish this effect. Sampling numbers were adjusted after consideration and group consultation depending on the workload of the microbiology department at OUCRU based at HTD, HCMC.

A stool specimen was collected in a sterile container for each patient as soon as possible after enrolment for both studies, and always within 24 hours of hospital admission to exclude the possibility of nosocomial infections and prior to any prescribed antimicrobial treatment. Samples were sealed separately in sterile plastic bags to avoid cross-contamination. A collection form had to be completed for each sample, and the patient was given a unique personal identification number, which was written on the sample collection container and the collection form, the questionnaire, the CRF and lab report forms. All participants were anonymous and identified only by the ID numbers in the database. Fresh stool specimens were used for classical microbiological identification and parasitic screening. Two aliquots of

each stool sample were then stored at -80 °C as 50 % (v/v) and 10 % (v/v) suspension in distilled PBS (Phosphate Buffered Saline), for further analyses and batched viral identification, respectively.

2.2.2.5. Questionnaire, GPS data and Case Report Form

2.2.2.5.1. Questionnaire

On admission, a parent or guardian of an enrolled child was asked to complete a questionnaire regarding information on socio-economic status, daily habits and potential sources of infection, and information on the duration of the illness and any pre-treatment prior to hospitalisation. The questionnaire for diarrhoeal patients (Appendix 9.3.1) contained the same questions as in the questionnaire for controls (Appendix 9.3.2) in addition to questions related to the disease history. The full address of enrolees was also requested in the questionnaire; this was taken in the form of district, ward, street and house number. These locations of the residents of all recruited individuals were mapped using a GPS (Global Positioning System) device and analysed using GIS (Geographic Information System) software as detail in section 2.4.6 below.

2.2.2.5.2. Case Report Form (CRF)

To understand the symptoms and disease duration, the correlation of the disease severity and causative agents and other clinical information, a basic standard Case Report Form (CRF) was recorded for all enrolled diarrhoeal patients, which were completed by the treating clinician (Appendix 9.4).

2.2.2.6. Data entry and management

All information in the CRFs, admission forms, questionnaires and lab report forms (Appendix 9.5) were entered in a confidential Microsoft Access database and verified. Enrolees could not be identified by their names in any documents/reports and at any stage of the study.

2.2.2.7. Meterological data and nutritional status assessment

Monthly average rainfall and temperature data for HCMC were obtained from Vietnam Southern Regional Hydro-Meteorological Station (HCMC). The nutritional status of all enrolled children were determined based on WHO guidelines and expressed as weight-for-age Z-score of the median WHO growth standards [329, 330]. Any children with a weight-for-age Z-score value below -2 were considered malnourished.

2.3. Microbiological methods

2.3.1. Microscopic smear observation and Gram stain

A fresh smear of each collected faecal specimen was prepared in PBS at the time when samples were received at the microbiology lab (Oxford University Clinical Research Unit, HTD, HCMC); 10 μl of which was examined at 400 times magnification under the microscope screening for the presence of WBC, RBC, and parasitic cysts including *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium* spp.

Another fresh smear of all faecal specimens was Gram stained and microscopically observed at 1,000 times magnification to check for bacterial morphology and Gram stain characteristics. For any suspected bacteria isolated on culture plates and for all colonies grown on *Campylobacter* selective media, a Gram stain was additionally performed.

2.3.2. Bacterial identification

2.3.2.1. Culture media

Culturing and bacterial identification were performed for all collected fresh stool samples on the day of sampling following the classical standard procedure in clinical microbiology [289]. Briefly, stool specimens were cultured on five different selective media to identify and isolate commonly typical diarrhoeal bacteria including *Shigella, Salmonella, Campylobacter*, *Yersinia*, *Plesiomonas* and *Aeromonas.* These media include Blood Agar (BA), MacConkey Agar (MC), Xylose-Lysine-Deoxycholate Agar (XLD), Selenite broth, and a *Campylobacter* blood-free selective agar (Oxoid, Basingstoke, UK). The BA, MC, XLD, and Selenite broth were incubated at 37 ^{o}C overnight and the *Campylobacter* plates were incubated micro-aerophilically (by supplying a gas mixture of 10 % CO_2, 5 % O_2 and 85 % N_2 in an anaerobic jar) at 42 ^{o}C for 48 hours. A full loop of the overnight Selenite broth was streaked on MC and XLD plates, followed by overnight incubation at 37 ^{o}C.

After the required incubation, all plates were checked for growth, colony morphology and characteristics on plates. Bacterial colonies (grey/yellowish, round and convex, or flat and spreading with irregular edge) growth at 42 ^{o}C on

Campylobacter blood-free selective plates that were identified as positive for oxidase and catalase enzymes and curved (seagull-shaped) Gram-negative bacilli (Figure 2-1), were reported as *Campylobacter* spp. Bacterial colonies that were non-lactose fermenting on MC and XLD plates were suspected of Gram-negative *Enterobacteriacaea*, and a sulphide production (colony with black centroid on XLD plate) was additionally indicative of *Salmonella* spp. Colonies of suspected Gram-negative *Enterobacteriacaea* pathogens on MC and XLD plates were sub-cultured onto nutrient agar for purity and further characterised using a short set of biochemical tests and commercial API 20E tests. The short set of biochemical tests provided preliminary indication of the organism identity; such results from short set tests were further checked (in case of bacterial colony with unclear identity) and confirmed by the commercial API 20E tests (bioMerieux, Paris, France). Subsequently, all confirmed *Campylobacter* spp., *Shigella* spp. and *Salmonella* spp. strains were serotyped according to the methods described in the section 2.3.2.4.

All isolated bacterial colonies were stored in skimmed milk at -20 ^{o}C for short-term storage, and lyophylised for long-term storage or until further required.

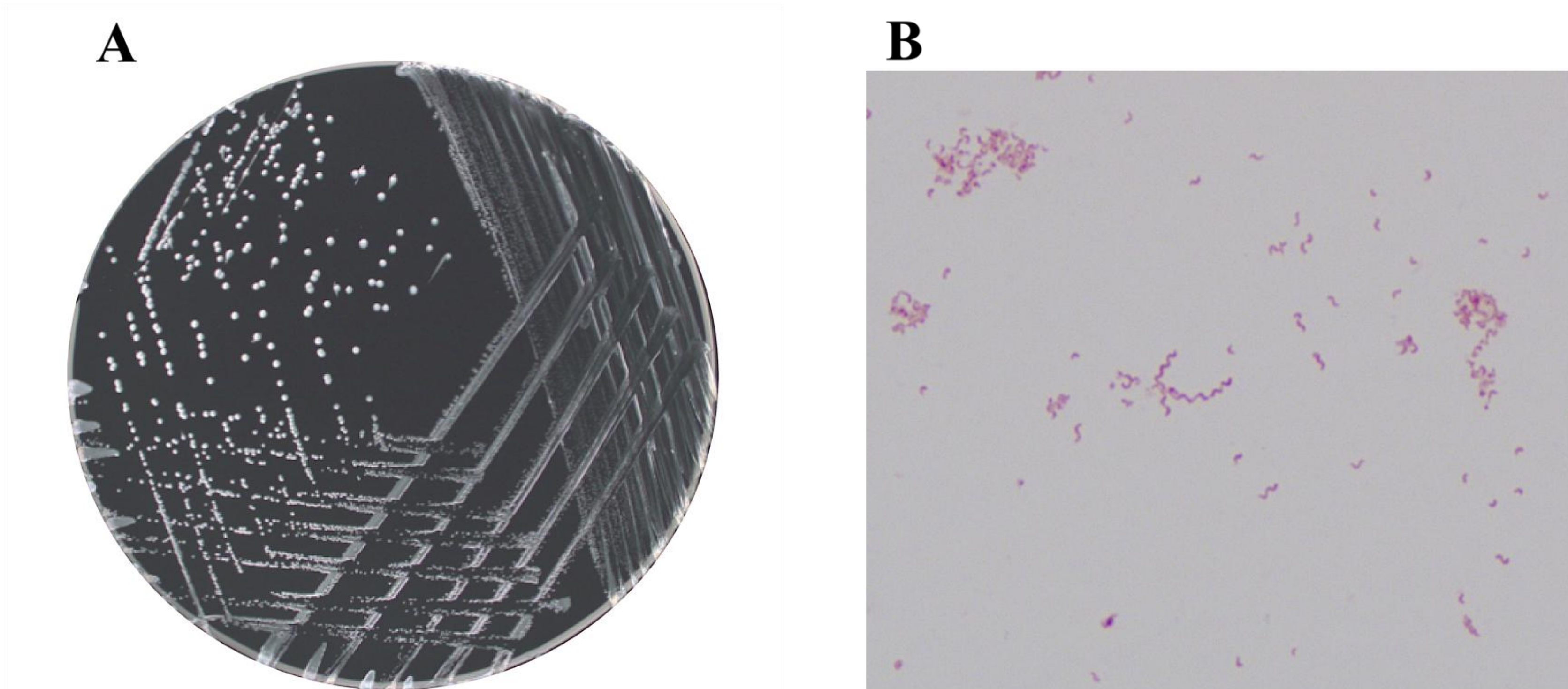


Figure 2-1. The morphology of *Campylobacter* spp.

Images showing *Campylobacter* (sample ID 30318) bacterial colony morphology (round and convex) on the *Campylobacter* blood-free selective agar (A) and corresponding bacterial morphology, under the microscope at 1,000 times magnification, illustrating typical small Gram-negative seagull-shaped bacilli (B).

2.3.2.2. Short sets of biochemical identification

A series of biochemical tests on sugar fermentation were used to characterise the bacteria including Kliger iron agar (KIA), citrate agar, urea agar, methyl red (MR), motility-indole (SIM) agar (Oxoid). Briefly, homogenous suspension from single colonies of test bacteria was prepared in the provided tube of nutrient broth, which was subsequently used for MR test. Full loops of the bacterial suspension were streaked with a straight wire on KIA, citrate and urea media, and also stab-inoculated to about two thirds of the KIA and SIM media tubes, all of which were incubated at 37 ^{o}C for 18 to 24 hours.

After incubation, results in five tubes were checked (Figure 2-2). KIA with glucose (upper layer) and lactose (lower layer) media tested on the fermentation of glucose or lactose as a source of sugar in the testing organisms. Sugar fermentation resulted in acid production, leading to a change in KIA media colour from red to yellow; no colour change suggested an overall alkalinity in the media (no sugar fermentation). An Alkaline/Acid (Alk/A) formation, red (upper) over yellow (lower) in the KIA, indicated that the organism could utilise glucose but not lactose. For motility testing, growth of non-motile organisms was confined to the stab inoculum in the SIM media; conversely, motile organisms migrated throughout the medium making it turbid. Indole is volatile and some organisms are capable of breaking down indole. Kovacs' reagent, serves as a solvent, was added into the SIM media after 18-24 hours of incubation to facilitate the indole breakdown reaction to occur; a colour change from yellow (original Kovacs colour) into red indicated positive result for indole test. Citrate utilisation was suggested by a dark blue colour after incubation; if citrate was not utilised, the media remained green (no turbidity). Similarly, urea utilisation by the organisms was indicated by a change in colour of urea media from

yellow into pink. MR test was used to distinguish bacteria that can ferment glucose vigorously, resulting in a rapid decrease in pH value. When MR was added in an overnight bacteria culture, a positive result for MR was indicated by a red colour inoculum (acidic reaction), and a negative result was indicated by a yellow colour of the inoculum.

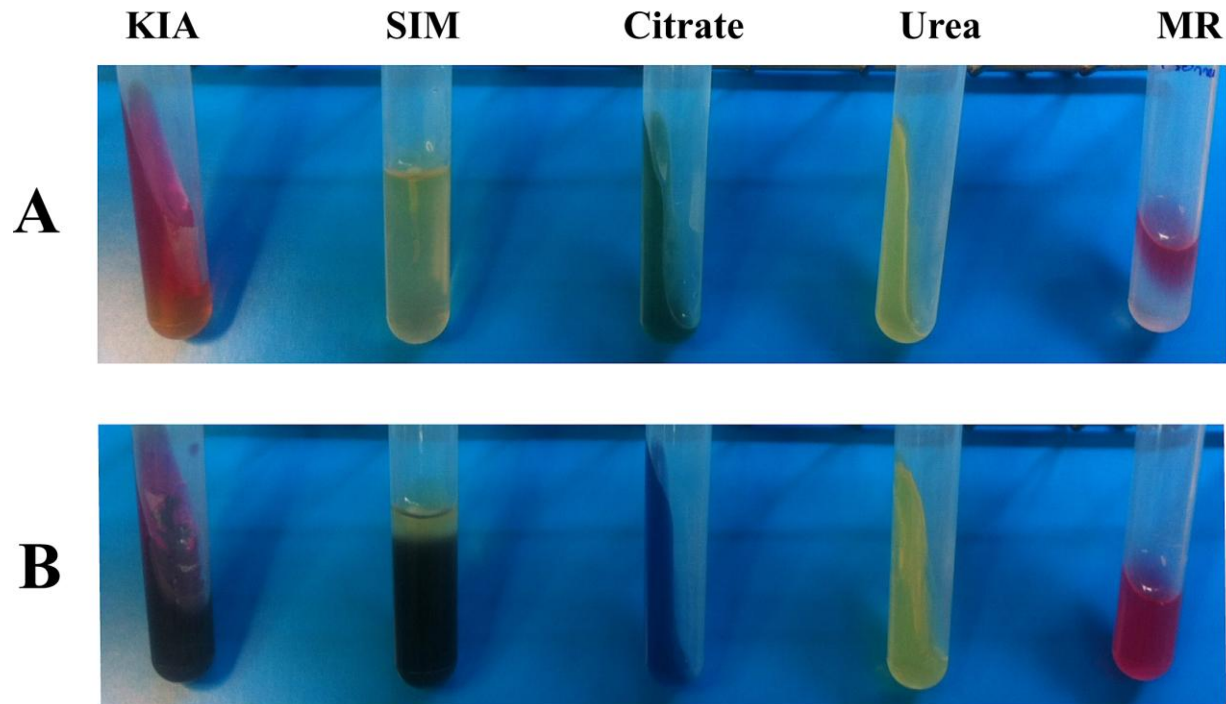


Figure 2-2. Short sets of biochemical testing of *Shigella* spp. and *Salmonella* spp.

Images showing short biochemical sets, after addition of Kovacs' reagent into SIM media and methyl red into overnight bacterial culture (MR), indicating (A) *Shigella* spp. (ATCC 25931): Alk/A formation in the KIA media (utilisation of glucose but not lactose); no gas and H_2S formation (KIA); no motility and negativity for indole (SIM); no utilisation of citrate (Citrate) and urea (Urea); and positivity for MR; (B) *Salmonella* spp. (ATCC 14028): Alk/A formation in the KIA media (utilisation of glucose but not lactose), gas production (bottom of the tube) and strong H_2S formation (KIA); negativity for indole but positivity for motility (SIM); utilisation of citrate but no utilisation of urea; and positivity for MR.

2.3.2.3. Identification of *Enterobacteriaceae* using the API 20E

Bacterial identity was further confirmed using the commercial API 20E test strip of biochemical reactions for the *Enterobacteriaceae* and other non-fastidious Gram-negative bacilli (bioMerieux, Paris, France). The test strip contains 20 individual microtubes of dehydrated substrates including ONPG, ADH, LDC, ODC, CIT, H2S, URE, TDA, IND, VP, GEL, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA (Table 2-1). The procedure was performed following the manufacturer's recommendations (bioMerieux). Briefly, single colonies of test bacteria were suspended in 5 ml sterile saline, mixed thoroughly to create homogenous bacterial suspension and then the tubes were filled (not the cupule unless otherwise indicated) of 20 test substrates. Precautions were taken for (i) CIT, VP and GEL tests by filling bacterial suspension in both tube and cupule; for (ii) ADH, LDC, ODC, H_2S and URE by overlaying the tests with mineral oil. About 5 ml of sterile water was spread evenly over the honeycombed wells of the test strip to provide sufficient humidity before the strip box was placed in the incubator at 37 ^{o}C overnight. After 24 hours of incubation, the strip results were read and the 21 test results, including oxidase (Ox) test to detect cytochrome c oxidase, were subsequently converted to a 7-digit numerical profile following the manufacturer's instructions (Figure 2-3).

Table 2-1. API 20E testing system.

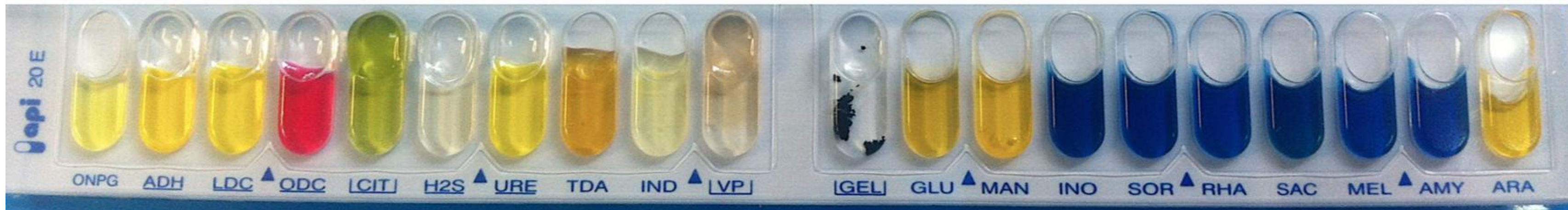
Test	Active ingredients	Note
ONPG	2-nitrophenyl-βD-galactopyranoside	
ADH †	L-arginine	
LDC †	L-lysine	
ODC †	L-ornithine	
CIT *	Trisodium citrate	
H_2S †	Sodium thiosulfate	
URE †	Urea	
TDA	L-tryptophane	After incubation, add 1 drop of TDA reagent and read the result immediately
IND	L-tryptophane	After incubation, add 1 drop of JAMES reagent and read the result immediately
VP *	Sodium pyruvate	After incubation, add 1 drop each of VP 1 and VP 2 reagents. Wait for 10 minutes and then read the result.
GEL *	Gelatin (bovine origin)	
GLU	D-glucose	
MAN	D-mannitol	
INO	Inositol	
SOR	D-sorbitol	
RHA	L-rhamnose	
SAC	D-sucrose	
MEL	D-melibiose	
AMY	Amygdalin	
ARA	L-arabinose	

* Fill both tube of cupule of these tests with the bacterial suspension

† Overlay these tests with mineral oil to create anaerobic environment

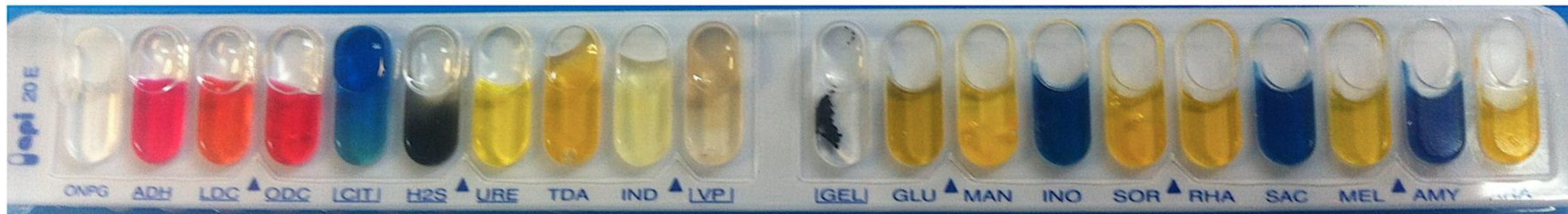
Every 3 tests of different reagents form 1 number, making up a 7-digit numerical profile for 21 tests.

A



+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	**Ox -**
1	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**4**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**0**
	1			**1**			**0**			**4**			**1**			**0**			**2**	

B



-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+	**Ox -**
0	**2**	**4**	**1**	**2**	**4**	**0**	**0**	**0**	**0**	**0**	**4**	**1**	**0**	**4**	**1**	**0**	**4**	**0**	**2**	**0**
	6			**7**			**0**			**4**			**5**			**5**			**2**	

Figure 2-3. The API 20E strip tests confirmation.

Images showing API 20E test strip results confirming *Shigella sonnei* ATCC 25931 (A; 1104102) and *Salmonella* spp. ATCC 14028 (B; 6704552).

2.3.2.4. Sero-grouping of bacterial isolates

For all isolates biochemically confirmed as *Shigella* spp. and *Salmonella* spp. (short sets and API 20E), serologic identification was performed by slide agglutination with antigen grouping sera and monovalent antisera for specific serotype identification (Denka Seiken, Japan). In short, single colonies of confirmed identities were picked from the nutrient agar plate and mixed with a drop of the appropriate testing agglutinating sera placed on a sterile glass slide. The slide was gently rotated following thorough mixing for 10 – 15 seconds, and checked for agglutination with naked eyes. *Shigella* spp. isolates were serotyped with polyvalent group A (*S.dysenteriae*), B (*S.flexneri*), C (*S.boydii*) and D (*S.sonnei*) according to the manufacturer's instructions (Denka Seiken, Japan). *Salmonella* spp isolates were serotyped with polyvalent O and Vi following the manufacturer' s protocols (Denka Seiken, Japan).

Campylobacter jejuni was differentiated from *Campylobacter coli* by sodium hippurate hydrolysis test. This test checks for the bacteria ability to hydrolyse hippurate to benzoic acid and glycine; the presence of glycine byproduct of the reaction is confirmed by an addition of ninhydrin reagent [331]. Briefly, a loopful of *Campylobacter* colonies sub-cultured on BA plates after overnight incubation was inoculated in an eppendorf tube containing 0.5 ml of 1 % aqueous sodium hippurate (Sigma-Aldrich, Singapore), followed by incubation at 37 ^{o}C in a water-bath for 2 hours. After the required incubation, 0.2 ml of ninhydrin agent solution (3.5 % ninhydrin in a 1:1 mixture of acetone and butanol (Sigma-Aldrich, Singapore) was slowly pipetted down the tube side to create an overlay (no mixing). Following an additional incubation in a water-bath at 37 ^{o}C for 10 minutes, the tube was examined for an appearance of dark blue/violet colour indicating a positive test; a negative test

result was indicated by a faint blue colour change or no colour change [331, 332]. A positive hydrolysis test confirmed *Campylobacter jejuni* strains, while negative hydrolysis classified *Campylobacter* strains as *Campylobacter coli*.

2.3.3. Antimicrobial susceptibility testing

2.3.3.1. Disc diffusion method

Antimicrobial susceptibility testing of all bacterial isolates was performed by Kirby Bauer disc diffusion method according to guidelines established by Clinical and Laboratory Standards Institute (CLSI) [333] using commercial antimicrobial discs (Oxoid). The disc diffusion was performed on Mueller-Hinton (MH) plates for bacteria isolates excluding *Campylobacter* spp. Plates of Mueller-Hinton with 5 % addition of sheep blood (MH-blood plates) were used for antimicrobial susceptibility testing for *Campylobacter* spp. isolates following similar procedures of disc diffusion method for testing other bacteria species. Single colonies from the overnight plated cultures were suspended in 1 ml of sterile distilled water and mixed thoroughly to obtain a homogenous suspension of 0.5 McFarland standard. The mixed inoculum was swabbed evenly over the surface of the agar plate in three different directions. Plates were set to dry on bench before placing antimicrobial discs. After incubating for 16 hours at 42 ^{o}C (for *Campylobacter* spp. isolates on MH-blood plates) or 37 ^{o}C (for all other bacterial species on MH plates), the diameter of the clear zones of inhibition around the antimicrobial discs on the growth was measured according to CLSI guidelines [333]. *Streptococcus pneumoniae* ATCC 49619 and *E. coli* ATCC 25922 were used as control strains for antimicrobial sensitivity assays for macrolides and for other antimicrobial classes, respectively. The breakpoints of antimicrobials used in this study are shown in Table 2-2.

2.3.3.2. Minimum inhibitory concentration (MIC) by E-tests

The minimum inhibitory concentration (MIC) of each bacterial isolate was determined to describe the efficacy of an antimicrobial agent against a particular bacterial strain, using commercial E-test strips (AB Biodisk, Solna, Sweden). A total of twelve selected antimicrobials were used for the test including Ampicillin (AMP 10 μg, MIC range 0.016-256 μg/ml), Amoxicillin-Clavulanic acid (AUG 20/10 μg, MIC range 0.016-256 μg/ml), Azithromycin (AZT 15 μg, MIC range 0.016-256 μg/ml), Ceftriaxone (CRO 30 μg, MIC range 0.016-256 μg/ml), Ceftazidime (CAZ 30 μg, MIC range 0.016-256 μg/ml), Chloramphenicol (CHL 30 μg, MIC range 0.016-256 μg/ml), Ciprofloxacin (CIP 5 μg, MIC range 0.002-32 μg/ml), Erythromycin (ERY 15 μg, MIC range 0.016-256 μg/ml), Gatifloxacin (GA 5 μg, MIC range 0.002-32 μg/ml), Gentamicin (CN 10 μg, MIC range 0.016-256 μg/ml), Ofloxacin (OFX 5 μg, MIC range 0.002-32 μg/ml), Nalidixic acid (NA 30 μg, MIC range 0.016-256 μg/ml), and Trimethoprim-sulfamethoxazole (SXT 25 μg, MIC range 0.002-32 μg/ml) (Table 2-2).

Similar to the disc diffusion method, bacterial suspension (0.5 McFarland standard of turbidity) was swabbed on MH-blood plates for *Campylobacter* spp. isolates and on MH plates for other isolated bacteria species. The plates were set to dry at room temperature before E-test strips were placed, 2 strips for each plate. Plates were incubated for 16 hours at 42 ^{o}C (MH-blood plates) or at 37 ^{o}C (MH plates). The MICs were read at the point of inhibition of all growth and interpreted according to CLSI guidelines [333] (Figure 2-4).

2.3.3.3. Test for ESBL production

All strains were subjected to phenotypic test to confirm production of extended-spectrum β-lactamase (ESBL) enzyme by double-disc synergy test, i.e. Ceftazidime (CAZ 30 μg) placed 20 mm (centre to centre) from Ceftazidime-Clavulanic acid (CAZ-CLA 30/10 μg), and Cefotaxime (CTX 30 μg) placed 20 mm from Cefotaxime-Clavulanic acid (CTX-CLA 30/10 μg). Isolates that showed an increase in diameter of inhibitory zone of ≥ 5 mm by the synergy of clavulanate were reported as ESBL positive [334]. If production of ESBL enzyme was confirmed in a bacterial isolate, the phenotypic susceptibility for AMP, AUG, CAZ and CRO of that isolate was reported as resistant irrespective of the diameter of zone of inhibition. *E. coli* ATCC 25922 was used as the control strain for antimicrobial sensitivity and ESBL producing test assays.

Table 2-2. Zone diameter interpretive standard according to CLSI guidelines.

Antimicrobial Agents		Disc content	Zone diameter, Nearest Whole (mm)			Equivalent MIC Breakpoints (µg/ml)	
			R	I	S	R	S
Ampicillin	AMP	10 µg	≤ 13	14 – 16	≥ 17	≥ 32	≤ 8
Amoxicillin-clavulanic acid	AUG	20/10 µg	≤ 13	14 – 17	≥ 18	≥ 32/16	≤ 8/4
Azithromycin	AZT	15 µg	≤ 13	14 – 17	≥ 18	≥ 2	≤ 0.5
Ceftriaxone	CRO	30 µg	≤ 13	14 – 20	≥ 21	≥ 64	≤ 8
Ceftazidime	CAZ	30 µg	≤ 14	15 – 17	≥ 18	≥ 32	≤ 8
Chloramphenicol	CHL	30 µg	≤ 12	13 – 17	≥ 18	≥ 32	≤ 8
Ciprofloxacin	CIP	5 µg	≤ 15	16 – 20	≥ 21	≥ 4	≤ 1
Erythromycin	ERY	15 µg	≤ 15	16 – 20	≥ 21	≥ 1	≤ 0.25
Gatifloxacin	GA	5 µg	≤ 14	15 – 17	≥ 18	≥ 8	≤ 2
Gentamicin	CN	10 µg	≤ 12	13 – 14	≥ 15	≥ 8	≤ 4
Ofloxacin	OFX	5 µg	≤ 12	13 – 15	≥ 16	≥ 8	≤ 2
Nalidixic acid	NA	30 µg	≤ 13	14 – 18	≥ 19	≥ 32	≤ 8
Trimethoprim-Sulfamethoxazole	SXT	1.25/23.75 µg	≤ 10	11 – 15	≥ 16	≥ 8/152	≤ 2/38

R: resistance; I: intermediate; S: sensitive.

Zone diameter interpretive standard and equivalent minimal inhibitory concentration (MIC) breakpoints for *Enterobacteriaceae* for used antimicrobials apart from Azithromycin and Erythromycin and *Streptococcus pneumoniae* ATCC 49619 (for Azithromycin and Erythromycin).

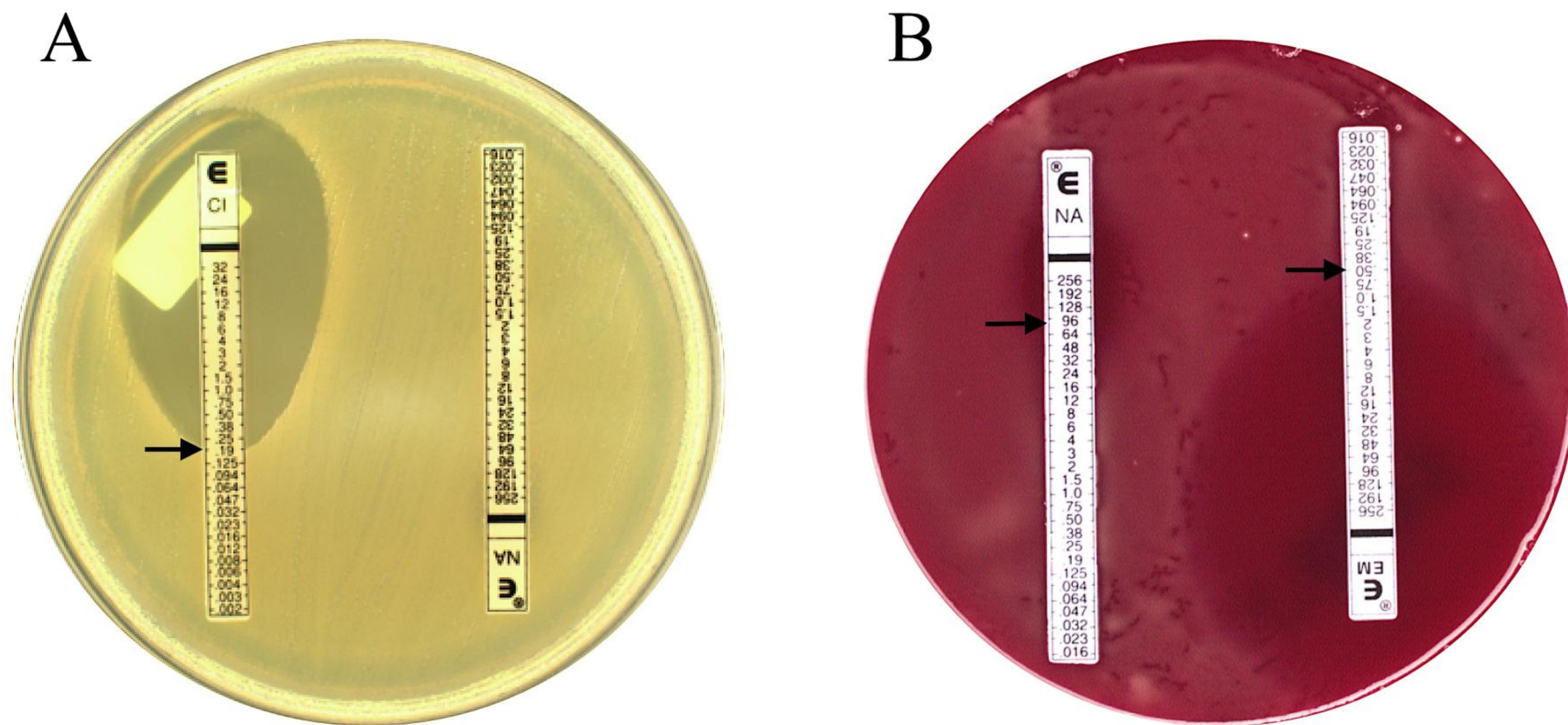


Figure 2-4. Representative images for MIC E-test for examining of antimicrobial agents.

(A) MH plate shows the MIC E-test of *E. coli* ATCC 25922 strain to NA (MIC range 0.016-256 μg/ml; MIC = 256 μg/ml) and CIP (MIC range 0.002-32 μg/ml; MIC = 0.19 μg/ml). (B) MH-blood plate shows the MIC E-test of *Campylobacter jejuni* strain ID C2115 to NA (MIC range 0.016-256 μg/ml; MIC = 96 μg/ml) and ERY (MIC range 0.016-256 μg/ml; MIC = 0.5 μg/ml). MIC read is indicated by the arrow.

2.4. Virus identification

2.4.1. Enzyme Immuno Assay (EIA) direct antigen detection

Stool samples were screened for group A RoV, NoV (GI and GII), enteric AdV and AstV using commercial enzyme immune assay (EIA) kits (IDEIA™) (Oxoid; Thermo Fisher Scientific, Ely, United Kingdom). Approximately 100 µl of each stool sample was diluted in 900 µl of sample diluent solution provided in the IDEIA™ kit, and the assays were performed according to the manufacturer's instructions (Figure 2-5).

The results of the assays (Figure 2-6) were determined by measuring the absorbance of each micro-well on the plate at 450 and 640 nm using the Bio-rad plate reader, model 550 (Bio-rad, Hertfordshire, UK). The positive/negative cut-off value was determined by adding 0.1 absorbance units to the negative control value. Stool specimens that had an absorbance value greater than the cut-off value were considered positive. Specimens with an absorbance value within 0.01 absorbance units of the cut-off value were interpreted as equivocal, and were tested further using EIA and RT PCR as recommended by the manufacturer.

A

B

Figure 2-5. The EIA kits for direct antigen detection of NoV (GI and GII) (A) and group A RoV (B).

Sources of images: www.oxoid.com. Each EIA kit contains a microtitration plate (96 micro-well plate coated with virus specific polyclonal antibody), sample diluent solution (Tris Buffered Saline [TBS] containing antimicrobial agent and red dye), positive control, negative control (TBS), conjugate solution, substrate TMB, stop solution and wash buffer (Phosphate buffered solution with antimicrobial agent and detergent). For EIA NoV (A) kit: http://www.oxoid.com/UK/blue/press/press.asp?art=Y&arch=N&pRef=PR0339&c=UK&lang=EN. For EIA RoV (B) kit:

http://www.oxoid.com/uk/blue/press/press.asp?art=Y&arch=&pRef=PRPROSPT&c=UK&lang=EN.

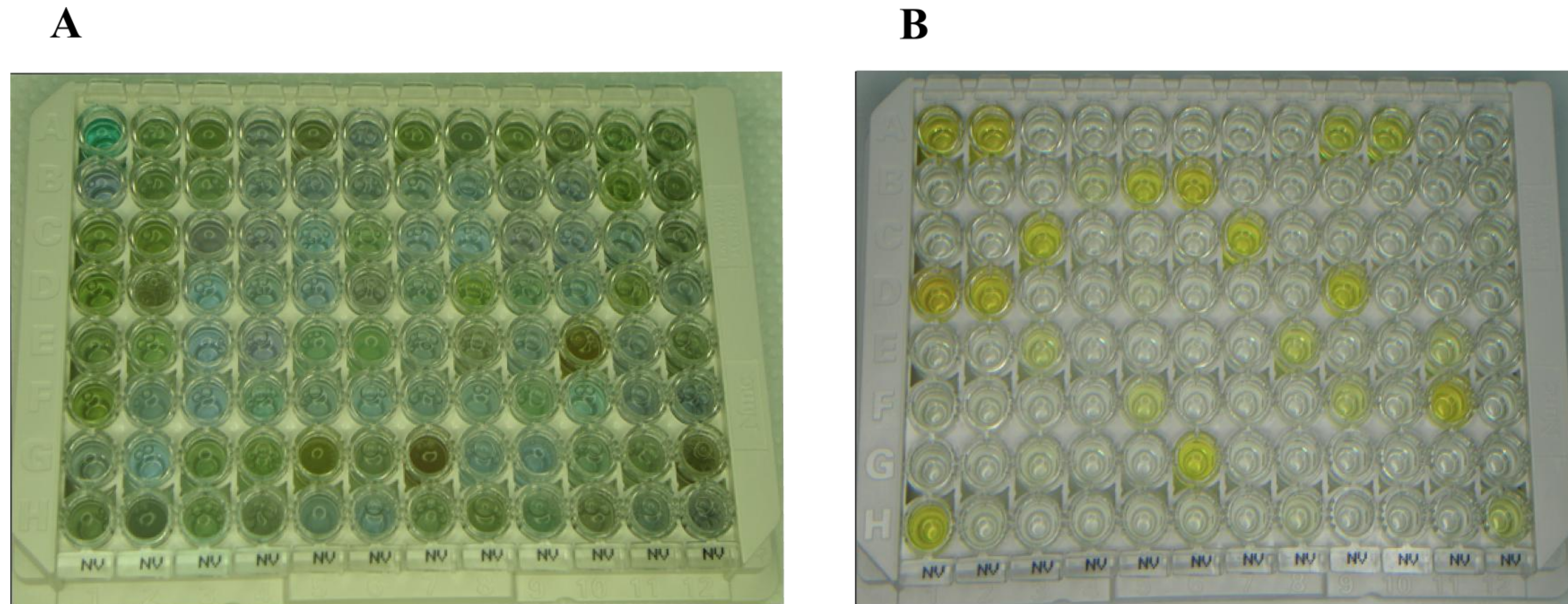


Figure 2-6. Illustration of the EIA assay plate for NoV.

Images showing the assay plates for NoV (GI and GII) after addition of conjugate substrate (A) and after addition of stop solution (B). For all assay plates, microtube A-1 is the positive control (top left microtube), and microtube B-1 is the negative control (second top left microtube). The microtubes may be assessed visually by comparing the colour intensity to the negative control B-1 (plate A). However, it is recommended by the manufacturer that the plate should be read photometrically to interpret the colour intensity (plate B; a yellow colour indicating a positive result) for a more accurate assessment, particularly for those microtubes in which the colour intensity is difficult to interpret when compared with the negative control. Dual wavelength photometrical reading is also suggested to exclude any potential interference such as dirt or marks on the optical surface of the microtubes.

2.4.2. Molecular detection of viruses using Polymerase Chain Reaction

2.4.2.1. Total viral RNA extraction and Reverse Transcription

Total viral RNA was extracted from 10 % (in PBS) faecal specimens using the QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany) and eluted in 60 µl Elution Buffer (EB) (provided in the kit) according to the manufacturer's recommendations. RNA preparations were converted to complementary DNA (cDNA) by reverse transcription (RT), and an aliquot of RNA of each sample was stored at -80 ^{o}C until required.

The total viral RNA extracted from stool samples was reverse transcribed into cDNA through two steps with reagents described in Table 2-3 for a total reaction volume of 20 µl. Briefly, 8 µl of extracted RNA was combined with 5 µl of Mix 1 (Table 2-3) containing random hexamers and dNTPs (Roche Diagnostics, Burgess Hill, UK) and distilled water. The RNA solution was heated to 97 ^{o}C for 5 minutes and snap cooled on ice for at least one minute. After cooling, the incubation solution was added to 7 µl of Mix 2 (Table 2-3) containing First Strand Buffer, DTT, RNase Inhibitor, SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and distilled water. This step was carried out on ice, and the final solution was reverse transcribed at 25 ^{o}C for 10 minutes, followed by 1 hour at 50 ^{o}C and 70 ^{o}C for 15 minutes. The resulting cDNA were stored at -80 ^{o}C until required.

Table 2-3. Reagents for reverse transcription PCR.

	Reagents	Stock concentration	Final concentration	Volume for 1 reaction	Supplier
Mix 1	Random hexamers	200 ng/µl	20 ng/µl	2 µl	Roche Diagnostics
	dNTPs	20 mM each	1 mM	1 µl	Roche Diagnostics
	Distilled water			2 µl	In-house
Mix 2	First strand buffer	5X	1X	4 µl	Invitrogen
	DTT	0.1 M	0.005 M	1 µl	Invitrogen
	RNase Inhibitor	40 U/µl	16 U	0.4 µl	Invitrogen
	SuperScript III reverse transcriptase	200 U/µl	40 U	0.2 µl	Invitrogen
	Distilled water			1.4 µl	In-house

2.4.2.2. RoV detection by Reverse Transcription Polymerase Chain Reaction (RT PCR)

2.4.2.2.1. Amplification of VP7 and VP4 using one-step RT PCR

Extracted total viral RNA was subjected to one step reverse-transcriptase PCR (RT PCR) in order to amplify the two outer capsid genes VP7 and VP4 using primers (Table 2-7) and amplification conditions as previously described for VP7 [335] and VP4 [336]. Amplification of VP7 and VP4 regions was performed individually, generating amplicons of approximately 881 bp and 663 bp, respectively.

The methodology for amplification was as follows; viral RNA (2.5 µl) was mixed with 13 µl of master mix containing 1 µl dNTP (20 mM each) (Roche Diagnostics), 1 µl of forward primer of 10 µM and 1 µl of reverse primer of 10 µM and 10 µl distilled water. The reaction mixture was heated to 97 ^{o}C for 3 minutes followed by immediate snap cooling on ice for 1 minute. The solution was then combined with another master mix comprised of 10 µl SuperScript III 5X buffer (Invitrogen), 3.5 µl DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, Singapore), 2.5 µl 0.1 M DTT (Invitrogen), 1 µl RNase Inhibitor (40 U/µl) (Invitrogen), 0.5 µl SuperScript III polymerase (200 U/µl) (Invitrogen), 0.5 µl Taq polymerase 5 U/µl (Bioline, US) and was brought up to 50 µl with distilled water.

2.4.2.2.2. Amplification of VP7 and VP4 using two-step RT PCR using cDNA

Viral cDNA was subjected to PCR amplification of rotavirus VP7 and VP4 genes using primers (Table 2-7) as described in section 2.4.2.2.1. PCR amplification was performed using 5 µl of cDNA in a 45 µl mixture containing 10xNH_4 buffer, 50

mM of $MgCl_2$, dNTPs (20 mM each), 10 µM of forward primer, 10 µM of reverse primer, 5 U/µl of Taq polymerase (Bioline, US) and distilled water (dH_2O) as described in Table 2-4. For each sample, PCR mixtures were prepared separately for VP7 and VP4 amplification following different thermal cycling programs listed in Table 2-5.

2.4.2.2.3. Agarose gel electrophoresis

PCR amplicons and DNA marker (Invitrogen) were run on 2 % agarose gels (2 % w/v DNA grade agarose, 0.5X Tris-borate-EDTA (TBE) buffer) for 30 – 45 minutes at 150 V. Gels were stained with 3 % ethidium bromide before visualised under UV light on UV transilluminator and photographed using Quantity One UVTech DNA documentation program.

Table 2-4. The PCR mixture preparation for RoV VP7 and VP4 amplifications.

Reagents	Stock concentration	Final concentration	Volume for 1 reaction	Supplier
NH_4 Buffer	10X	1X	5 µl	Bioline
$MgCl_2$	50 mM	2 mM	2 µl	Bioline
dNTPs	20 mM each	0.32 mM	0.8 µl	Roche Diagnostics
Forward primer *	10 µM	0.12 µM	0.6 µl	Sigma
Reverse primer *	10 µM	0.12 µM	0.6 µl	Sigma
Sample cDNA			5 µl	
Taq polymerase	5 U/µl	2.5 U	0.5 µl	Bioline
dH_2O up to 50 µl			35.5 µl	In-house
Total volume			50 µl	

* The amplification primers for each corresponding gene (VP7 or VP4) were listed in Table 2-7.

Table 2-5. The thermal cycling programs for amplications of RoV and NoV.

	RoV VP7 gene	**RoV VP4 gene**
	1 cycle of 95 ^{o}C for 5 minutes	
35 cycles	94 ^{o}C for 1 minute	94 ^{o}C for 1 minute
	42 ^{o}C for 2 minutes	50 ^{o}C for 2 minutes
	68 ^{o}C for 1 minute	72 ^{o}C for 1 minute
	1 cycle of 72 ^{o}C for 10 minutes	
	Held the reaction at 12 ^{o}C	

2.4.3. NoV detection by RT PCR

Consensus primers G1SKF/G1SKR and COG2F/G2SKR were used to detect NoV GI and GII, respectively, in separate conventional RT PCR reactions [171, 337] (Table 2-7). The G1SKF/G1SKR primers targeted a region between position 5,342 and 5,671 (330 bp) relative to the genome of NoV GI Norwalk/68 (GenBank accession no. M87661), containing 17 bp overlap of 3' end ORF1 and 313 bp of 5' end ORF2 [171] (see Figure 1-4 for schematic genome organisation of NoV GI and GII). The COG2F/G2SKR primers amplified a region between position 5,003 and 5,389 (387 bp) relative to the genome of NoV GII Lordsdale/93 (GenBank accession no. X86557), containing a 83 bp overlap of 3' end ORF1 and 304 bp of 5' end ORF2 [171, 337] (see Figure 1-4). The amplifying regions contained the genomic region C of the ORF2 for routine NoV diagnostics and genotyping (as mentioned in the NoV classification, section 1.2.2.2).

The PCR mixtures for amplifying GI and GII were prepared separately with concentrations of each reagent (Bioline, US) as listed in Table 2-6. The DNA amplification thermal cycling was performed as following program:

i. 1 cycle of 95 ^{o}C for 5 minutes.

ii. 35 cycles of 94 ^{o}C for 30 seconds, 50 ^{o}C for 30 seconds, and 72 ^{o}C for 30 seconds.

iii. 1 cycle of 72 ^{o}C for 10 minutes.

iv. Held at 12 ^{o}C.

Agarose gel electrophoresis and DNA amplicons visualisation were performed as described in aforementioned section 2.4.2.2.3.

Table 2-6. The PCR mixture preparation for NoV GI and GII amplifications.

Reagents	Stock concentration	Final concentration	Volume for 1 reaction	Supplier
NH_4 Buffer	10X	1X	5 µl	Bioline
$MgCl_2$	50 mM	2 mM	2 µl	Bioline
dNTPs	20 mM each	0.24 mM	0.6 µl	Roche Diagnostics
Forward primer *	10 µM	0.08 µM	0.4 µl	Sigma
Reverse primer *	10 µM	0.08 µM	0.4 µl	Sigma
Sample cDNA			6 µl	
Taq polymerase	5 U/µl	2.5 U	0.5 µl	Bioline
dH_2O up to 50 µl			35.1 µl	In-house
Total volume			50 µl	

* The amplification primers for each corresponding GI and GII were listed in Table 2-7.

Table 2-7. Oligonucleotide primers used for RoV (VP7 and VP4) and NoV (GI and GII) RT PCR detection.

Gene target	Primer name	Sequence 5' to 3'	Binding site	Size of amplicon	Reference
RoV-**VP7**	VP7F	ATGTATGGTATTGAATATACCAC	49-71	881 bp	[335]
	VP7R	AACTTGCCACCATTTTTTCC	932-914		[335]
RoV-**VP4**	VP4F	TATGCTCCAGTNAATTGG	132-149	663 bp	[336]
	VP4R	ATTGCATTTCTTTCCATAATG	795-775		[336]
NoV-**GI**	G1-SKF	CTGCCCGAATTYGTAAATGA	5,342-5,361	330 bp	[171]
	G1-SKR	CCAACCCARCCATTRTACA	5,671-5,653		[171]
NoV-**GII**	COG2F	CARGARBCNATGTTYAGRTGGATGAG	5,003-5,028	387 bp	[337]
	G2-SKR	CCRCCNGCATRHCCRTTRTACAT	5,389-5,367		[171]

List of degenerate nucleotides: R=A/G; Y=C/T; B=C/G/T; H=A/C/T; N=any nucleotide.

2.4.4. Direct DNA sequencing

PCR amplicons from successful PCR amplifications were DNA sequenced using amplification primers. PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN, Germany) (section 2.4.4.1), and DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, United Kingdom) (section 2.4.4.2). Direct DNA sequencing (section 2.4.4.3 below) was performed using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's recommendations.

2.4.4.1. PCR purification

PCR amplicons were purified using the QIAquick PCR purification kit following the manufacturer's instructions (QIAGEN, Germany). All amplicons were applied on the PCR purification column and were eluted in 30 µl of distilled water.

2.4.4.2. DNA quantification by spectrophotometer

Purified DNA were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.), which is a cuvette free spectrophotometer. Distilled water was used as a blank. The DNA concentration was calculated by the computer based on the following formula: $c = \frac{A260\ x\ 50}{0.1}$

Where c is the nucleic acid concentration in ng/µl, A260 is the absorbance in AU, and the path length is 0.1 cm. The wavelength-dependent extinction coefficient of double stranded DNA is 50 ng-cm/µl.

2.4.4.3. DNA sequencing

2.4.4.3.1. PCR reaction for sequencing

Amplification for sequencing was performed using purified PCR products with determined concentration of around 20-30 ng/μl, following the below

Reagents	Stock concentration	Final concentration	Volume for 1 reaction
ABI Buffer *	5X	1X	2 μl
BigDye Premix *	2.5X	1X	4 μl
Primer	10 μM	0.3 μM	0.3 μl
PCR product		20-30 ng	varied
Distilled H_2O up to 10 μl			varied
Total volume			10 μl

* Buffer and BigDye premix were supplied in the BigDye Terminator Cycle Sequencing kit (Applied Biosystems).

DNA amplifications were performed using the following programme:

v. 1 cycle of 96 °C for 1 minute.

vi. 30 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes.

vii. Held at 12 °C.

2.4.4.3.2. Ethanol precipitation and DNA sequencing

PCR amplicons from section 2.4.4.3.1 were purified by ethanol precipitation. All the procedure steps were performed on ice. Briefly, for each 10 µl of a sequencing reaction, 2 µl of stop solution, composing 3M NaOAc (1 µl) and 100 mM EDTA (1 µl), were added. The solution was mixed thoroughly followed by an addition of 35 µl of cold 95 % ethanol. The pellet was collected after centrifugation at 14,000 rpm for 15 minutes in a tabletop microcentrifuge (Eppendorf) at 4 ^{o}C and washed with 200 µl of cold 70 % ethanol for two times. For each wash, the microfuge tube was drained of all liquid solution and was centrifuged immediately at 14,000 rpm for 2 minutes at 4 ^{o}C. The DNA pellet was allowed to dry for 3 minutes before being rehydrated in 10 µl of sample loading solution (Hi-Di) and was incubated at room temperature for at least 15 minutes before loading on the plate for sequencing in the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, US).

2.4.5. Sequence analysis

2.4.5.1. Sequence comparisons and validation

DNA sequences were generated using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, US), and the resulting sequences were assembled using DNA Baser Sequence Assembler v3.0.17 (Heracle Biosoft, Pitesti, Romania).

2.4.5.2. RoV genotype determination

All RoV VP7 and VP4 sequences were genotyped using the online RotaC v2.0 rotavirus genotyping tool (available at http://rotac.regatools.be) [44] according to the recommendations of the Rotavirus Classification Working Group (RCWG).

All RoV sequences for Chapter 3 were submitted to EMBL (GenBank accession numbers, VP4: FR820957 to FR821065 and VP7: FR822209 to FR822321). The resulting VP4 and VP7 strains were additionally compared with other corresponding genotype sequences using BLASTn (available at https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4.5.3. NoV genotype determination

NoV genotypes were assigned based on the amplification of partial ORF2 sequences using the online Norovirus Automated Genotyping Tool v1.0 (available at http://www.rivm.nl/mpf/norovirus/typingtool), on the RIVM-NoroNet page [160]. All NoV sequences (N = 315) for Chapter 6 were submitted to EMBL (GenBank accession numbers, HE716437 to HE716751).

2.4.5.4. Sequence alignment using ClustalW and Se-AL

Coding sequences were initially aligned using ClustalW function in Mega 5 program [338], from which the alignment was manually refined using Se-AL v2.0a11 (available at http://tree.bio.ed.ac.uk/software/). Additional sequences (global sequences of comparing genotypes or strains retrieved from GenBank) were trimmed to correspond with the sequences identified in this thesis to maximise sequence homology.

2.4.5.5. Phylogenetic construction using RAxML

Maximum likelihood (ML) trees for each of the RoV genotypes (G1, G12 and

P[8]) (Chapter 3) were inferred using RAxML v7.0.4 [339] employing the general-time reversible model of nucleotide substitution with a γ-distribution of among-site rate variation (GTR+Γ), which was indicated by jModelTest [340] according to AICs (Akaike Information Criterion) to be the best-fit model to the data. One thousand bootstrap replicates were implemented using a rapid bootstrap algorithm available in RAxML. For VP7 G1 genotype, 81 sequences amplified (Chapter 3) using the primers described for RoV VP7 amplification (section 2.4.2, Table 2-7) in addition to 392 global RoV G1 sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov/), all of which were trimmed to 850 bp, were included for ML tree construction. For VP7 G12 genotype, 19 sequences detected (Chapter 3) using the amplification primers for VP7 (section 2.4.2, Table 2-7) and 137 global G12 sequences (from GenBank), trimmed to 847 bp, were included in this phylogenetic construction. For VP4 P[8] genotype, 162 global P[8] sequences (from GenBank) in addition to 96 sequences (Chapter 3) generated from VP4 amplifications (section 2.4.2, Table 2-7), all of which were trimmed to 639 bp, were included for ML tree construction.

ML trees were also inferred for NoV using RAxML v7.0.4 [339], employing the general-time reversible model of nucleotide substitution with a γ-distribution of among-site rate variation (GTR+Γ), again determined as the best-fit model using jModelTest according to AICs [340]. One thousand bootstrap replicates were implemented in the rapid bootstrap algorithm available in RAxML [339]. All GI and GII sequences identified from NoV-positive cases and controls (N = 315; Chapter 6) using the amplification primers for GI and GII (section 2.4.3, Table 2-7) were included in the primary ML tree construction. From this primary phylogenetic tree, a secondary phylogenetic tree was constructed based on sub-sampled strains (N = 109

GII strains) by removing GI sequences and identical GII sequences, to enable the focus on distinguishing viruses identified from cases or controls.

All RoV (VP7 G1 and G12, and VP4 P[8]) and NoV (GI and GII) sequences used for ML tree constructions, generated using amplification primers (section 2.4.3.3), were partial coding sequences; thus global sequences retrieved from GenBank were trimmed to correspond with the study sequences for phylogenetic construction. Global strains were retrieved from GenBank and selected to encompass the greatest diversity of the comparing genotype or strain. Resulting trees were visualized in FigTree v1.3.1 (available at http://tree.bio.ed.ac.uk/software/figtree/) and mean (uncorrected) pairwise genetic distances were estimated in using HyPhy v2.0 (available at http://www.datam0nk3y.org/hyphy/doku.php) [341] (Chapter 3) or in Mega 5 [338] (Chapter 6).

2.4.5.6. BEAST analysis for NoV GII.4

All available NoV GII.4 of either partial (genomic region C) or full length ORF2 sequences were retrieved from GenBank, from which 269 strains encompassing the global diversity of GII.4 variants were selected. In addition, available archived stool samples that were EIA-positive for NoV (section 2.4.1) from the work in Chapter 3 were re-confirmed by RT PCR, sequenced and genotyped using method described in section 2.4.3, 2.4.4, and 2.4.5.3; based on which a selection of GII.4 sequences (10 strains) were included for this BEAST analysis. The time of isolation for each selected global NoV strains was retrieved from GenBank or the publication associated with the sequence, and the year of isolation was used to

estimate a substitution rate due to inadequate dates information available for all selected sequences.

All sequences (N = 526) were aligned (see section 2.4.5.4) and trimmed to 378 bp for the construction of the phylogenies. Phylogenetic reconstructions of evolutionary relationships among GII.4 variants, compared with global GII.4 sequences, were performed using the Bayesian Markov Chain Monte Carlo (MCMC) method as implemented in BEAST [342]. A GTR substitution model with γ-distribution of among-site rate variation (GTR+Γ), and a relaxed uncorrelated lognormal clock model with a constant population size were employed. The MCMC analysis was run for 50 million generations (with a burn-in of 5 million) and analysed using Tracer (available at http://tree.bio.ed.ac.uk/software/tracer/). Maximum clade credibility (MCC) trees were summarized using TreeAnnotator v1.6.1 (provided within the BEAST packages) and visualised in FigTree v1.3.1. The weighted average substitution rate across all nucleotide sites in the alignment was assessed using Tracer. This represents the evolutionary rate for each branch weighted according to the time represented by that branch, averaged across the tree [343].

2.4.6. Spatiotemporal analysis for NoV (Chapter 6)

2.4.6.1. GPS location

The location of each enrolee's residence was recorded using an eTrex Legend GPS device (Garmin, United Kingdom) and verified by an additional member of the study team. Latitude and longitude of each residence (recorded in decimal degrees) were entered along with patient metadata in Microsoft Excel (Microsoft, Redmond,

US). Location data were converted to KML format and locations were visualized and validated in Google Earth version 5 (available at http://www.google.com/earth /index.html).

2.4.6.2. Mantel tests

Mantel tests were performed to assess potential correlations between genetic, temporal, and spatial distances separately for GII strains and variants within the GII.4 genocluster, using the *ade4* package of R software [344] using available scripts (available at http://www.ats.ucla.edu/stat/r/faq/mantal_test.htm). Mantel tests compare spatial, temporal and genetic distances; more specifically comparing Euclidean distance (latitude/longitude) with temporal distance (days), Euclidean distance with genetic distance, and phylogenetic distance versus temporal distance. These tests were performed for the NoV strains with available GPS coordinates and separately for the GII.4 clade (N = 239) and for GII sequences (N = 279). Matrices of geographical distances (kilometres), and temporal distances (days of isolation) were created in Microsoft Excel (Microsoft, Redmond, US). Corresponding matrices of genetic distances (number of substitutions per site) for NoV GII.4 and NoV GII were separately prepared in Mega 5 [338] based on the nucleotide alignments and subsequently exported into Microsoft Excel format. Two matrices were reported as correlated if the simulated *P* value from Mantel test, based on 9,999 replicates, was ≤ 0.05 with $\alpha = 0.05$.

2.4.6.3. SaTScan test

A Bernoulli model was used to examine spatiotemporal clusters of GII.4-2010, using all non-GII.4-2010 to represent the background distribution NoV population using SaTScan v9.1.1 software (available at http://www.satscan.org/). A spatial scan statistic uses a circular window to represent potential geographic clusters. By continuously varying the size of the window centred sequentially on all locations in the dataset, the geographic area is scanned over time for potential localized clusters without incorporating prior assumptions about size or location. A likelihood ratio is calculated for the hypothesis that there is an increased risk of disease inside the circle against the null hypothesis that there is not. For the purposes of the work in chapter 6, the upper limit for cluster detection was specified as 10 % of the study population over 10 % of the study duration. The most likely cluster was that with the largest likelihood ratio, the cluster least likely to be due to chance. The significance of the detected clusters was assessed by a likelihood ratio test, with a *P* value obtained by 999 Monte Carlo simulations generated under the null hypothesis of a random spatiotemporal distribution.

2.4.7. Statistical analysis

For statistical analyses in all chapters, Chi-squared test (categorical variables) or Fisher’s exact test (categorical variables with a number of observation < 5) were used to compare proportions between groups, and Mann Whitney U test (numeric variables, non-parametric data) to compare median values between different groups, as appropriate.

For Chapter 3, all statistical analyses were performed in R version 2.9.0 [344]; *P* values ≤ 0.05 were considered statistically significant.

For the multi-site hospital-based study (Chapter 4), confidential data was exported into Microsoft Excel (Microsoft, US), and subsequently cleaned and analysed in STATA 9.2 (StataCorp, College Station TX, US). Simple comparisons of demographic, clinical and laboratory characteristics among cases and controls were performed using Chi-squared test or Fisher's exact test, and Mann Whitney U test, as appropriate. Spearman's rank correlation coefficient (Spearman's rho, for non-parametric data) (Chapter 4) or Pearson's correlation coefficient (for parametric data) (Chapter 5) were used to examine the association between the proportion of pathogen detected and the mean temperature/average rainfall accumulation by month. Two-sided *P* values ≤ 0.05 were considered statistically significant throughout.

In Chapter 5, simple tabulations of socio-demographic and behavioural factors were performed. Univariate odds ratios (ORs) were generated using logistic regression to assess exposures associated with symptomatic NoV infection. The presence of interaction was assessed using the test of homogeneity of odds ratios across strata. Subsequently, multivariate logistic regression was performed to identify factors that were independently and significantly associated with symptomatic NoV infection. This analysis simultaneously controlled for not only the three *a-priori* defined founders (age, sex and household income level) but also the other risk factors that were significant (*P* ≤ 0.05) in the univariate analysis. These variables were normally distributed for logistic regression analysis. Two-sided *P* values ≤ 0.05 were considered statistically significant throughout. A limited sensitivity analysis comparing NoV-positive cases to NoV-negative controls and NoV-negative cases to NoV-negative controls to evaluate whether identified risk

factors were NoV-infection specific as opposed to general risk factors for diarrhoeal disease.

2.4.8. Calculations for sensitivity and specificity for RT PCR detection of RoV

Using RoV EIA as a reference standard method for RoV identification, the sensitivity and specificity of RT PCR method for detection of RoV (VP7 and VP4) were calculated based on proposed calculations for evaluating diagnostic tests [345].

By definition, sensitivity refers to the proportion of truly infected samples that were positive when using the RT PCR method; specificity refers to the proportion of truly uninfected samples that were negative under the RT PCR conditions. The sensitivity and specificity measures are expressed as a percentage. Sensitivity and specificity evaluations often include the 95 % confidence interval (95 % CI) ranges.

Similarly important evaluations of method performance are positive predictive value (PPV) and negative predictive value (NPV). PPV measures the probability that those testing positive by the particular method are truly infected, while NPV measures the probability that those testing negative by the test are truly uninfected. Both of these measurements, PPV and NPV, measures are expressed as percentages.

Formulae for calculation of sensitivity, specificity, PPV, NPV and 95 % CI are as follows:

Test under evaluation	**Reference standard test**		**Total**
	Positive	Negative	
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	

Test sensitivity = $\frac{a}{a+c}$

Test specificity = $\frac{d}{b+d}$

PPV = $\frac{a}{a+b}$

NPV = $\frac{d}{c+d}$

95 % CI = $p \pm 1.96\ X\ \frac{\sqrt{p(1-p)}}{n}$

a = true positive; b = false positive; c = false negative; d = true negative

p = sensitivity (or specificity) measured as a proportion (not a percentage)

n = number of samples from infected people or from uninfected people, respectively for sensitivity or for specificity.

3. THE PREVALENCE OF ENTERIC VIRUSES CAUSING ACUTE DIARRHOEAL DISEASE IN CHILDREN IN HCMC AND DT PROVINCE, VIETNAM

3.1. Abstract

Diarrhoea in young children represents an on-going public health challenge, particularly in low and middle-income countries where the burden is greatest, and the majority of these infections are attributed to enteric viruses. The aim of this chapter was to compare the relative prevalence of the major enteric viruses in an urban and a rural location in southern Vietnam. Over a one-month long period, 362 children hospitalised with acute watery diarrhoea in HCMC and DT province were simultaneously enrolled, from which collected faecal specimens were screened for four major viral gastrointestinal pathogens. The results demonstrated that RoV was the predominant virus identified, followed by NoV. A disproportional distribution of enteric viruses was observed between the urban and rural locations. Furthermore, the phylogenetic analysis of RoV sequences was performed to differentiate the strains by the sampling location. Findings from this chapter reported the emergence of RoV G12 virus, provided evidence of differential distribution of viruses by geographical locations and continual importation of new RoV strains into the southern Vietnam.

3.2. Introduction

Among 11 dsRNA segments of RoV genome, genetic divergence of the two capsid proteins, VP7 and VP4, forms the basis of dual genotyping system that differentiate individual RoV strains [44, 45]. Globally, among diverse strains circulating, RoV G1P[8] is the most frequent GP genotype identified from symptomatic human RoV illnesses [33, 46, 56, 113, 114].

The complex transmission and distribution of RoV are suggestively influenced by interactions between various social, demographic and environmental factors [117, 118]. Vietnam is a typical example of a rapidly developing country where the spectrum of infectious agents is concurrently changing [277]. Existing data on enteric viruses, although being sparse, are available in HCMC between 1998 and 2007 [314, 315, 318, 320, 321, 323, 346]. The detection and identification of enteric pathogens are not performed routinely in hospitals, which consequently lead to limited information about the prevalence of enteric viruses and the circulating strains in different geographic and demographic settings in Vietnam.

The aim of this chapter was to investigate the distribution of common enteric viruses (RoV, NoV, enteric AdV, AstV) and RoV genotypes causing diarrhoea in paediatric patients in distinct urban and rural locations in southern Vietnam.

3.3. Results

3.3.1. The prevalence of four enteric viruses

During a one-month period, 362 children from the two locations with acute watery diarrhoea were simultaneously recruited from HCMC and DT (see section 2.2.1), including 252 patients from HCMC and 110 patients from DT. The presence

of major enteric viruses, RoV, NoV, AdV or AstV was detected in 195 samples (53.9 %) using EIA (as described in section 2.4.1) and 8 children had more than one viral pathogen in their stools (Table 3-1). Among samples originating from HCMC (N = 252), RoV was identified in 75 (29.8 %) samples and NoV was identified in 34 (13.5 %) samples (Table 3-1). In 110 samples collected from patients from DT, 72 (65.5 %) and 4 (3.6 %) samples were found positive for RoV and NoV, respectively. A limited proportion of all the acute diarrhoeal cases were positive for enteric AdV and AstV in HCMC (2.4 % and 2.8 %, respectively), and in DT (2.7 % and 1.8 %, respectively).

The age distribution of recruited patients showed similar age structure in both sampling locations. In particular, patients originating from HCMC and from DT had a mean age of 15.8 months (median 13 months; range 2 – 96 months) and 15.3 months (median 10 months; range 1.5 – 156 months), respectively. The preponderance of patients in HCMC (90.6 %) and in DT (94.9 %) was under 24 months of age, with the most common 6-month age group being 7 – 12 months which consisted of 43.6 % (51/117) and 48.7 % (38/78) of the positive samples from HCMC and from DT, respectively. Among cases originating from HCMC, the prevalence of viral diarrhoea was significantly lower in patients < 6 months and > 24 months of age compared to patients in the intermediate age group ($P < 0.001$ for each comparison, Chi-squared test). The prevalence of viral positive samples in cases originating from DT was lower in those > 18 months of age compared to those between 0 and 18 months of age ($P < 0.001$, Chi-squared test). Additionally, in the group of patients less than 6 months of age, the frequency of RoV infections was significantly higher in DT than in HCMC ($P = 0.0289$, Chi-squared test). Conversely, the proportion of RoV-positive detected in the group of patients aged

between 19 and 24 months in HCMC was significantly higher than the proportion detected in the corresponding age group of patients in DT (P = 0.009457, Chi-squared test).

Table 3-1. Results of EIA detection of four enteric viruses in stool specimens collected from recruited paediatric patients with acute diarrhoea in HCMC and DT.

Viral Assay	No. positive samples in HCMC (%) *	No. positive samples in DT (%) †
Group A RoV ¶	75 (29.8)	72 (65.5)
NoV (GI and GII)	34 (13.5)	4 (3.6)
Enteric AdV	6 (2.4)	3 (2.7)
AstV	7 (2.8)	2 (1.8)
Multiple positive	5 (4.3)	3 (3.8)
RoV/AstV	3 (2.6)	1 (1.3)
RoV/AdV	1 (0.85)	1 (1.3)
RoV/NoV	1 (0.85)	0
NoV/AdV	0	1 (1.3)
Total viruses detected	122	81
Total positive samples	117 (46.4)	78 (73.6)

* N = 252. † N = 110. ¶ Equivocal results were found in two samples originating from HCMC.

3.3.2. Evaluation of sensitivity and specificity of RT PCR for RoV detection

Conventional RT PCR for RoV was performed on all stool samples (as described in section 2.4.2). The stool samples were recorded as positive for RoV when: (1) the negative control for that set of samples was negative; and (2) the positive control was positive for VP7 and VP4; and (3) amplicons for either VP7 or VP4 or both genes were amplified from that sample (Figure 3-1 and Figure 3-2). None of the samples that were negative for RoV using EIA were positive by RT PCR, and 133 of 157 samples of the positive EIA samples gave a PCR amplicon for one or both of the target loci. The sensitivity and specificity of RT PCR method were calculated as described in section 1.4.8. Compared to EIA, RT PCR demonstrated comparable sensitivity (90.48%) and specificity (100%) with NPV of 93.89 and PPV of 100 as shown Table 3-2.

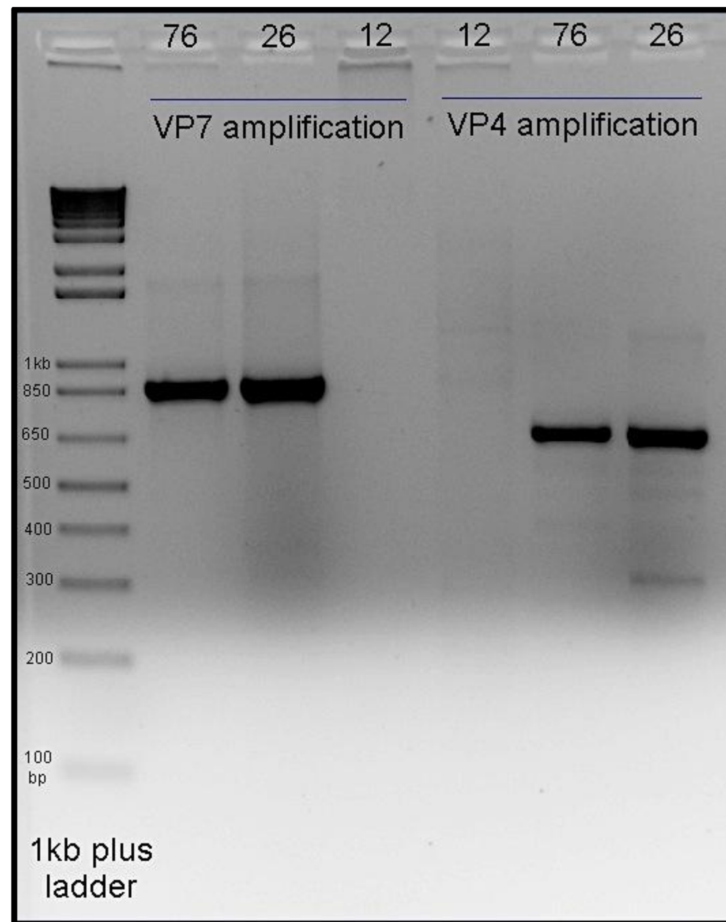


Figure 3-1. Agarose gel electrophoresis confirming PCR amplification of RoV VP7 and VP4 genes

Image showing agarose gel electrophoresis of RT PCR amplicons amplifying VP7 and VP4 genes of RoV (as described in section 2.4.2.2). For each set of RT PCRs, one sample was included as negative control and one sample was included as positive control. Samples were numbered according to patient ID, ie 76 equivalent to patient ID 76 with positive VP7 and VP4 amplicons (881 bp and 663 bp respectively), confirming RoV infection in this patient; sample ID 26 was RoV positive by EIA and served as a positive control from RNA extraction to RT PCR steps; sample ID 12 was RoV negative by EIA and served as a negative control from RNA extraction to RT PCR steps.

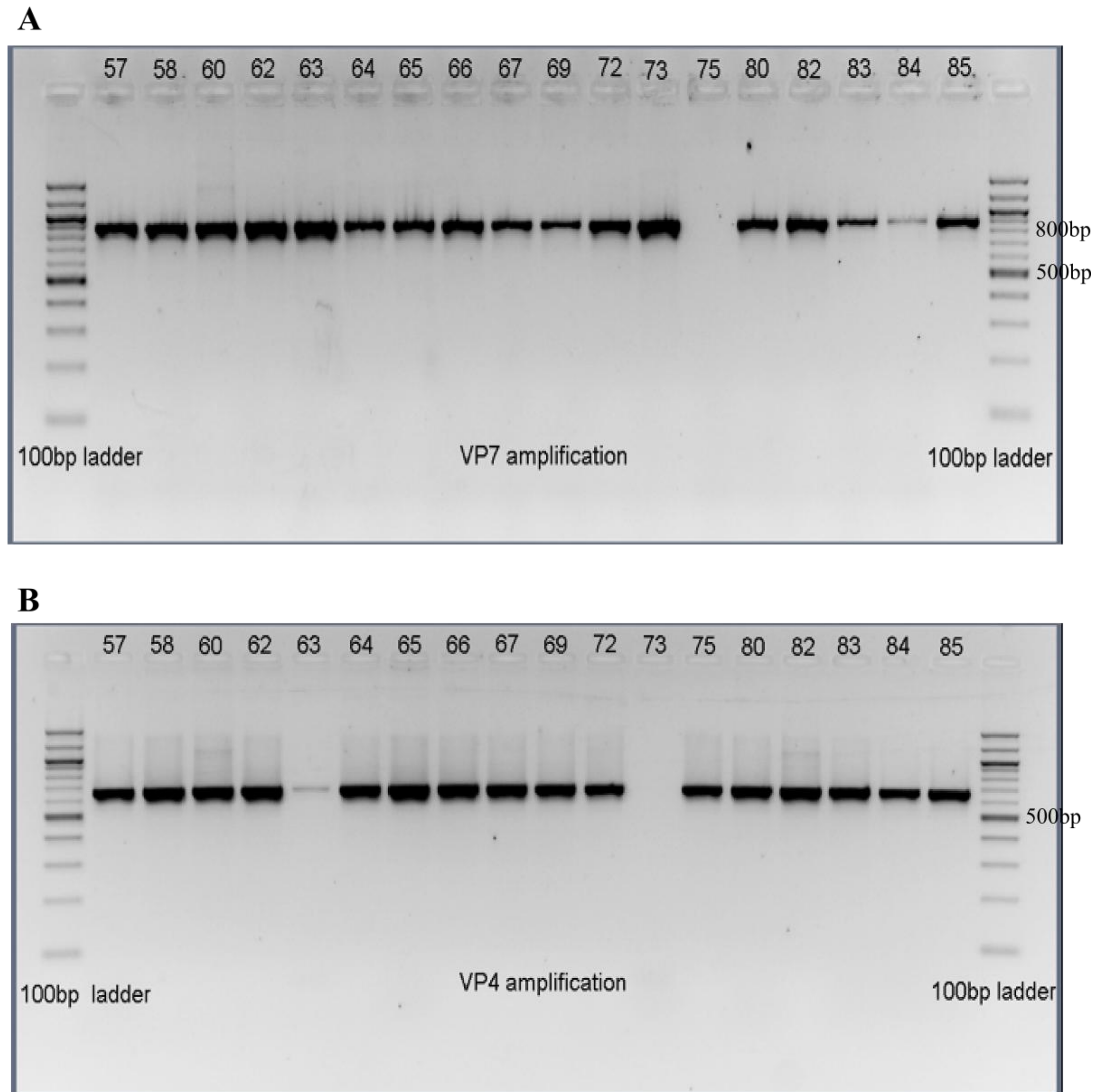


Figure 3-2. Agarose gel electrophoresis confirming PCR amplification of RoV VP7 and VP4 genes

Images showing agarose gels of RT PCR amplicons of the VP7 (A) and VP4 (B) genes of RoV (as described in section 2.4.2.2). Samples were labelled according to patient ID. Samples were recorded as positive for RoV if positive amplification was found for either VP7 (881 bp amplicon) or VP4 (663 bp). For example, sample ID 73 was recorded as RoV positive, ie VP7 amplification was detected in sample ID 73 although VP4 amplification was negative in this sample. Similar interpretation was applied for sample ID 75 in this set of samples.

Table 3-2. The sensitivity and specificity of RT PCR detection of RoV in stool specimens from paediatric diarrhoeal patients in southern Vietnam (HCMC and DT) in comparison to commercial EIA kit

RT PCR	**EIA**		**Total**
	Positive	Negative	
Positive	133	0	133
Negative	14	215	229
Total	147	215	362
Sensitivity % (95 % CI)	90.48 (84.54 – 94.70)		
Specificity % (95 % CI)	100 (98.30 – 100)		
NPV (%) *	93.89		
PPV (%) †	100		

* NPV = Negative Predictive Value

† PPV = Positive Predictive Value

3.3.3. Distribution of RoV genotypes

After the amplification of VP4 and VP7 fragments, all successful amplicons were DNA-sequenced (as described in section 2.4.4). Among 133 successful amplicons, 118 amplicons produced a sequence consistent with the VP4 region (118/133), and 109 produced a sequence consistent with the VP7 region (109/133). Genotypes were assigned based on generating sequences for VP7 and VP4 as described in section 2.4.5.2. Various G genotypes were found, among which, G1 was the predominant G-type circulating, representing 69.5 % (82/118) of all typeable G types (Figure 3-3). The second most common G genotype was the novel G12 genotype, accounting for 16.1 % (19/118) of all G types. Other G types identified in the remainder positive samples included G2, G3, and G4, comprising of a limited proportion (Figure 3-3). Among all typeable P types, P[8] was the most prevalent type, representing 88.1 % (96/109) of all P types, followed by P[4] (10.1 %; 11/109) and P[6] (1.8 %; 2/109) (Figure 3-3).

RoV G1P[8] was the dominant GP combination, detected in 78.5 % (73/93) of all positive samples for VP7 and VP4 genes. Other globally diffuse GP combinations were also identified, yet in a limited number of samples, including G2P[4] (10.8 %; 10/93) and G3P[8] (2.2 %; 2/93). Substantial variations in the distribution of G and P types were noted between the two sampling locations. In particular, G2 and G3 types were detected in RoV-positive samples originating from HCMC, yet neither was present in samples positive for RoV originating from DT. Although RoV G12 was detected in both sampling locations, its frequency of detection was greater in samples originating from DT than from HCMC, accounting for 28.3 % and 3.4 % of all typeable G in these locations, respectively ($P = 0.0016$, Chi-squared test). Furthermore, while P[4] was prevalent at the frequency of 20.8 %

of P types detected in HCMC samples, the presence of this P type was not found in samples originating from DT. Likewise, RoV P[6] was detected in a few samples originating from DT, but not in any samples from HCMC.

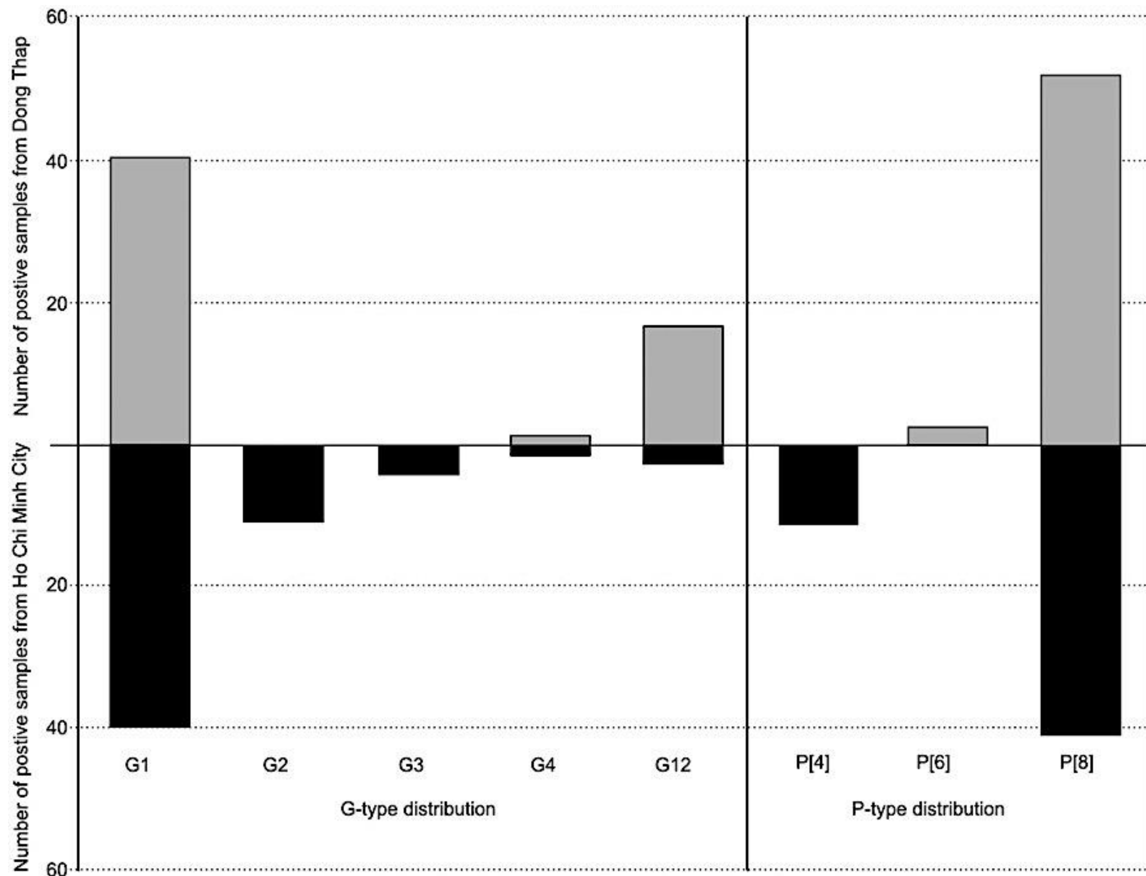


Figure 3-3. The distribution of RoV G and P types detected in faecal samples collected from recruited diarrhoeal patients in HCMC and DT.

Graph showing the distribution of diverse G and P types including G-types 1, 2, 3, 4, and 12 and P-types [4], [6], and [8] in 118 and 109 VP7 and VP4 PCR amplification positive samples, respectively. The graph is sub-divided to show G (N = 60) and P types (N = 56) from DT (upper, grey), and G (N = 58) and P types (N = 53) from HCMC (lower, black).

3.3.4. Phylogenetic analysis of RoV sequences

Phylogenetic analyses was performed separately on the G1, G12 and P[8] sequences generated from VP7 and VP4 amplifications, comparing them with global sequences retrieved from GenBank (as described in section 2.4.5). The G1 sequences from DT and HCMC exhibited extensive genetic diversity [maximum genetic distance (uncorrected) = 0.0774], falling into three phylogenetically distinct lineages (Figure 3-4). A significant phylogenetic association identified between the HCMC and DT RoV G1 sequences highlighted the circulation of closely related RoV strains in both locations. A comparison of these G1 data generated from this study with previous RoV sequence data originating in Vietnam was also performed. One single lineage was identified to share close phylogenetic relationship to other G1 also derived from HCMC, suggesting the persistence of this particular lineage in the local population (Figure 3-4) [347]. Comparable with the distribution pattern observed in detected G1 strains, the distribution of P[8] sequences also exhibited extensive genetic diversity [maximum genetic distance (uncorrected) = 0.159] (Figure 3-5). These P[8] strains could be separated into four phylogenetically distinct lineages, which are closely related to other P[8] strains isolated from different locations around Asia (Figure 3-5). The G12 lineage containing sequences primarily identified in samples from DT was phylogenetically closely related to the G12 sequences isolated from Thailand and India (Figure 3-6). Conversely to the extensive genetic diversity observed in G1 and P[8] sequences, G12 strains showed less overall genetic diversity [maximum genetic distance (uncorrected) = 0.0130]), with only one major lineage identified (Figure 3-6), suggesting the potentially recent introduction of this variant into the local population.

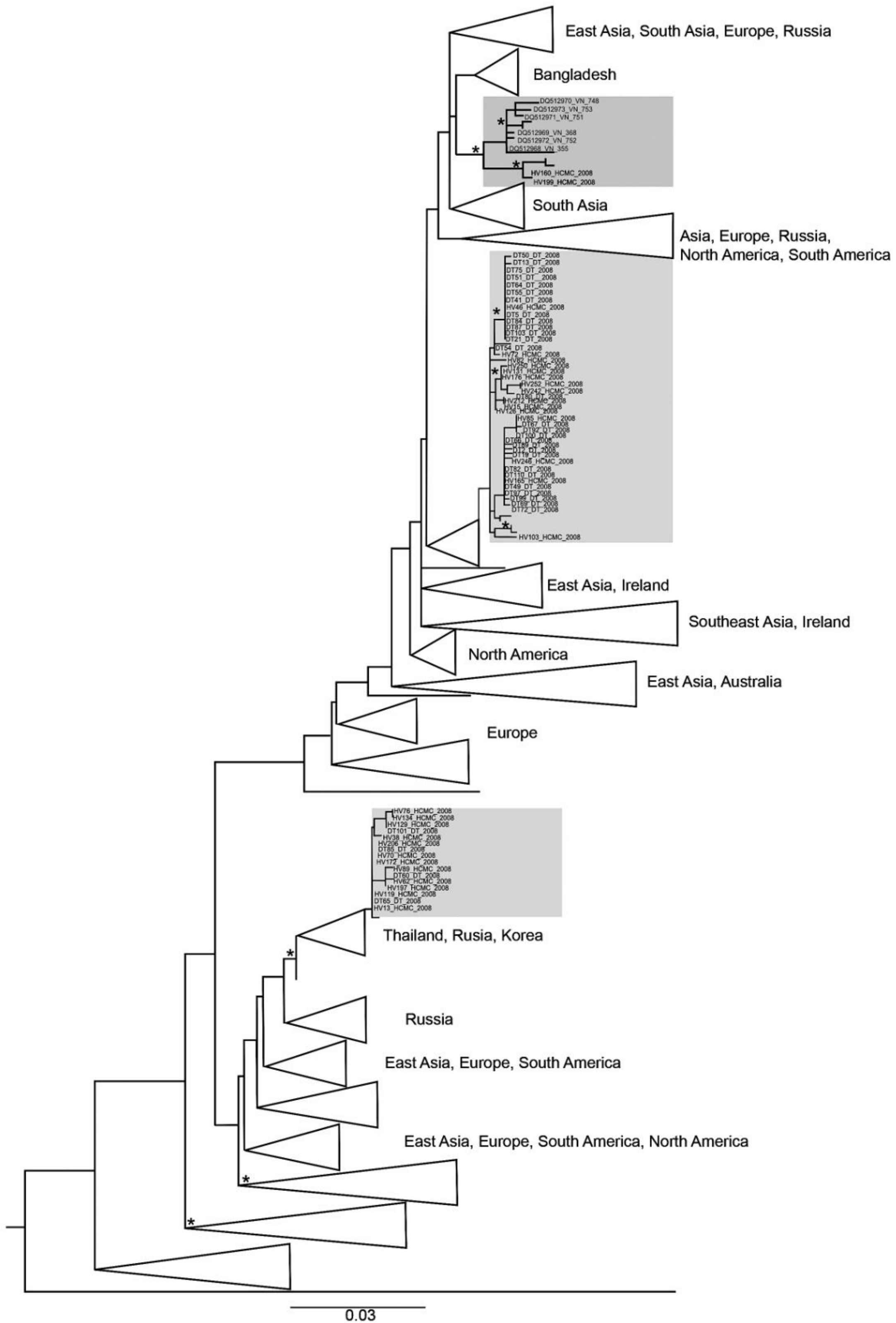


Figure 3-4. Phylogenetic tree of 81 RoV G1 strains comparing with global G1 sequences.

Maximum likelihood phylogenetic tree [VP7 gene] constructed from 81 G1 sequences (section 2.4.5.5), comparing with 392 representative global RoV G1 sequences. Sequences generated from this study and from previous studies in Vietnam are indicated in black and grey, respectively. The tree is mid-point rooted, with all horizontal branch lengths drawn to the scale of a nucleotide substitution per site. Bootstrap values > 85 % are indicated by asterisks, and triangles represent compressed regions of the tree.

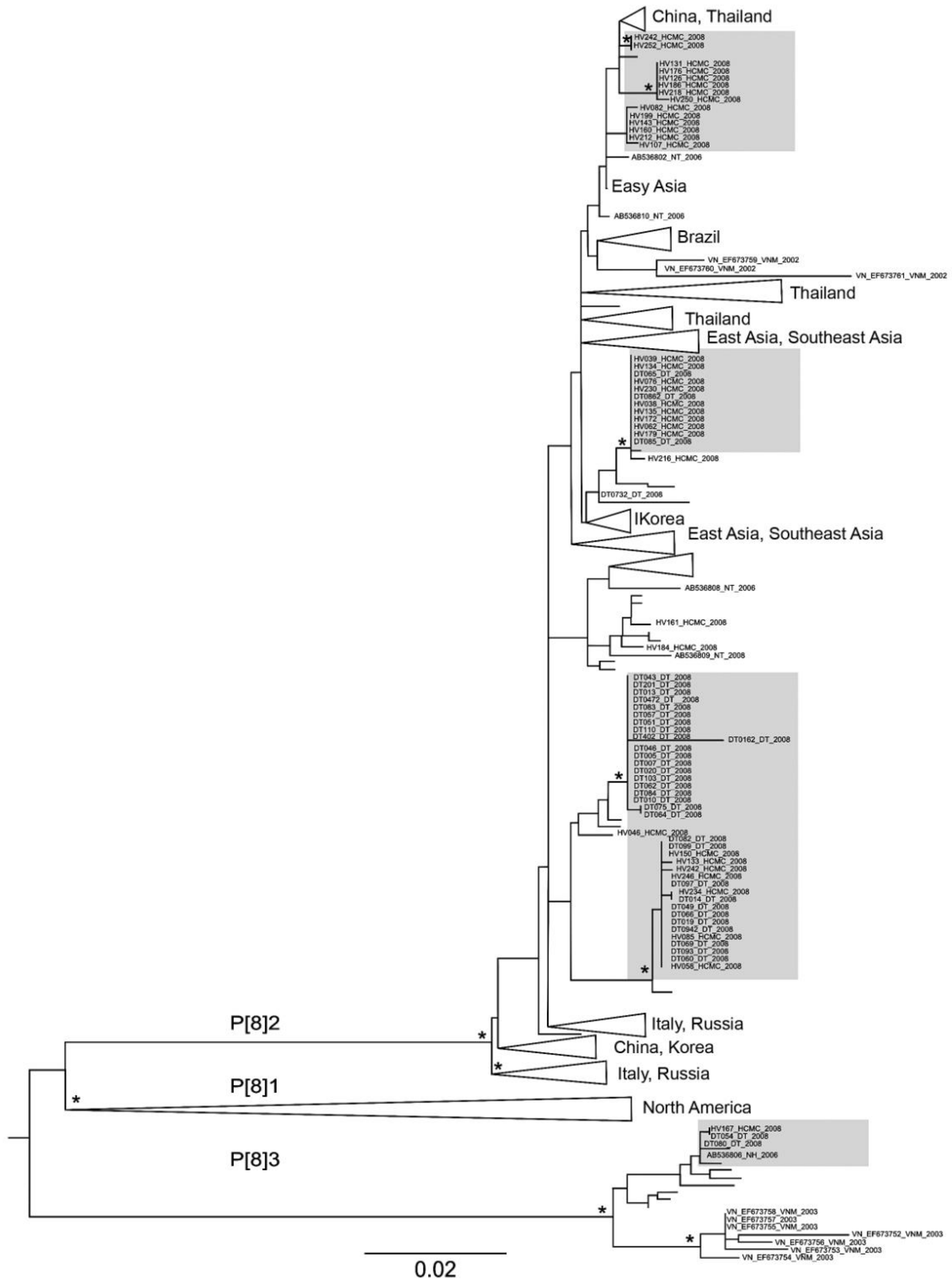


Figure 3-5. Phylogenetic tree of 96 RoV P[8] strains comparing with global P[8] sequences.

Maximum likelihood phylogenetic tree [VP4 gene] constructed from P[8] sequences and 162 representative global sequences of RoV P[8] strains retrieved from GenBank (as described in section 2.4.5.5). Tree mid-point rooting, bootstrap cut-off values, branch lengths, and font correspond to those factors presented Figure 3-4. .

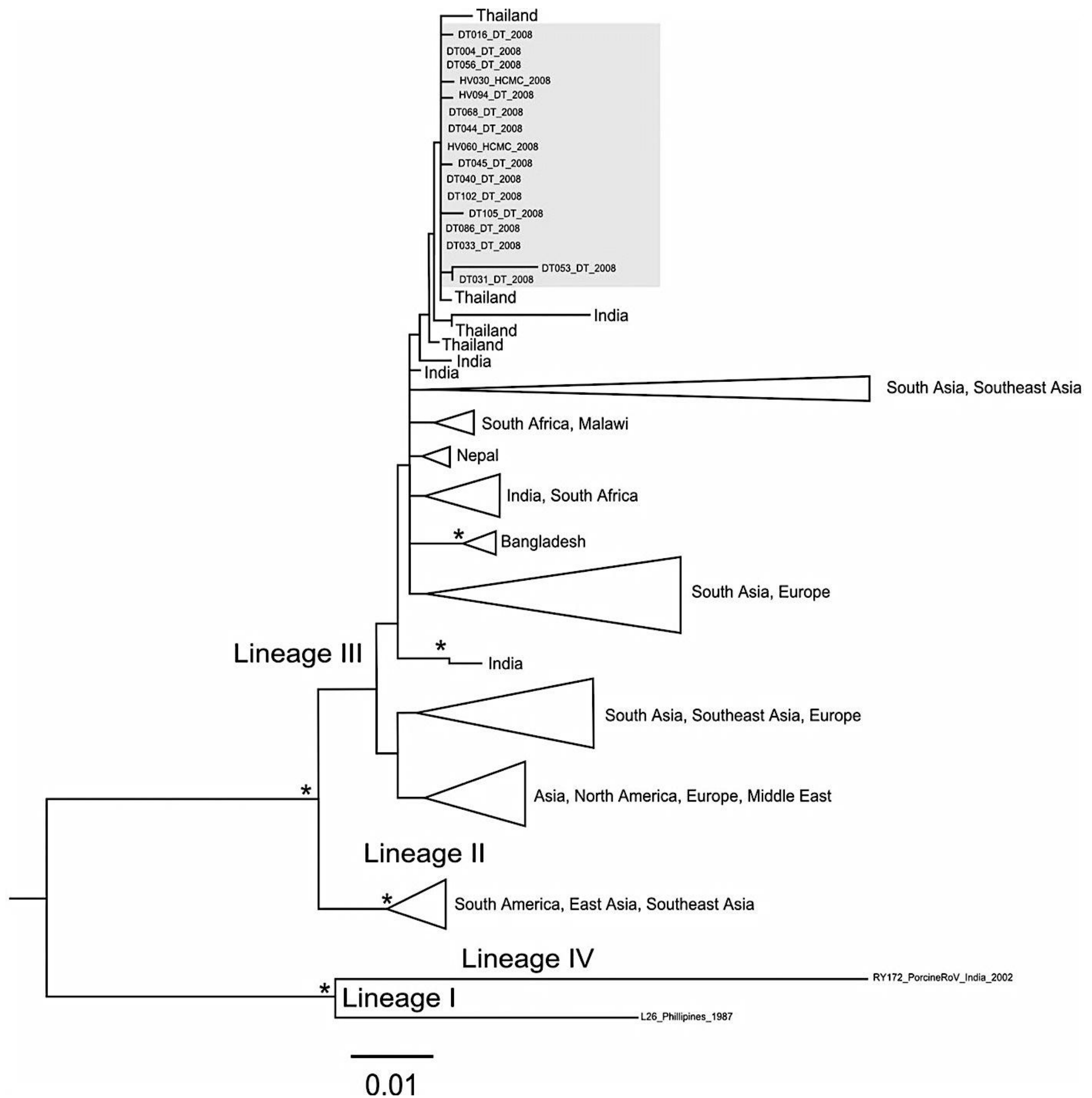


Figure 3-6. Phylogenetic tree of 19 RoV G12 sequences from HCMC and DT comparing with global G12 sequences.

Maximum likelihood phylogenetic tree [VP7 gene] constructed from G12 strains and 137 representative global sequences of RoV G12 retrieved from GenBank (as described in section 2.4.5.5). Tree mid-point rooting, bootstrap cut-off values, branch lengths, and font correspond to those factors presented in Figure 3-4.

3.4. Discussion

Enteric viruses play a prominent role in acute childhood diarrhoea in Vietnam [252, 312, 320, 321, 327, 348-350]. This chapter was aimed to investigate the prevalence and distribution of four main enteric viruses in paediatric diarrhoeal patients attending two defined healthcare centres, one rural and one urban location in southern Vietnam. This pilot study, despite representing only a one-month long snapshot, demonstrated the predominant aetiological role of viral pathogens in acute childhood diarrhoea in this setting, a view that is consistent with previous studies in Vietnam [252, 312, 320, 348]. Moreover, the distribution of enteric viruses varied in diarrhoeal inpatients recruited from two distinct locations, urban versus rural, indicating various pathogens co-circulating and corresponding differential infection risks.

The results from this work showed that viral pathogens were responsible for a large proportion of diarrhoea with RoV being the overall dominant viral agent among recruited patients in both sampling locations. Although there were relatively a small number of samples collected during a limited temporal distribution, a dramatic genetic diversity was identified among RoV-positive stool samples. Such great diversity observed within RoV G1 sequences is proposed to support the hypothesis of multiple introductions of G1 genotype to the local population. Additionally, phylogenetic relationship was identified in only one of the three G1 lineages to previous G1 strains from HCMC identified in 2002-2005, adding evidence to an on-going strain introduction with limited *in situ* evolution in this region [347]. The predominated frequency of P[8] reported in this work was comparable to the prevalence reported in North America, Australia and Europe but higher than previously reported prevalence in Vietnam [56, 252, 312, 313, 320]. Similar to G1

sequences, phylogenetic analysis of P[8] strains showed extensive genetic heterogeneity, which is likely caused by multiple strain introduction phenomenon rather than a clonal expansion of this particular genotype.

The results additionally demonstrated a differential distribution of enteric viral pathogens between the urban and rural settings. A significantly larger proportion of RoV G12 was identified in samples originating from the rural location compared with the urban location. This study reports the first identification of a RoV genotype G12 in Vietnam; and nearly a decade since the primary detection in the Philippines in 1987, RoV G12 has become increasingly prevalent worldwide particularly in countries around Asia [351-365]. Given the dramatic genetic diversity among global G12 strains and a great proportion of G12 isolated in Southeast Asia, it was suggested that the global distribution of this genotype stemmed from G12 viruses in Southeast Asia through increased transportation of humans and animals [114]. Notably, this variant was detected at a high rate (28.3 % of all G types in DT), underscoring the capacity of this virus to spread and become fixed in a local population. This finding has direct implications for RoV immunisation, because protection provided by either available vaccines (Rotarix™ and RotaTeq™) against RoV G12 is as yet determined. However, provided a high evolutionary rate of the VP7 gene (1.66×10^{-3} substitutions per site per year), the introduction of either vaccine may impose a selective pressure on circulating strains in the population, which consequently accelerate evolutionary rates and facilitate the rapid emergence and spread of new variants [113]. Such factors emphasize the need for on-going modification and development of RoV vaccines and continued surveillance for genotype circulation.

Secondary data supporting variations in geographical distribution of enteric viruses is the distribution of NoV in the two distinct settings. NoV was the second most commonly identified virus in collected stool specimens, which concurs with NoV literature [232]. NoV constituted 29 % of positive stool sample originating from HCMC compared with only 5.1 % in DT. These data suggests different epidemiological risk factors related to this organism in these locations. NoV is highly contagious and is related to outbreaks in developed countries [133]. HCMC is more densely populated and has undergone a greater level of urbanisation and development with respect to the surrounding province, such as DT. Transmission and the corresponding exposure to NoV are likely to be concurrent with such a developmental change. In parallel to RoV infections, the majority of NoV-infected patients were in the 7 – 12 months age group. This 6-month age group is, hence, suggested to be the key age group for children with diarrhoeal infections and poses the greatest number of epidemiological questions. Decreased rates of enteric viruses detected in patients outside this key age group may be related to risks, exposure, healthcare-seeking behaviour, maternal immunity, and natural post-infection immunity. Additional studies on the effect of maternal antibody or immunity from previous exposure to NoV should help guide vaccine development and usage within this age group.

The study design does have several caveats to be considered. Firstly, although the age distribution of recruited patients was similar in both locations, such sampling was open to selection bias. The aim of this work was to examine the distribution of viral diarrhoeal pathogens and not determine risk factors; therefore, a limited period of sampling may bias the prevalence of the various agents. Moreover, about 5 % of control healthy patients (defined as inpatients for non-infectious causes without

diarrhoea in the previous 2 weeks) between the age of 3 months and 5 years may carry viral pathogens in their stool specimens; thus additional sampling is necessary to understand the transmission dynamics and asymptomatic carriage of enteric viruses in children [327]. There were some dual viral infections (4.1 %; 8/195), but it was not possible to elucidate the impact in pathogenesis and their clinical significance, which also may be an issue when other bacterial and parasitic agents are considered.

3.5. Conclusion

This chapter demonstrated the first identification of RoV G12 in Vietnam, and a differential distribution of the major enteric viral pathogens and RoV genotypes in acute gastroenteritis in two distinct locations in southern Vietnam. The data presented in this chapter provided necessary information for the design of subsequent studies in the next chapter, and also highlight the need for a longitudinal research of enteric viruses and continued monitoring of circulating RoV strains for effective prevention and vaccination strategies.

4. DIARRHOEAL DISEASE IN CHILDREN UNDER THE AGE OF FIVE IN THREE HOSPITALS IN HCMC, A PROSPECTIVE MULTI-SITE HOSPITAL-BASED STUDY

4.1. Abstract

This chapter presents data from a prospective multi-centre hospital-based study investigating the aetiology, clinical characteristics, demographics, and antimicrobial susceptibilities of enteric pathogens in children under 5 years of age residing in HCMC from May 2009 to December 2010. Known diarrhoeal pathogens were identified in stool samples from 1,067/1,419 (75.2%) of cases and from 81/609 (13.3%) of controls. RoV was the most commonly found pathogen in the stools of cases, followed by NoV. Bacterial diarrhoeal pathogens belonging to the genera *Salmonella*, *Campylobacter* and *Shigella* were isolated from much smaller proportion of diarrhoeal cases. Concomitant viral and bacterial infections were commonly observed. The most common presentation on hospital admission was loose watery diarrhoea with vomiting and fever. Children infected with *Shigella* spp. were significantly older than children infected with other pathogens. Extensive antimicrobial resistance was identified in bacterial diarrhoeal pathogens, most notably to (fluoro)quinolones and third generation cephalosporins. In conclusion, multiple pathogens are responsible for paediatric diarrhoeal disease in this setting with RoV/NoV playing the key roles, suggesting that RoV vaccination would have a dramatic impact on the incidence of diarrhoeal disease in Vietnamese infants. The remarkably high level of antimicrobial resistance among all the identified bacterial genera should alert policy makers in Vietnam, as antimicrobial usage in hospitals and in the community clearly requires urgent evaluation.

4.2. Introduction

Diarrhoeal disease has a significant impact on global health and is often overlooked as a major global health issue and does not command the same attention as many of the other "big infections". Low-income countries in Africa and Asia bear the brunt of the global disease burden of paediatric diarrhoeal disease [7, 10], where infections can be severe and may require hospitalisation [7]. The rate of disease mortality has declined since the introduction of oral rehydration therapy in the 1980s [366], yet the disease remains the second most common cause of childhood mortality worldwide, responsible for around 800,000 deaths in children under five as estimated in 2010 [6].

Diverse viral, bacterial and parasitic pathogens are responsible for childhood diarrhoea in developing countries, such as RoV, NoV, *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., pathogenic *E.coli*, *Cryptosporidium* spp. and *Giardia lamblia* [12]. In Vietnam, routine aetiological diagnosis is not performed on stools taken from individuals hospitalised with diarrhoeal disease, leading to limited data on the prevalence of the main agents of diarrhoeal disease from this part of Asia. Furthermore, the clinical manifestations of diarrhoeal disease caused by differing pathogens can be indistinguishable, which, together with a lack of routine pathogen identification, may confound appropriate therapy and hinder future prevention strategies.

This aim of the study presented in this chapter was to identify the most common causes and characteristics of children hospitalised with diarrhoeal disease in HCMC. Here, some of the clinical features, epidemiological characteristics and the antimicrobial susceptibility profiles of diarrhoeal pathogens in this rapidly

developing Asian city are described, providing a cross-sectional resource on which further diarrhoeal disease research can be conducted. .

4.3. Results

4.3.1. Demographic characteristics of cases and controls

Over the study period, 1,419 diarrhoeal cases (referred hereon as cases) and 609 non-diarrhoeal controls (referred hereafter as controls) were enrolled (as described in section 2.2.2); the demographic characteristics of enrolled cases and controls are shown in Table 4-1. Both cases and controls were more frequently male (63.8 % and 52.9 %, respectively) and had a combined median age of 12 months. Controls were more likely to have a poor weight-for-age Z-score than cases (12.5 % versus 6.6 %; $P < 0.001$, Chi-squared test). Seventy per cent (70 %) of cases and controls were frequently breastfed as infants, yet the regular use of milk formula and probiotics was more common among the controls (82 %; 498/609 and 64 %; 304/473, respectively) than cases (58 %; 819/1,419 and 14 %; 121/861, respectively) ($P < 0.001$ for each comparison, Chi-squared test). Day-care attendance or nursery school was limited in both cases (15.9 %; 226/1,419) and controls (15.4 %; 94/609). The majority of families of the enrolees (> 80 %) resided within the urban districts of HCMC, as opposed to the peri-urban/rural areas, and reported a monthly income equivalent of < $500 USD. The households of more than half of all enrolees used a government pipeline as their major household water source and there were no significant differences in household water source between cases and the controls.

Table 4-1. Demographic characteristics of cases and controls, N (%)

Characteristic N (%)	Cases N = 1,419	Controls N = 609
Male gender	905 (63.8)	322 (52.9)
Median age (IQR) months	13 (8 – 19)	12 (8 – 20)
Poor weight-for-age Z-score *	93 (6.6)	76 (12.5)
Breastfed	1,017 (71.7)	465 (76.4)
Attends daycare/nursery school	223 (15.9)	93 (15.4)
Median household size (range)	6.5 (2 – 31)	6.4 (3 – 26)
Income bracket		
< $145	422 (29.7)	136 (22.3)
$145-242	532 (37.5)	211 (34.6)
$243-483	326 (23)	168 (27.6)
$484-725	90 (6.3)	61 (10.1)
> $725	49 (3.5)	33 (5.4)
Household water source		
Government pipeline	835 (59.0)	359 (59.0)
Well	501 (35.4)	223 (36.6)
Other $^{\Delta}$	81 (5.7)	27 (4.4)
Residence location †		
Rural/peri-urban	261 (18.4)	76 (12.5)
Urban	1,158 (81.6)	533 (87.5)

IQR: Interquartile Range.

* Weight-for-age Z-score < -2 [329, 330].

$^{\Delta}$ Other household water sources include rainwater, well water and water bought from governmental truck dispenser.

† Rural and urban districts are classified according to HCMC Statistical Office [367].

According to the World Bank data on the average annual income per person for Vietnam in 2009, the income bracket of below $243 USD per month (~$5,000,000 VND) was classified as poor income, from $243-483 as low-middle income, $484-725 as middle income, and above $725 as high income (http://databank.worldbank.org/data/views/reports/tableview.aspx).

4.3.2. Prevalence of enteric pathogens

At least one enteric pathogen was identified in 75.2 % (1,067/1,419) of the stool samples from the cases and in 13.3 % (81/609) of the stool samples from the controls (Table 4-2). The majority of enrolees with a pathogen positive stool sample (cases; 91 %; 970/1,067 and controls; 94 %; 76/81) were infected with a single pathogen (as described in sections 2.3 and 2.4). Ninety-seven (9 %) cases were infected with two pathogens; including combinations of bacteria (0.4 %; N = 5), viruses (2.3 %; N = 32), or virus and bacteria (4.2 %; N = 60). RoV and NoV were identified in the stools of 46.8 % (664/1,419) and 20.6 % (293/1,419) of the cases, respectively (Table 4-2). From these RoV/NoV positive stools, 32 were a mixed RoV/NoV infection, 18 were mixed NoV/bacterial infections and 42 were mixed RoV/bacterial infections. The bacterial genera Salmonella, Shigella, and Campylobacter were cumulatively isolated from 14.4 % (204/1,419) of the stools from the cases, of which 67 cases had an additional pathogen (Table 4-2).

In contrast to the dominance of viral infections among cases, bacterial enteric pathogens were identified in a greater proportion than viruses among the stool samples from the controls. The most commonly found bacterial genus in the stool samples of the 609 controls was *Salmonella*; isolated from 39 (6.4 %) stool samples (Table 4-2); none of the control stool samples were culture positive for *Shigella* spp. NoV and RoV were found in the stools of asymptomatic controls on 17/609 (2.8 %) and 13/609 (2.1 %) occasions.

Table 4-2. Enteric pathogens identified in the stools of cases and controls

Organism	**Cases** (N = 1,419)	**Controls** (N = 609)	***P* value***
NoV	241 (17.0)	15 (2.5)	**<0.001**
RoV	590 (41.6)	10 (1.6)	**<0.001**
Campylobacter	31 (2.2)	16 (2.6)	0.544
jejuni	19 (1.3)	11 (1.8)	0.424
coli	12 (0.8)	5 (0.8)	0.955
Salmonella	57 (4.0)	34 (5.6)	0.118
Group B	35 (2.5)	12 (2.0)	0.496
Group C	8 (0.6)	0 (0.0)	0.115
Group D	4 (0.3)	1 (0.2)	1.000
spp.	9 (0.6)	21 (3.4)	**<0.001**
arizonae	1 (0.1)	0 (0)	1.000
Shigella	48 (3.4)	0 (0)	**<0.001**
flexneri	4 (0.3)	0 (0)	0.323
sonnei	44 (3.1)	0 (0)	**<0.001**
Other bacteria	2 (0.1)	1 (0.2)	1.000
Parasites	1 (0.1)	0 (0)	1.000
Mixed viral RoV/NoV	32 (2.3)	1 (0.2)	**<0.001**
Mixed viral bacterial	60 (4.2)	3 (0.5)	**<0.001**
Mixed bacteria	5 (0.4)	1 (0.2)	0.675
Total	1,067 (75.2)	81 (13.3)	**<0.001**

* *P* values from Chi-squared test or Fischer's exact test as appropriate, boldface indicates statistical significance. .

4.3.1. Clinical manifestations

The clinical characteristics, type of diarrhoeal stool and the presence of RBC and WBC in stools (by microscopy) were recorded for all cases on admission (Table 4-3). Loose watery diarrhoea was the most commonly recorded stool type (79 %; 1,118/1,419), which was most prevalent among children who had a viral enteric pathogen in their stool (89 %; 766/863). Approximately half of the cases infected with *Shigella* (49 %; 23/47), *Campylobacter* (58 %; 18/31) and *Salmonella* (49 %; 28/57) presented with bloody or mucoid diarrhoea. The majority of cases had a moderate (37.2 to 39 ^{o}C) (52 %; 744/1,419) or severe fever (> 39 ^{o}C) (22 %; 312/1,419) in addition to vomiting (78 %; 1,100/1,419). Children with a viral enteric pathogen were more likely to present with dehydration and vomiting, and to have had diarrhoea for a longer period prior to hospitalisation, than those with a bacterial enteric pathogen.

Cases with an enteric bacterial pathogen in their stool were more likely to present with abdominal pain, severe fever and to have blood cell positive stool smears. The median age of cases was comparable when stratified by the pathogen(s) found in the stools, with the exception of children with a *Shigella* infection (median: 31 months; IQR: 20 – 36 months), who were considerably older than cases with other enteric pathogens in their stool (median: 13 months; IQR: 8 – 19 months) ($P < 0.001$, Mann Whitney U test) (Figure 4-1).

Table 4-3. Clinical manifestations of viral and bacterial associated diarrhoea

Characteristic, N (%) or median (IQR)	**Viral** N = 863	**Bacterial** N = 143	**Mixed** N = 60	**Unknown** N = 352	*P* values ^
Bloody diarrhoea	4 (0.5)	12 (8.4)	0 (0)	21 (6.0)	**<0.001**
Mucoid diarrhoea	93 (10.8)	60 (42.0)	16 (26.7)	94 (26.7)	**<0.001**
Watery diarrhoea	558 (64.7)	59 (41.3)	33 (55.0)	170 (48.3)	**<0.001**
Watery diarrhoea+solids	208 (24.1)	12 (8.4)	11 (18.3)	67 (19.0)	**<0.001**
Mild fever	480 (55.6)	73 (51.0)	33 (55.0)	158 (44.9)	0.319
Severe fever	165 (19.1)	45 (31.5)	4 (6.7)	98 (27.8)	**0.001**
Dehydration	92 (10.7)	4 (2.8)	6 (10.0)	15 (4.3)	**0.002**
Vomiting	730 (84.6)	89 (62.2)	46 (76.7)	235 (66.8)	**<0.001**
Cough	274 (31.7)	38 (26.6)	20 (33.3)	98 (27.8)	0.242
Abdominal pain	40 (4.6)	30 (21.0)	3 (5.0)	44 (12.5)	**<0.001**
Anorexia	533 (61.8)	74 (51.7)	38 (63.3)	190 (54.0)	**0.027**
WBC+ stool	191 (22.1)	100 (69.9)	27 (45.0)	173 (49.1)	**<0.001**
RBC+ stool	94 (10.9)	78 (54.5)	16 (26.7)	107 (30.4)	**<0.001**
Average daily episodes	4 (3-7)	5 (3-8)	5.5 (3-8.5)	4 (3-6)	0.4143
Maximum daily episodes	10 (6-13)	10 (6-13)	10 (6-12)	8 (5-10)	0.9037
Length of illness*	2 (2-3)	2 (1-3)	2 (1-3)	2 (2-3)	**<0.001**
Length of stay $^{\Delta}$	5 (3-7)	4 (3-7)	5 (2.5-7)	5 (3-6)	0.5247

One parasitic infection was excluded from this section.

* Prior to hospitalisation (days)

$^{\Delta}$ Hospitalisation duration (days)

^ Comparison of viral/bacterial diarrhoea. Fisher's exact, Chi-squared or MWU: Mann-Whitney U test, as appropriate, boldface indicates statistical significance.

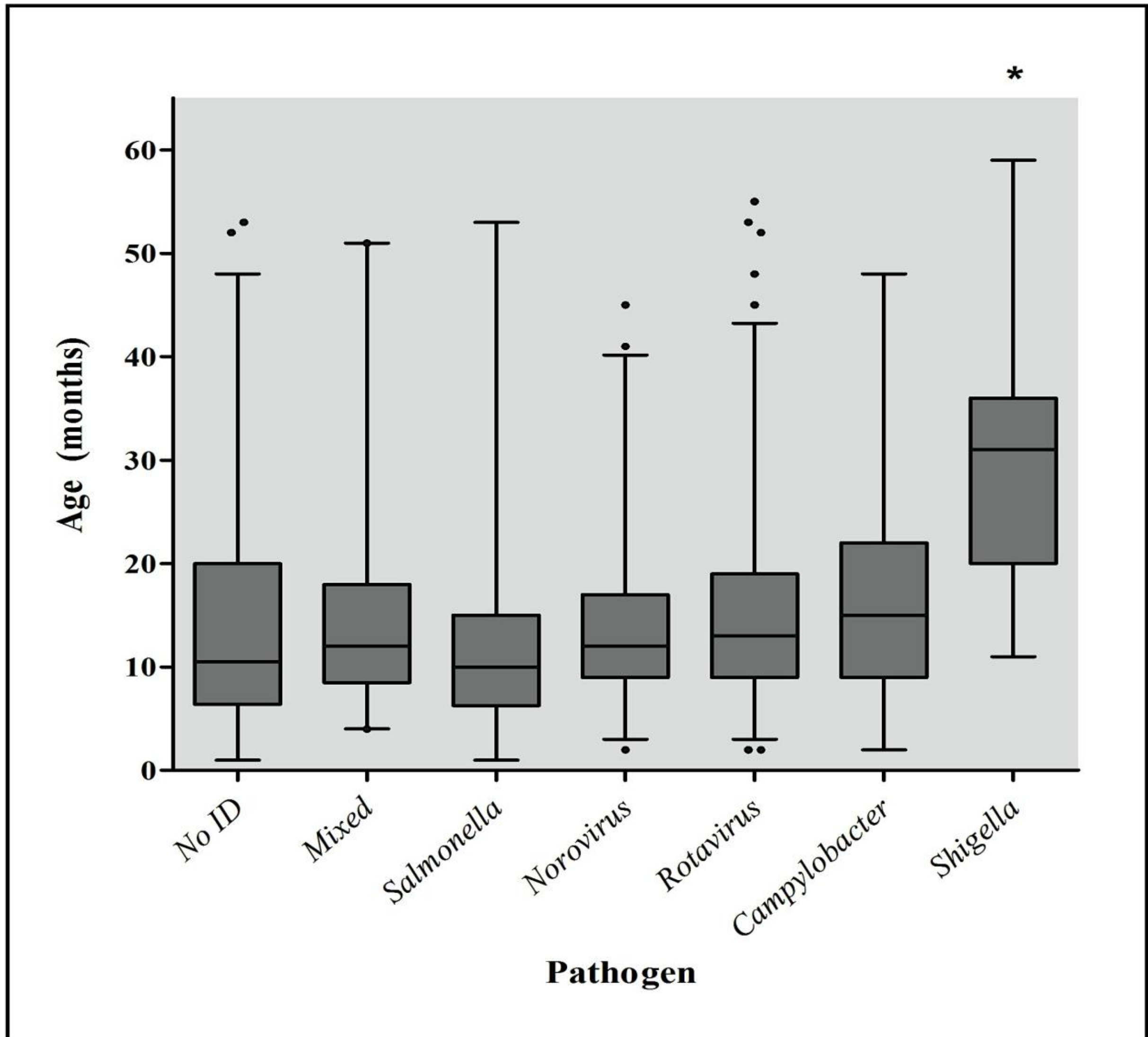


Figure 4-1. The age distribution of hospitalised diarrhoeal patients by pathogen.

Box plots showing the age (in months) distribution of children hospitalised with diarrhoea, stratified by isolated pathogens. Each dark box indicates the IQR (from the 25th to the 75th percentile) of the ages of diarrhoeal patients infected by individual targeted pathogen (*Salmonella*, Norovirus, Rotavirus, *Campylobacter* and *Shigella*), by mixed pathogens, or unidentified pathogens (No ID). The horizontal line in the middle of each box represents the median age (the 50th percentile) in months in each category. Each whisker bar on both sides of the box represents the 5 % to 25 % (lower whisker) and 75 % to 95 % (upper whisker) ranges of age. The dots represent age observations (in months) out of the whisker ranges, including the smallest and largest observations. The asterisk indicates statistically significant difference in the median age of *Shigella*-infected patients compared with the median age of non-*Shigella* patients ($P < 0.001$, MWU test).

4.3.2. Diarrhoeal treatment regimes

Patients were treated following standard Vietnamese treatment guidelines based on clinical observations and prior to any microbiological diagnostic report. Generally, common treatment includes rehydration therapies (RT), probiotics and zinc supplement (Zn); antimicrobials (Abx) are suggested for prescription only in suspected dysentery or cholera or severe cases with potential systemic infections. Diarrhoeal patients were commonly treated with RT (51.9 %; 736/1,418), probiotics (67.5 %; 956/1,418) and Zn (73.2 %; 1,038/1,418) (Table 4-4); yet, an Abx was prescribed in approximately half of enrolled cases (47.2 %; 670/1,418). Significantly more bloody (100%; 37/37) and mucoid (71.6 %; 189/264) diarrhoea were treated with an Abx compared to watery diarrhoea ($P < 0.001$ for each comparison, Chi-squared test). Additionally, patients presented with severe fever and/or abdominal pain were more likely to had Abx treatment ($P < 0.001$ for each comparison, Chi-squared test). Approximately similar proportions of patients with or without dehydration and/or vomiting were treated with an Abx.

Extrapolating treatment data to pathogen profiles, those with a confirmed bacterial infection were more likely to be prescribed an Abx on presentation (74 %; 106/143) than those with a viral infection (38 %; 324/862) ($P < 0.001$, Chi-squared test). Approximately half (48 %; 29/60) of those with a combined bacterial/viral infection were prescribed an Abx, and 60 % (210/352) of those with diarrhoea of unknown origin were treated with Abx. The most commonly used groups of antimicrobials were fluoroquinolones (61 %; 438/714) and cephalosporins (22 %; 160/714). Zn and probiotics were prescribed more frequently to those who had a confirmed viral infection (74 %; 635/862 and 69 %; 591/862, respectively) than those who had a bacterial infection (66 %; 95/143 and 62 %; 88/143, respectively).

Table 4-4. Treatment prescription for diarrhoeal cases

Characteristic	Case count	Abx N (%)	RT N (%)	Probiotics N (%)	Zn N (%)
Diarrhoea	**1,418**	**670 (47.2)**	**736 (51.9)**	**956 (67.5)**	**1,038 (73.2)**
Bloody	37	37 (100)	5 (13.5)	3 (8.1)	33 (89.2)
Mucoid	264	189 (71.6)	136 (51.5)	175 (66.3)	173 (65.5)
Watery	819	319 (39)	433 (52.9)	585 (71.5)	616 (75.2)
Watery+solids	298	125 (42)	162 (54.4)	193 (64.8)	216 (72.5)
Fever					
Moderate	743	321 (43.2)	398 (53.6)	515 (69.3)	569 (76.6)
Severe	313	214 (68.4)	150 (47.9)	212 (67.9)	256 (70.7)
No	362	135 (37.3)	188 (51.9)	229 (63.3)	256 (70.7)
Dehydration					
Moderate	117	59 (50.4)	73 (62.4)	68 (58.1)	82 (70.1)
Severe	1	1 (100)	1 (100)	0 (0)	1 (100)
No	1,300	610 (46.9)	662 (50.9)	888 (68.4)	955 (73.5)
Vomiting					
Yes	1,100	491 (44.6)	603 (54.8)	723 (65.8)	812 (73.8)
No	318	179 (56.3)	133 (41.8)	233 (73.3)	226 (71.1)
Abdominal pain					
Yes	117	86 (73.5)	45 (38.5)	63 (53.8)	82 (70.1)
No	665	267 (40.2)	431 (64.8)	407 (61.3)	467 (70.2)

The total treatments were recorded for 1,418 cases (total cases enrolled: 1,419) with one self-discharge prior to determination of treatment.

Abx: Antimicrobial prescription; RT: Rehydration therapies; Zn: Zinc supplement.

4.3.3. Temporal and spatial distribution of enteric pathogens

The monthly distribution of pathogens detected over the case enrolment period, including both monoinfections and mixed infections, from May 2009 through April 2010 was shown in Figure 4-2. Peak of NoV infections occurred in September – November, while RoV infections peaked from November through January. Peaks of *Salmonella* infections were found in August, November and March; *Shigella* infections appeared to be most common in May and June.

HCMC possesses a tropical climate with a rainy season (May to October) and a dry season (Novermber to April), average monthly temperature ranging from 27.3 ^{o}C to 30.3 ^{o}C. No association was found between the frequency of cases caused by any of the identified pathogens and the monthly mean temperature using Spearman's rank correlation coefficient test (see method section 2.4.7). Regarding association with monthly average rainfall, a weak positive correlation was observed between rainfall and the isolation of *Shigella* spp. (Spearman's rho = 0.592, P = 0.043); and, surprisingly, all other pathogens exhibited a non-significant but positive correlation with rainfall, except RoV, which had a slightly negative association (Spearman's rho = -0.417, P = 0.178) (Figure 4-3).

Additionally, the proportion of identified pathogens stratified by HCMC district is shown in Figure 4-4, which demonstrated the predomination of RoV and NoV in all districts of the city. No association was identified between the relative proportion of each pathogen and the population density or urban/peri-urban locations.

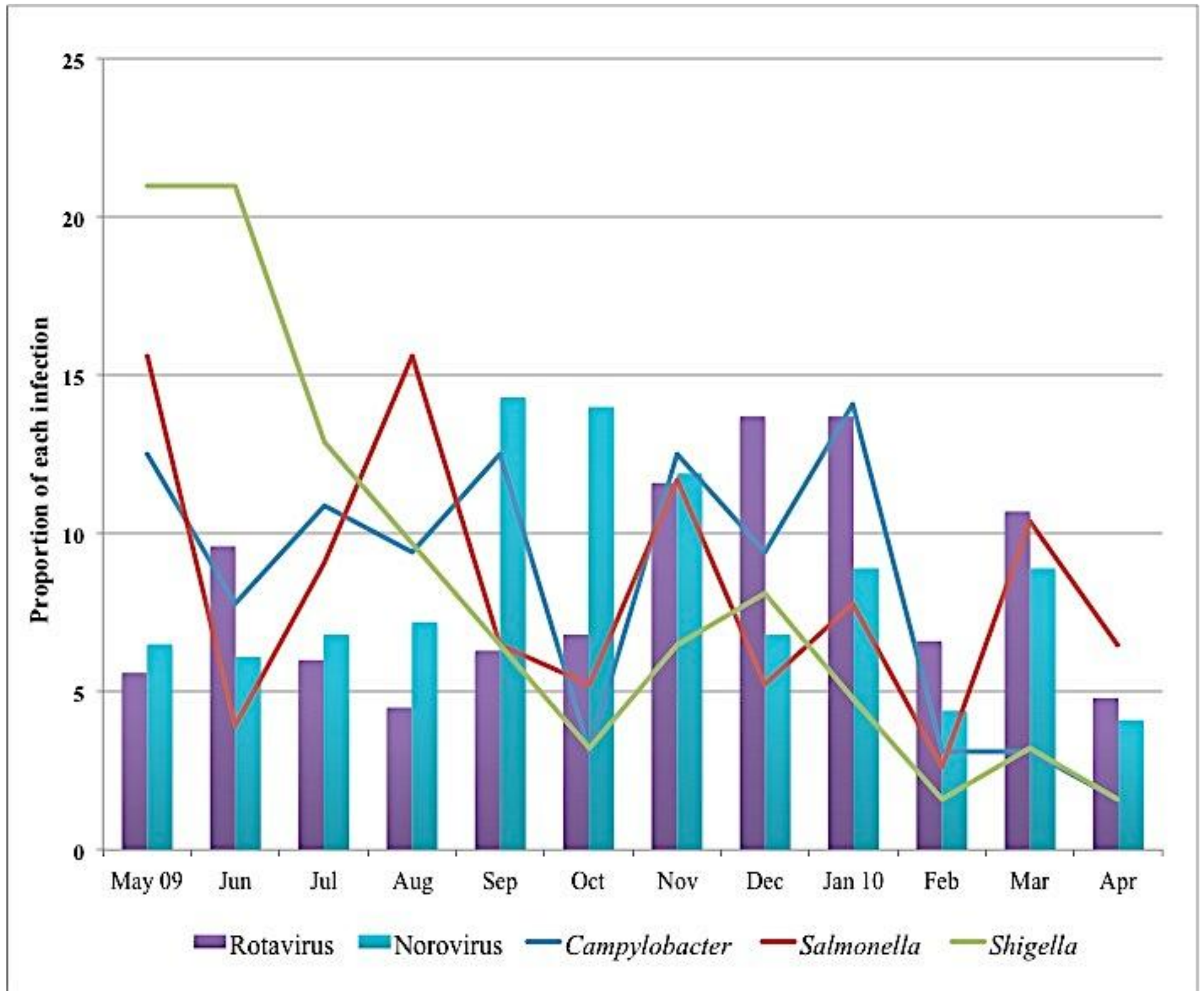


Figure 4-2. The distribution of pathogens identified in diarrhoeal cases over the case enrolment period, including monoinfections and mixed infections.

Each value represents the total number of a particular pathogen isolated in a month (eg *Shigella* cases in May) divided by the total number of isolates (eg all *Shigella* from the study).

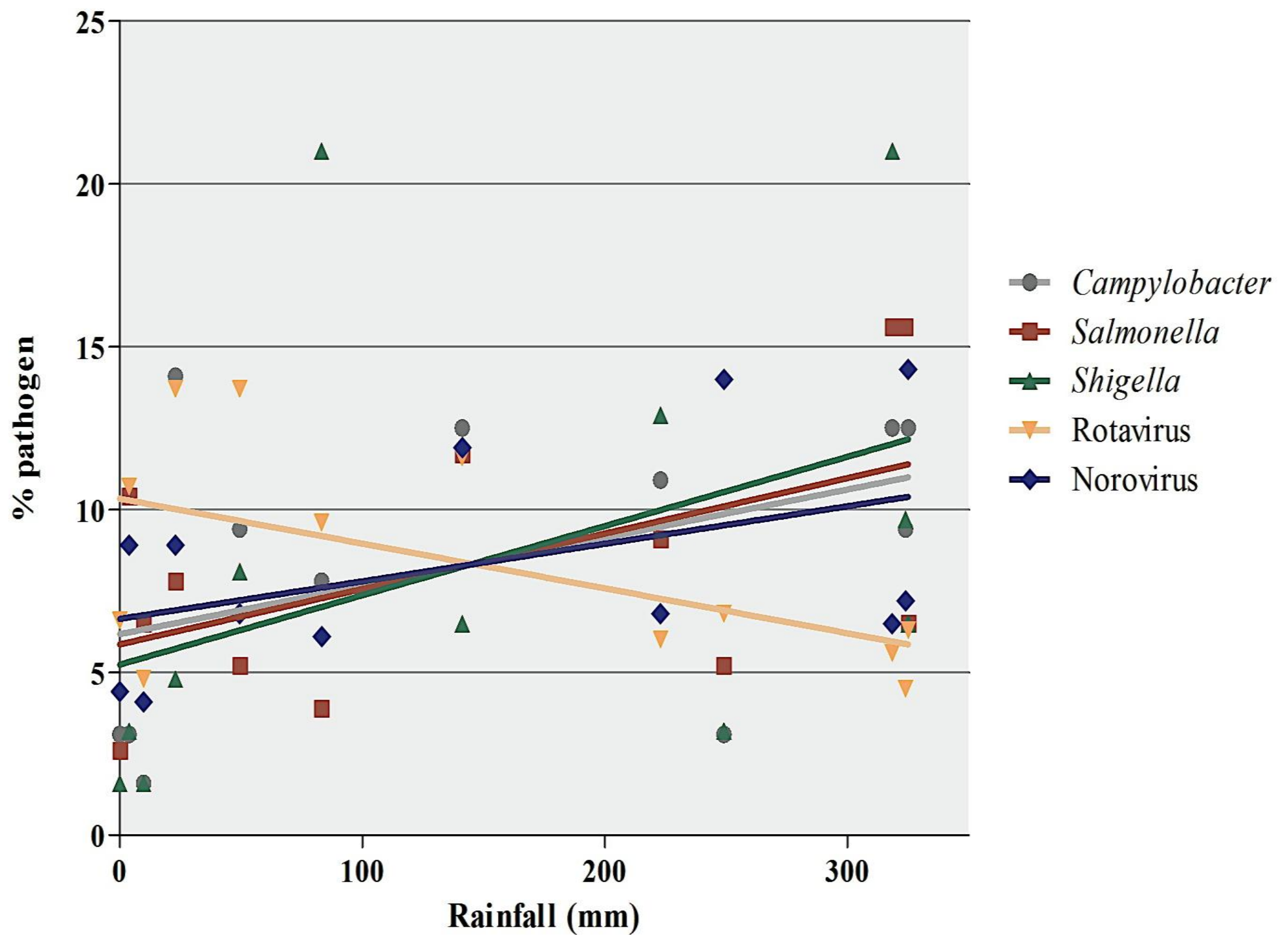


Figure 4-3. The correlation of proportion of each pathogen per month and average monthly rainfall (mm), with lines showing linear regression.

Spearman's rho (*P* value) for each pathogen evaluating association between rainfall and % pathogen, *Campylobacter*: 0.483 (0.112); *Salmonella*: 0.415 (0.18); *Shigella*: 0.592 (0.043); Rotavirus: -0.417 (0.178); Norovirus: 0.481 (0.114).

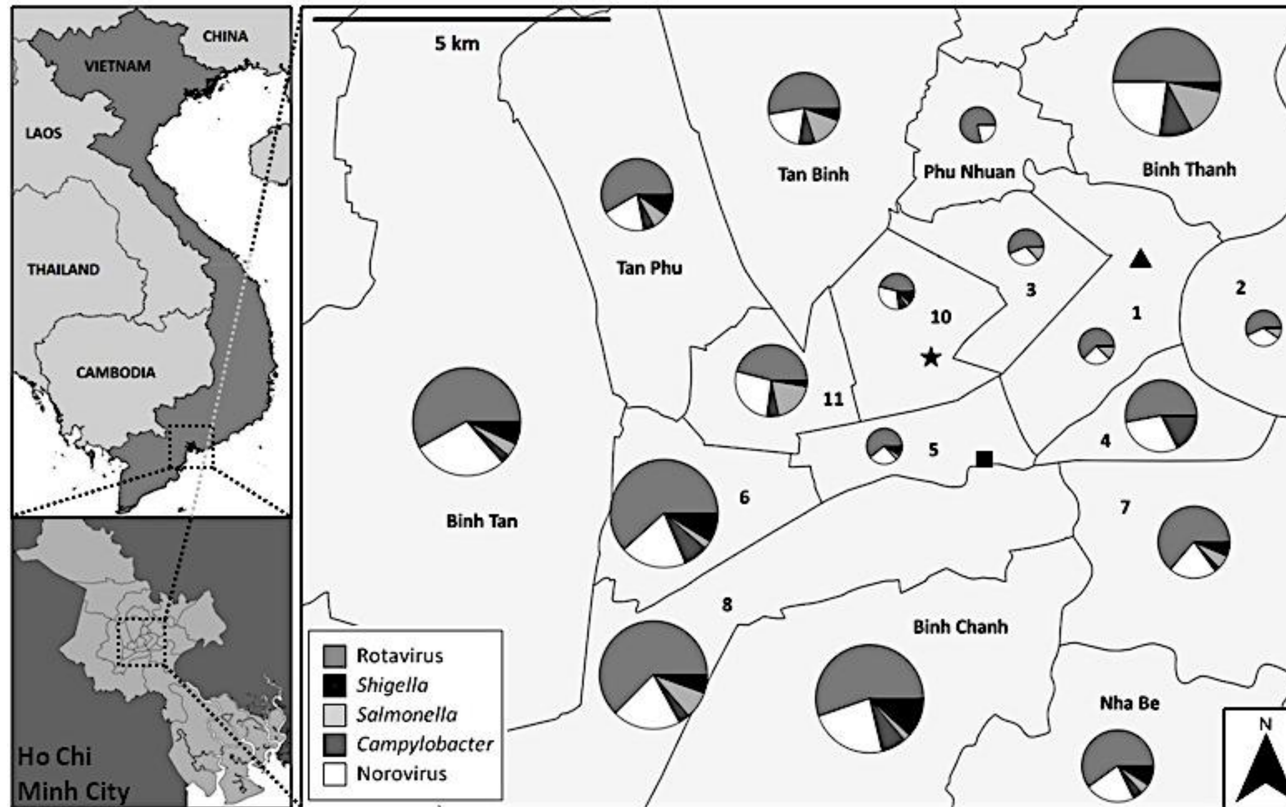


Figure 4-4. The distribution of diarrhoeal pathogens by district in HCMC.

Map of HCMC showing the distribution of enteric pathogens (pie charts) by individual districts. The size of the pie chart indicates the number of cases identified with at least one pathogen in that location (small: < 30, medium: 30 – 60, large: > 60 diarrhoeal cases). The pie charts are divided to show proportions of cases identified with NoV, RoV, *Campylobacter*, *Shigella*, and *Salmonella*. The HCMC districts are named and separated by black bordering lines. Symbols are used to represent the geographical locations of three study sites, star; CH1 (District 10), triangle; CH2 (District 1), and square; HTD (District 5).

4.3.4. Antimicrobial Susceptibility

The isolated bacteria in the genera of *Campylobacter*, *Shigella* and *Salmonella* exhibited remarkable resistance using disc diffusion method (as described in section 2.3.3.1), particularly to (flouro)quinolones and third generation cephalosporins (Figure 4-5). The majority of *Campylobacter* isolates (N = 64) were resistant to CRO (84 %; 54/64), CIP (80 %; 51/64), NA (84 %; 54/64) and SXT (98 %; 63/64). Similarly, isolated *Shigella* (N = 62) also showed extensive resistance to CRO (76 %; 47/62), NA (94 %; 58/62) and SXT (98 %; 61/62). Resistance in the *Salmonella* isolates (N = 78) was generally much lower for these antimicrobials, CRO (12 %; 9/78), CIP (4 %; 3/78), NA (24 %; 19/78) and SXT (39 %; 30/78).

The MIC (method section 2.3.3.2) distribution of the *Campylobacter*, *Shigella* and *Salmonella* isolated from the cases against selected antimicrobials (Figure 4-6) showed comparable results to the data of disc diffusion method. The majority of the *Campylobacter* isolates exhibited resistance to NA (84 %; 54/64), CRO (80 %; 51/64) and CIP (80 %; 50/63). Correspondingly, a large proportion of the 62 *Shigella* isolates were also resistant to NA (90 %; 56/62) and CRO (75 %; 47/62). However, *Salmonella* isolates were comparatively susceptible to both (fluoro)quinolones and cephalosporins, with very few isolates displaying resistance to CRO (8 %; 6/77), CIP (4 %; 3/77), or NA (18 %; 14/77). We found that significantly more *Salmonella* isolates exhibited resistance to CHL (40 %; 31/77) and CN (26%; 17/65) than *Campylobacter* (1.5 %; 1/64 and 1.9 %; 1/51, respectively) or *Shigella* (6.55 %; 4/61 and 0 %; 0/36, respectively) ($P < 0.001$ for each comparison, Fisher's exact test).

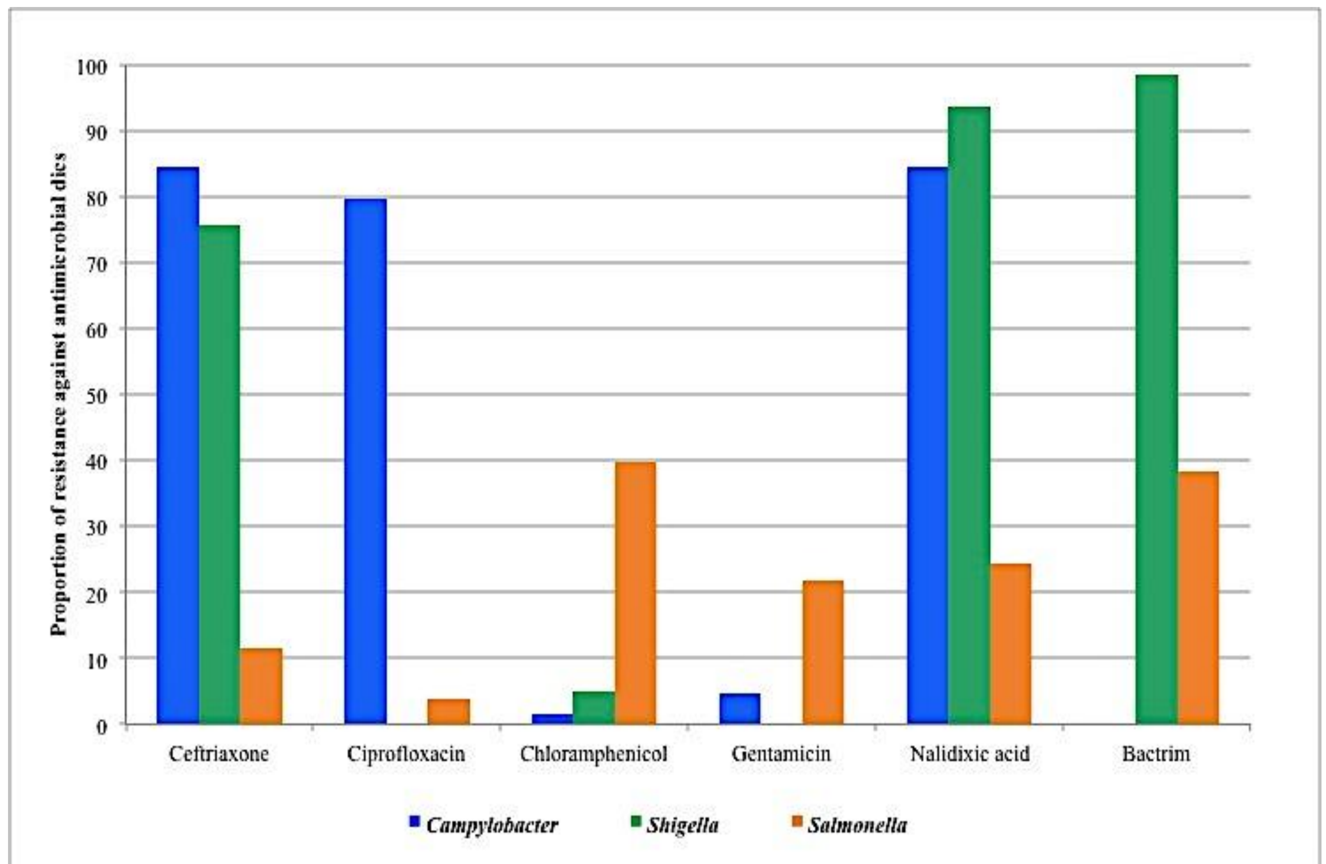


Figure 4-5. The proportion of resistance against antimicrobial discs

Resistance for *Campylobacter*, *Shigella* and *Salmonella* isolates against antimicrobial discs was determined based on CLSI guideline cut-off values for disc diameter for resistance and susceptibility [333] (see section 2.3.3.1, Table 2-2).

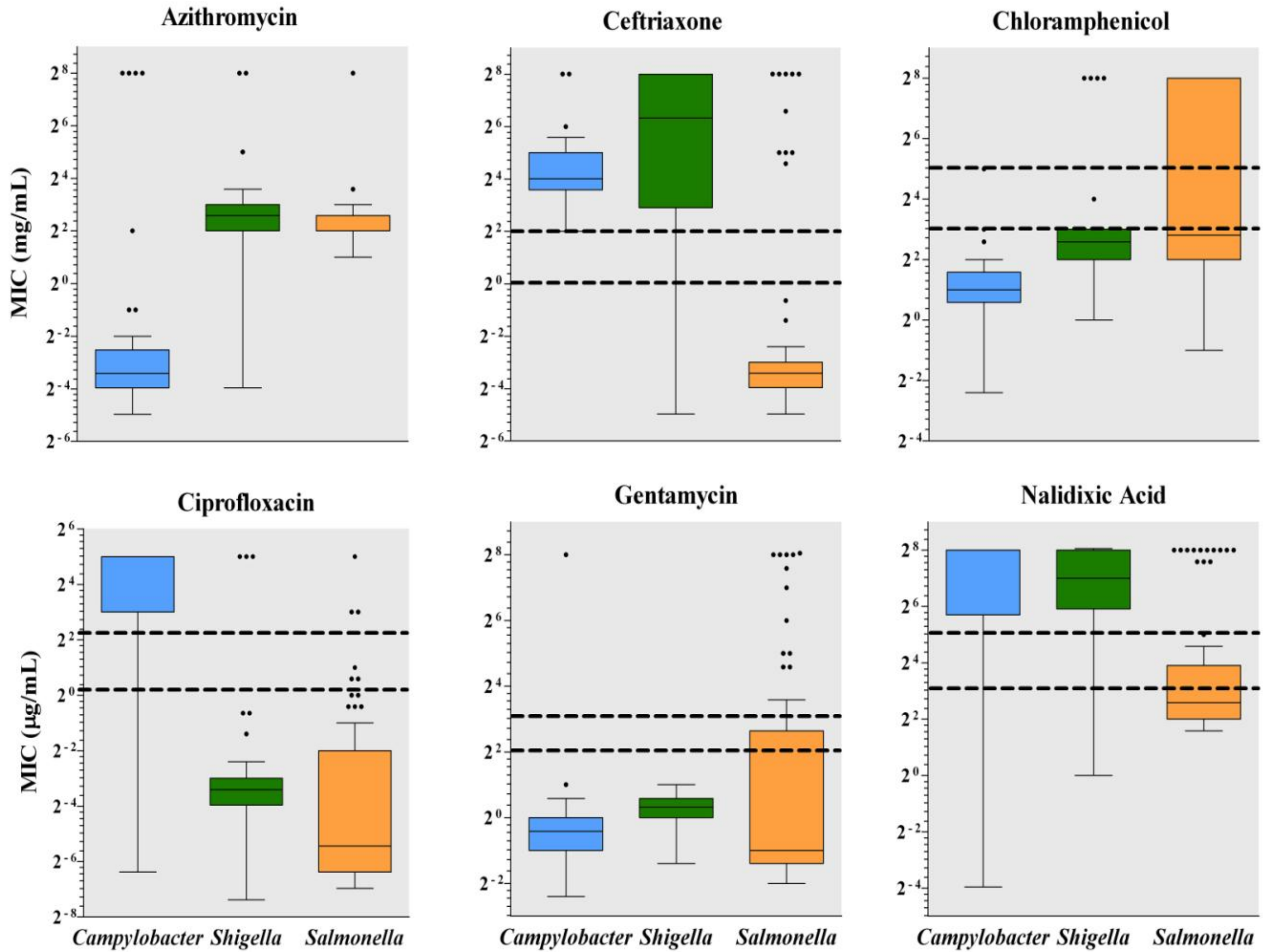


Figure 4-6. The minimum inhibitory concentrations of enteric bacterial pathogens to selected antimicrobials.

Box plots showing the Log_2 of the MICs (μg/ml) for *Campylobacter* (blue), *Shigella* (green) and *Salmonella* (orange) isolates (cases) against Azithromycin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamycin, and Nalidixic Acid. The upper broken line represents the CLSI guideline cut-offs MIC for resistance (Table 2-2, section 2.3.3.2), the lower broken line represents the cut-off value for susceptibility [333]. The length of each box corresponds with the interquartile range and the horizontal line within the box represents the median value for each bacterial category.

The majority of the *Campylobacter* (91 %; 58/64) and *Shigella* (90 %; 56/62) isolates demonstrated resistance to ≥ 3 classes of antimicrobials and a high proportion were ESBL positive (*Shigella*; 75 %; 47/62; *Campylobacter*; 78 %; 50/64). In contrast, a negligible proportion of *Salmonella* isolates exhibited ESBL activity (3 %; 2/78).

Generally, a lower proportion of pathogens isolated from stools of asymptomatic controls demonstrated antimicrobial resistance than the corresponding genera from diarrhoeal cases. Specifically, all the *Salmonella* isolates from the controls were susceptible to CIP, CRO, CN and CAZ, and resistance to CHL was low (2.6 %; 1/39). However, many of the *Campylobacter* isolates from the control stool samples were resistance to CIP (68.7 %; 11/16), NA (62.5 %; 10/16) and CRO (100 %; 16/16), and CHL (18.7 %; 3/16).

4.4. Discussion

This work represents the first investigation of the aetiology, clinical features and the antimicrobial susceptibility profiles of enteric pathogens in paediatric diarrhoeal patients and diarrhoea-free controls in HCMC. A known enteric pathogen was detected in 75 % of all diarrhoeal cases. This proportion is greater than the proportion calculated by a previous case-control study conducted in northern Vietnam (67.3 %; N = 587) [346] and studies in other developing countries such as Tanzania (67.1 %; N = 280) [368], Libya (51 %; N = 239) [369], and Thailand (70 %; N = 236) [370]. The remaining 25 % of cases, in which a pathogen was not detected, may correspond with disease caused by alternative pathogens, limited diagnostic sensitivity, unreported pre-treatment with antimicrobials, or other causes,

such as food allergy, malabsorption or maldigestion [10]. Thus, it is suggested that the higher rate of pathogen isolation reported here, compared to others studies, is dependent on improved molecular methods for NoV and RoV detection.

Diarrhoeal disease in young children in developed countries is predominantly caused by viral pathogens, while bacterial and parasitic diarrhoea are generally considered more prevalent in developing countries [11]. In this study, viral pathogens were identified more frequently than bacterial pathogens in diarrhoeal cases, reflecting the effect of economic transition on the epidemiology of enteric pathogens in HCMC [277]. Specifically, this economic transition has led to marked improvements in sanitation and access to clean water and health services. In addition, increasing population density and changing human contact patterns are thought to alter the transmission of many human pathogens [371], and are likely play a role in the shifting spectrum of the pathogens detected.

The high prevalence of children hospitalised with RoV-induced diarrhoea in this setting predicts that the use of RoV vaccines would have a considerable impact on the diarrhoeal disease burden in this population. The cost of RoV immunisation is prohibitive ($75 USD for a full course of Rotarix™ or RotaTeq™ vaccination in Vietnam) and neither licensed vaccine is currently included in Vietnam's childhood immunisation schedule. It is proposed that the integration of RoV vaccines into the National Immunisation Program, which would provide vaccine for children free of charge or at subsided cost, should be considered as a matter of necessity [29]. A potential way forward for RoV vaccine implementation in Vietnam would be through mass production with alternative, lower cost, RoV vaccines. A live-attenuated monovalent G1P[8] (Rotavin-M1) vaccine is in development in Vietnam and is currently undergoing clinical trials [316, 317]. The regional production of a generic

RoV vaccine would substantially reduce the cost to the Vietnamese health service and may allow for greater national coverage and greater impact.

One of the key findings from this study was the detection of *Salmonella* spp. in healthy controls at higher prevalence (6.4 %) to that of diarrhoeal cases (5.5 %). Furthermore, the rate of *Campylobacter* spp. isolation from controls (2.6 %) was almost half that isolated from cases (4.5 %). These findings contrast with a previous case-control study conducted in Hanoi [327], which found no asymptomatic *Campylobacter* spp. and a negligible frequency of *Salmonella* spp. (1 %) in 249 non-diarrhoeal controls. However, recent case-control studies conducted in Southeast Asia have found a high prevalence of *Campylobacter* spp. and *Salmonella* spp. from healthy controls in rural Thailand (25 % and 9 %, respectively, N = 236) [370] and Cambodia (8 % and 15 %, respectively, N = 578) [372], which implies that asymptomatic infection with these organisms is more common than previously recognized. Due to the study aims and design, it was unable to assess whether these asymptomatic individuals were "carriers" or "super-shedders" as the asymptomatic controls were not followed longitudinally, suggesting that the presence of pathogens in the stool could have been detected in the incubation period or clinically recovered cases with prolonged faecal shedding. While the clinical relevance of these asymptomatic *Salmonella* and *Campylobacter* infections is, as yet, unknown, the presence of these pathogens in otherwise healthy children in HCMC likely represents an important but largely undocumented mobile reservoir of the disease and antimicrobial resistance genes. Further investigation is clearly warranted to determine their role in transmission and pathogen maintenance within the local community.

A remarkably high frequency of multi-drug resistance was identified in this setting, with > 90 % of *Campylobacter* and *Shigella* and > 50 % of *Salmonella* isolates demonstrating resistant to three or more antimicrobial classes. Reduced susceptibility and resistance to broad spectrum antimicrobials in enteric pathogens is increasingly reported across Asia, and the results of this study support that resistance to multiple antimicrobial groups is common across multiple genera of enteric pathogens [373]. For example, an exceptionally high prevalence of ciprofloxacin-resistance was demonstrated in *Campylobacter*, which has been recognized in several Asian countries, including Cambodia (31 % in *C.jejuni*, N = 64; and 57 % in *C.coli*, N = 23) [372], India (71.4 %, N = 49) [374] and China (up to 100 %, N = 44) [375]. Antimicrobial therapy is not generally recommended to treat non-dysenteric diarrhoea, although ciprofloxacin is commonly used as a first line antimicrobial in the case of severe bouts of diarrhoea [22]. In hospitals in HCMC, routine identification of the causative agents of diarrhoea is not routinely performed; hence, antimicrobial regimes are prescribed according to clinical manifestations, which do not reliably distinguish between viral and bacterial infections. Additionally, the overall clinical effect of antimicrobial resistance in the identified groups of enteric pathogens is undetermined. Future work should be focused on measuring the effect of antimicrobials on those infected with a multi-drug resistant enteric pathogen to provide evidence on the potential benefits of antimicrobial regimes for treating acute diarrhoea.

Ceftriaxone, a third generation cephalosporin, is recommended by the WHO as an alternative treatment for severe infectious diarrhoea and shigellosis [22, 276]. Yet the prevalence of ceftriaxone resistance has increased markedly, particularly in *Shigella* spp., in this location in the past decade. The first ESBL-mediated

ceftriaxone resistant *Shigella* in southern Vietnam was isolated from a paediatric diarrhoeal patient in 2001 at HTD (HCMC) [376]. The extent of ceftriaxone-resistant *Shigella* strains found in this study (75 %) is triple that of the 2007-2008 period in paediatric diarrhoeal patients in the same hospital (23 %; N = 103) [277]. The prevalence of these resistant *Shigella* strains may be overlooked due to a lack of routine diagnosis and antimicrobial resistance surveillance in the region; yet still demonstrates a rapidly increasing pattern in resistance to the third generation cephalosporins. Moreover, it could postulate that antimicrobial-resistant bacteria are circulating in commensal enteric microbiota in the community at high levels [377]. This hypothesis would increase the likelihood of antimicrobial resistance gene transfer, thus increasing the rate of emergence of multi-drug resistant strains and limiting effective therapeutic regimes for treating patients with severe or life-threatening bacterial infections [376]. These observations reiterate that regulating antimicrobial usage in the community and improved control of hospital prescription practices may be a potential strategy of slowing the rate of increasing antimicrobial resistance in this location.

This study has some limitations. Firstly, the proportion of pathogen-positive stool samples may be underestimated because our methods of detection targeted a limited group of pathogens, potentially missing other enteric pathogens such as enteric AdV, AstV and helminths. More specifically, we did not screen for pathogenic *E. coli* variants in the stools of enrolees due to limited technical staff and insufficient financial support. Additionally, the nature of study design as hospital-based surveillance relies on a passive enrolment, and case detection was entirely dependent on healthcare-seeking behaviour. Therefore, the study may inevitably skew results toward the severe end of the disease spectrum, as the bulk of mild

infections remain undetected in the community. Finally, the majority of the controls were children attending a nutritional clinical for health checks, which may result in a bias in nutritional status between the cases and the controls. Furthermore, parents taking their children for a nutritional health check may be from a wealthier socio-economic group than those taking their children to hospital for diarrhoeal disease; poor socio-economic status is a well-described risk factor for diarrhoeal disease [378, 379]. Notwithstanding these limitations, the clinical observations, aetiology and prevalence data are informative and likely to be broadly representative of hospitalised diarrhoea in this setting and other economically transitioning regions in Asia.

4.5. Conclusion

In this study, a known enteric pathogen was identified in 75 % of cases of hospitalised paediatric diarrhoea in HCMC, with RoV and NoV being the most frequently identified. These data suggest that a recent and rapid economic transition in HCMC is responsible for "industrialising" breakdown in enteric pathogens detected herein. The alarming rates of antimicrobial resistance are likely to contribute to the burden of hospitalised diarrhoea in this setting, a situation that demands stricter regulation on the prescription and sale of antimicrobials in the community. As an additional control measure, a prompt evaluation of RoV vaccine into the infant immunisation program should be performed; the introduction of RoV vaccine may substantially reduce the burden of what remains a prevalent but largely neglected cause of childhood morbidity in Vietnam.

5. THE CLINICAL, SOCIO-DEMOGRAPHIC CHARACTERISTICS, AND RISK FACTORS OF SYMPTOMATIC NOROVIRUS INFECTIONS IN YOUNG CHILDREN IN HCMC

5.1. Abstract

This aim of the work presented in this chapter was to investigate the prevalence, clinical manifestations and risk factors of NoV gastroenteritis in children less than 5 years of age in HCMC, Vietnam. Among 1,419 diarrhoeal inpatients and 609 asymptomatic control children enrolled over May 2009 to December 2010, NoV was detected in the stool specimens of 20.6 % (N = 293) and 2.8 % (N = 17) of cases and controls, respectively. NoV GII was predominant in both groups of children; yet, the proportion of NoV GI was significantly higher in controls (26.7 %) than in symptomatic cases (1.2 %). Most NoV-positive cases exhibited classical acute watery diarrhoea with vomiting and low-grade fever, and a substantial proportion of patients were treated with antimicrobials (35.3 %) or probiotics (70.8 %). Risk factors of endemic symptomatic NoV infections included residential crowding and symptomatic contacts, highlighting the likely importance of person-to-person transmission of NoV in Vietnam. These data highlight a significant burden of endemic NoV infections in paediatric gastroenteritis in HCMC.

5.2. Introduction

The ingestion of diarrhoeagenic pathogens in water and inadequate sanitation and hygienic practices are widely acknowledged to be among the major risk factors for childhood diarrhoea in developing countries [380]. There is compelling evidence that transmission and incidence of diarrhoeal infections are intertwined with sustained environmental contamination [7, 380] and that reductions in diarrhoeal risk can be achieved through improvements in water quality, adequate sanitation and through the promotion of better household hygiene practices [7, 381].

NoV is one of the leading causes of acute gastroenteritis in children under 5 years old [232]. While the role of NoV as an important cause of sporadic and epidemic gastrointestinal infections in developed countries has been researched extensively [209], the contribution of this pathogen to the endemic burden of diarrhoeal disease in developing countries is not well characterised [133, 232]. Until a safe and efficacious vaccine is available for NoV (vaccine candidates are currently in advanced stage clinical trials as summarised in section 1.2.2.6), the control of NoV infections remains a challenge for the public health community particularly in developing countries. Thus, gaining insight into the epidemiology of NoV infections in children in such countries is important for developing disease management strategies.

In developing countries like Vietnam, the lack of routine identification of any diarrhoeal pathogens limits our understanding of the epidemiology of NoV, and the burden of NoV infections in this area is probably under-recognised as a result. To address the lack of data on risk factors for endemic NoV infections in low-income countries, the work in this chapter aimed to provide data to define the epidemiological characteristics, clinical aspects and risk factors of endemic NoV

infections in paediatric gastroenteritis patients from the multi-centre surveillance (Chapter 4).

5.3. Results

5.3.1. Demographic characteristics of NoV infections in HCMC

NoV was detected in the stools of 20.6 % (293/1,419) of children with a symptomatic diarrhoeal infection and 2.8 % (17/609) of children without a symptomatic diarrhoeal infection (Table 5-1). Of the 293 positive samples from cases and 17 positive samples from controls, a bacterial and/or viral pathogen was identified the stools of 52 (17.7 %) cases and 2 (11.8 %) controls; these were excluded for the purposes of the subsequent analyses to avoid affects that may be attributable to additional pathogens. The mean age of hospitalised children with a symptomatic NoV monoinfection (N = 241) was 13.3 months (range 2 – 45 months), the mean age of children with an asymptomatic NoV monoinfection (N = 15) was 15.8 months (range 2.3 – 52 months) and mean age of the asymptomatic healthy children without detectable NoV in the stool (N = 592) was 16.8 months (range 0 – 60 months). Additional baseline characteristics between the three groups were comparable (Table 5-1). The majority of NoV-positive cases (77.6 %; 187/241), NoV-negative cases (70.2 %; 790/1,126), NoV-positive controls (73.3 %; 11/15) and NoV-negative controls (76.4 %; 452/592) were breast-fed. NoV infections are often associated with outbreaks in enclosed environments yet we found that attendance in either day-care centres or nursery school was not common in any of the three groups, with the majority of children staying home under the care of a family member.

NoV GII strains were detected in 239/241 (98.8%) and 11/15 (73.3%) of samples from symptomatic and asymptomatic children, respectively. The remainder of symptomatic patients were NoV GI, and one individual was infected with both a GI and GII NoV. Conversely, GI was more common in the 15 asymptomatically NoV infected individuals, with four (26.7 %) infected with NoV GI than in symptomatically NoV infected patients (1.2 %; 2/241) ($P < 0.001$, Chi-squared test).

Table 5-1. Baseline characteristics of NoV-positive cases and controls, and NoV-negative cases and controls, N (%).

Characteristic	Cases		Controls	
	NoV-positive (N = 241)	**NoV-negative (N = 1,126)**	**NoV-positive (N = 15)**	**NoV-negative (N = 592)**
Male sex	147 (61.0)	724 (64.3)	8 (53.3)	314 (53.0)
Age in months (mean, range)	13.3 (2-45)	15.8 (1-59)	15.8 (2.3-52)	16.8 (0-60)
Age groups (months)				
0-6	24 (10.0)	165 (14.7)	3 (20.0)	98 (16.6)
7-12	102 (42.3)	375 (33.3)	4 (26.7)	208 (35.1)
13-18	76 (31.5)	245 (21.8)	3 (20.0)	113 (19.1)
19-24	25 (10.4)	147 (13.1)	3 (20.0)	56 (9.5)
> 24	14 (5.8)	194 (17.2)	2 (13.4)	117 (19.8)
Poor Z-score *	18 (7.5)	73 (6.5)	1 (6.7)	75 (12.7)
Breastfed	187 (77.6)	790 (70.2)	11 (73.3)	452 (76.4)
Daily Activity				
Daycare/Nursery	30 (12.5)	194 (16.4)	4 (26.7)	89 (15.2)
Home	211 (87.6)	938 (83.6)	11 (73.3)	498 (84.8)

* Weight-for-age Z-score calculated based on WHO Child Growth Standards guidelines as described in method section 2.2.2.7. Z-score below -2 was considered to be under-nutrition [329, 330].

5.3.2. Clinical characteristics of NoV infections in HCMC

Clinical presentations were consistent within all symptomatic NoV infections, with the majority (61.4 %; 148/241) being admitted with acute watery diarrhoea with or without solids (27 %; 65/241) (Table 5-2). Mucoid diarrhoea was less common (10.8 %; 26/241). Atypically, two individuals with NoV infections had diarrhoea with macro-bloody stool type (0.8 %); smear microscopy of one in these two stool samples showed presence of red and white blood cells. The NoV patients had diarrhoeal symptoms for a median of two days (IQR 2 – 3 days) prior to hospitalisation and the diarrhoeal cases remained in hospital for a median of five days (IQR 3 – 7 days) until symptoms ceased. During the period of hospitalisation, NoV patients were observed to have an average of four diarrhoeal episodes per day (IQR 3 – 6 episodes), with a median maximum number of 10 diarrhoeal episodes per day (IQR 6 – 12 episodes). Vomiting was the most common additional symptom, observed in 214/241 (88.8 %) cases, followed by anorexia, recorded in 60.2 % (145/241) of all cases. More than half (66.8 %; 161/241) of the NoV cases developed a fever (> 37.5 ^{o}C) during their hospitalisation, of which 26 (10.8 %) cases developed a severe fever in excess of 39 °C. Dehydration was not a common manifestation in symptomatic individuals, as only 6.2 % (15/241) of patients demonstrated any sign of either moderate or severe dehydration.

Table 5-2. Clinical description of NoV-positive cases.

Diarrhoea type	**N**	**%**
Bloody	2	0.8
Mucoid	26	10.8
Watery	148	61.4
Watery with solids	65	27.0
Diarrhoea characteristics	**Median**	**IQR**
Length of illness* (days)	2	2-3
Length of hospital stay (days)	5	3-7
Average number of episodes/day	4	3-6
Maximum number of episodes/day	10	6-12
Symptoms	**N**	**%**
Abdominal pain	9	3.7
Anorexia	145	60.2
Cough	85	35.3
Dehydration‡	15	6.2
Vomiting	214	88.8
Mild Fever (37.2 - 39 ^{o}C)	135	56.0
Severe Fever (> 39 ^{o}C)	26	10.8

N = 241

* Prior to hospitalisation.

‡ Includes 1 severely and 12 moderately dehydrated patients.

5.3.3. Treatment regimes of NoV infections in HCMC

Prescribed therapy across the NoV cases was highly variable and was performed based on presenting symptoms and prior to any aetiological diagnostic evaluation. Zinc supplements 78.3 % (188/241), probiotics (lactobacilli reconstituted in water) 70.8 % (170/241) and RT 54.6 % (131/241) were commonly prescribed for the symptomatic NoV patients (Table 5-3). Furthermore, an Abx was prescribed for 35.3 % (85/241) of the diarrhoeal patients with NoV, of which fluoroquinolones and cephalosporins were the most commonly used, administered to 18.7 % (45/241) and 10.8 % (26/241) of those receiving Abx, respectively. Other antimicrobials including macrolides, aminoglycosides and quinolones, were also used, but infrequently.

Table 5-3. Treatment prescription of NoV-positive cases

Prescriptions	**N**	**%**
Abx	**85 ***	**35.3**
Aminoglycoside	1	1.1
Cephalosporin	26	29.2
Fluoroquinolone	45	50.6
Macrolide	16	18.0
Quinolone	1	1.1
Zn	**188**	**78.3**
RT	**131**	**54.6**
Probiotics	**170**	**70.8**

N = 241

*Four patients were prescribed 2 antibiotics each, so although 85 patients were prescribed an antibiotic, there were 89 total prescribed drugs.

5.3.4. Correlation of NoV incidences and climatic factors in HCMC

Monthly average rainfall and temperature data in HCMC were used to evaluate NoV infection seasonality (see method section 2.2.2.7) (Figure 5-1). NoV infections were consistently detected throughout the yearlong period of investigation, yet infections were more prevalent in the rainy season. The minimal monthly proportion of NoV cases observed among all diarrhoeal patients was in December, corresponding with 13.42 % (20/149) of all diarrhoeal infections. The proportion of diarrhoeal patients infected with NoV peaked in September and October; during which up to 35.59 % (42/118) and 34.17 % (41/120), in each respective month, of all admitted cases were positive for NoV. This peak in infection correlated with a secondary peak of rainfall. The proportion of NoV infections declined in subsequent months, yet remained continuously prevalent, at approximately 18 % of diarrhoeal patients throughout the remainder of the year. Whilst there was a correlation with NoV infections and rainfall (Pearson's correlation coefficient r = 0.626, P = 0.029), NoV infections did not correlate with variation in average temperature (range 22.1 ^{o}C – 37.8 ^{o}C) (Pearson's correlation coefficient r = -0.308, P = 0.330).

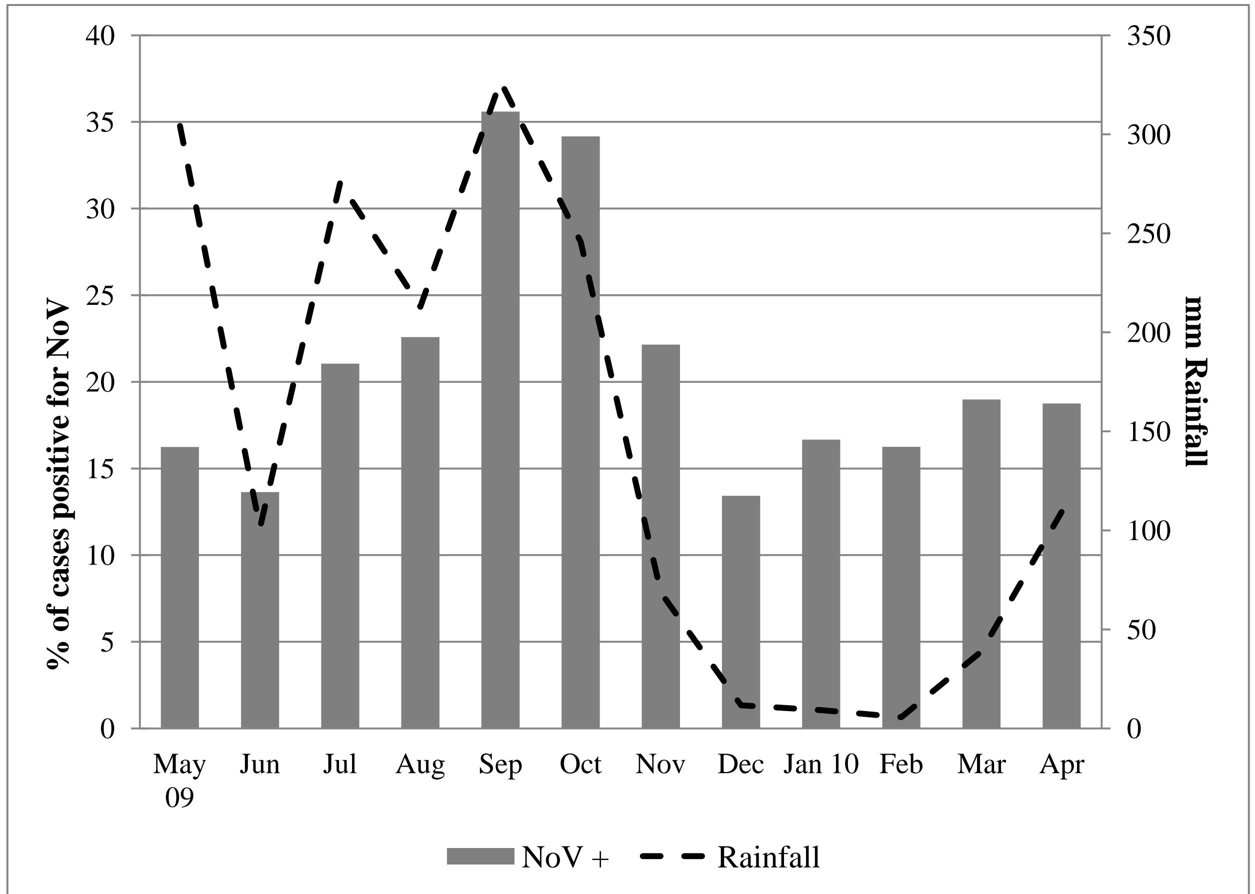


Figure 5-1. The seasonality of NoV-positive cases in HCMC

The proportion of patients that were positive for NoV (monoinfection) (dark bars) by month compared to average monthly rainfall accumulation measured in mm (black line) in HCMC, Vietnam. Climatic data was obtained from Vietnam Southern Regional Hydro-Meteorological Station (see method section 2.2.2.7).

5.3.5. Risk factors of symptomatic NoV infections in HCMC

A risk factor analysis was performed comparing stratified variables between those with NoV-positive cases (N = 241) and NoV-negative controls (N = 592) (as described in method section 2.4.7) (data presented in Table 5-4). Crude associations were found between NoV infection and age (odds ratio [OR] 0.97; 95 % CI 0.96-0.99, $P < 0.001$), male sex (OR 1.38, 95 % CI 1.0-1.9, $P = 0.037$), weight-for-age Z-score (OR 0.56, 95 % CI 0.3-0.9, $P = 0.033$), living in a household with ≥ 3 children (OR 1.62, 95 % CI 1.0-2.5, $P = 0.035$), owning a refrigerator (OR 0.59, 95 % CI 0.4-0.9, $P = 0.006$), living in a household where meat and vegetables are purchased primarily at outdoor markets (OR 3.77, 95 % CI 2.6-5.5, $P < 0.001$), drinking bottled water compared to drinking water from a government pipeline supply (OR 1.63, 95 % CI 1.1-2.3, $P = 0.008$), using an outdoor toilet (OR 0.32, 95 % CI 0.2-0.5, $P < 0.001$) and having recent contact with a individual (child or adult) that had had a diarrhoeal episode (OR 14.23, 95 % CI 6.5-31.0, $P < 0.001$).

After controlling for confounding factors, including the *a-priori* factors of sex, age and household income level, several risk factors remained significantly and independently associated with symptomatic NoV infections. These risk factors were younger age (adjusted OR [AOR] 0.96, 95 % CI 0.94-0.98, $P < 0.001$), living with ≥ 3 other children in the household (AOR 1.70, 95 % CI 1.0-2.9, $P = 0.052$), living in a household where food is regularly purchased from outdoor markets (AOR 4.99, 95 % CI 3.1-7.9, $P < 0.001$), drinking bottled water compared to drinking water from a government pipeline supply (AOR 2.18, 95 % CI 1.4-3.4, $P < 0.001$), using an outdoor toilet (AOR 0.22, 95 % CI 0.1-0.4, $P < 0.001$) and having contact with recent symptomatic individual (AOR 26.14, 95 % CI 10.4-65.9, $P < 0.001$).

Table 5-4. Univariable and multivariable analysis of risk factors for symptomatic NoV infections.

Risk factor	**NoV-positive cases**	**NoV-negative controls**	**OR**	**95%CI**	**AOR**	**95%CI**
Age in months (mean, range)	13.3 (2-45)	16.8 (0-60)	**0.97**	**0.96-0.99**	**0.96**	**0.94-0.98**
Male sex	147 (61.0)	314 (53.0)	**1.38**	**1.0-1.9**	1.38	0.9-2.0
Poor Z-score	18 (7.5)	75 (12.7)	**0.56**	**0.3-0.9**	0.61	0.3-1.1
Low income *	150 (62.2)	335 (56.6)	1.26	0.9-1.7	0.89	0.6-1.3
≥ 5 adults in hh	72 (29.9)	158 (26.7)	1.17	0.8-1.6	ni	
≥ 3 children in hh	36 (14.9)	58 (9.8)	**1.62**	**1.0-2.5**	**1.70**	**1.0-2.9**
Own a refrigerator	187 (77.6)	506 (85.5)	**0.59**	**0.4-0.9**	0.73	0.5-1.2
Market food	201 (84.1)	345 (58.4)	**3.77**	**2.6-5.5**	**4.99**	**3.1-7.9**
Water source for activities						
Pipeline †	132 (54.8)	347 (58.6)	1.00	-	ni	
Well	96 (39.8)	220 (37.2)	1.15	0.8-1.6		
Other ‡	13 (5.4)	25 (4.2)	1.37	0.7-2.8		
Drinking water source						
Pipeline †	116 (48.1)	334 (56.4)	1.00	-	1.00	-
Bottled water	69 (28.6)	122 (20.6)	**1.63**	**1.1-2.3**	**2.18**	**1.4-3.4**
Well	42 (17.4)	109 (18.4)	1.11	0.7-1.7	0.94	0.6-1.5
Other ‡	14 (5.8)	1.49	0.8-2.9	0.25	1.45	0.6-3.2
Toilet type						
Indoor †	213 (90.6)	446 (75.9)	1.00	-	1.00	-
Outdoor	22 (9.4)	142 (24.2)	**0.32**	**0.2-0.5**	**0.22**	**0.1-0.4**
Hand washing *§*	130 (85.0)	291 (89.5)	0.66	0.4-1.2	ni	
Daycare/Nursery school	30 (12.5)	89 (15.2)	0.80	0.5-1.2	ni	
Symptomatic contact	38 (16.5)	8 (1.4)	**14.23**	**6.5-31.0**	**26.14**	**10.4-65.9**
Rural ¶	36 (14.9)	75 (12.7)	1.21	0.8-1.9	ni	

N (%) unless otherwise noted; OR: Odds ratio; AOR: Adjusted odds ratio; ni: not included in multivariable analysis; hh: household; **Boldface** indicates statistical significance at $P \leq 0.05$

* Classified as making less than the Gross National Income ($232/month) based on World Bank (http://data.worldbank.org/indicator/NY.GNP.PCAP.CD).

† Reference group.

‡ Rain water, water from a truck provided by the government or other water source.

§ After the child uses the toilet.

¶ Binh Chanh, Can Gio, Cu Chi, Hoc Mon and Nha Be districts.

Table 5-5. Sensitivity analysis of risk factors comparing NoV-positive cases and controls as well as NoV-negative cases.

Factor	NoV-positive cases versus NoV-negative controls			NoV-negative cases versus NoV-negative controls		
	AOR	**95%CI**	***P* value**	**AOR**	**95%CI**	***P* value**
Age in months	0.96	0.94-0.98	**<0.001**	0.99	0.98-0.99	**0.02**
Male sex	1.38	0.9-2.0	0.08	1.50	1.2-1.9	**<0.001**
Poor Z-score	0.61	0.3-1.1	0.11	0.44	0.3-0.7	**<0.001**
Low household income	0.89	0.6-1.3	0.56	1.27	1.0-1.6	**0.05**
>3 children in household	1.70	1.0-2.9	**0.05**	1.42	1.0-2.0	**0.04**
Own a refrigerator	0.73	0.5-1.2	0.18	0.78	0.6-1.1	0.11
Market food	4.99	3.1-7.9	**<0.001**	3.60	2.8-4.7	**<0.001**
Drinking water source						
Pipeline	1.00	-	-	1.00	-	-
Well	0.94	0.6-1.5	0.82	0.81	0.6-1.1	0.17
Bottled	2.18	1.4-3.4	**<0.001**	1.25	0.9-1.7	0.12
Other	1.45	0.6-3.2	0.38	1.46	0.9-2.4	0.15
Toilet type						
Indoor	1.00	-	-	1.00	-	-
Outdoor	0.22	0.1-0.4	**<0.001**	0.55	0.4-0.7	**<0.001**
Symptomatic contact	26.14	10.4-65.9	**<0.001**	8.87	4.2-18.8	**<0.001**

A limited sensitivity analysis was performed (see section 2.4.7). Several risk factors were identified specifically for NoV infection (bottled water), compared to risk factors for all-cause diarrhoea (male sex, poor weight-for-age Z-score, low income). However, there were also similarities in risk factors for the two comparisons including young age, household crowding, market food, outdoor toilet and contact with symptomatic individual.

5.4. Discussion

The high prevalence (20.6 %) of NoV presented here is higher than a pooled international estimate [232] and previous studies in HCMC [250, 252, 319], yet was lower than a study conducted in northern Vietnam, Hanoi (36 %) [251]. Although there is a paucity of generalizable data in this region, it appears that there has been a gradual increase in the rate of NoV detection among hospitalised children in HCMC over the past decade. Taking available data, the relative frequency of NoV infections in HCMC was observed from 5.4 % in 1999-2000 [319], to 5.5 % in 2002-2003 [252], to 6.4 % in 2005-2006 [250] to 13.5 % in 2008 (Chapter 3), and finally to the level reported here. Despite differences in study settings, data strongly suggest that NoV is endemic in HCMC. Increasing frequencies of NoV detection may reflect the pattern in the city but may also be attributed to confounding factors such as better sample collection procedures and improved viral identification methods.

Previous studies on NoV prevalence in various settings have suggested that asymptomatic infections are present in the community [232]. The duration of longitudinal NoV carriage was not assessed in healthy children in Vietnam, yet asymptomatic NoV infections were reported in 2.8 % of diarrhoea-free children in HCMC. This asymptomatic assessment is comparable to a pooled median prevalence of 4 % (range: 0 – 16 %) from a recent meta-analysis using data from both developing and developed countries [232]. The clinical relevance of these asymptomatic infections presented in this work was not determined, but these asymptomatic infections may be pre-symptomatic cases in the incubation phase or clinically recovered cases with prolonged shedding [219]. Alternatively, these cases

may be truly asymptomatic infections caused by transient NoV passing through the gastrointestinal tract, representing a mobile reservoir of infectious viruses [382].

NoV infections are commonly associated with outbreaks in enclosed environments [133], yet it was found that attendance in day-care centres and nursery schools was not common; with the majority of children remaining at home during the day. However, several factors were significantly and independently associated with symptomatic NoV infections after controlling for several confounding factors. Person-to-person transmission of NoV has been shown to be the most common route of infections during sporadic outbreaks [383-386], which can occur within a household setting [387] or through external contact with symptomatic individuals [383, 388]. The two key risk factors for infection determined in this study were residential crowding particularly with children, and recent contact with symptomatic individuals. Young children are not only at high risk to NoV infections [133] but are also too young to know about suitable hygiene practices which leads to increased transmission within enclosed environments [389]. As most of enrolled patients did not attend day-care, the design of future public health prevention measures for NoV in HCMC should consider household education.

NoV can be transmitted via many routes; these include foodborne transmission and poor food hygiene through infectious handlers [236, 239-243]. The analysis found that living in a household where meat and vegetables were purchased primarily at outdoor markets, rather than supermarkets, was strongly associated with symptomatic NoV infections in children. Consuming bottled water, rather than governmental pipeline water, proposes a risk of symptomatic NoV infections. However, this factor requires further validation because those drinking municipal water also reported boiling or filtering water prior to consumption, whilst those using

bottled water did not, provided that bottled water quality is highly variable and is poorly regulated in this setting. An unexpected finding was the protective nature of outdoor toilets, which may be resulted from the sterilising capabilities of sunlight or containing faecal contamination outside the residence during the pre-walking period of infancy.

Monthly stratification of NoV cases demonstrated that infections peaked in the rainy season, which has also been described in NoV epidemiological studies conducted in tropical environments [252, 390]. The data in this chapter showed a positive linear correlation between NoV infections and monthly rainfall, but no similar correlation with temperature. NoV illnesses are classically associated with the winter season in developed countries in temperate zone [260, 391], the association of NoV infections with the tropical rainy season may reflect differential transmission between different climatic regions, between tropical versus more temperate climates [391].

This study has certain caveats that need to be considered before placing the findings into a generalizable context. Firstly, the work represented only a snapshot of the overall disease prevalence and was not comprehensive and largely dependent on health-care seeking behaviour of families that may be dependent on disease severity and income in this setting. The controls may not be entirely representative of the population from which the cases arose since a large proportion of the controls were visiting the hospital for nutritional advice, which may impact on diarrhoeal disease risk [378]. However, a limited sensitivity analysis comparing NoV-positive cases to NoV-negative controls and NoV-negative cases to NoV-negative controls (Table 5-5) showed several differences in risk factors, suggesting that the identified risk factors are associated with NoV rather than healthcare-seeking behaviour.

Additionally, the hospital surveillance (Chapter 4) investigated diarrhoea only and children with NoV may present with vomiting as the sole symptom [389, 392], so it is possible that some NoV-infected but otherwise diarrhoea-free children were unintentionally missed. Moreover, real-time RT PCR is considered to be more sensitive than conventional RT PCR [337], hence the conventional method used in this study may have led to a number of false negatives, leading to an underestimation of the overall disease prevalence.

5.5. Conclusion

This chapter demonstrated that NoV is endemic with a rainfall-referral pattern and an important cause of hospitalisation in children under 5 years of age suffering from diarrhoea in HCMC. It was possible to detect a small yet important proportion of asymptomatic NoV infections in diarrhoea-free children. There are limited publications on the prevalence and epidemiology in this part of Asia, and NoV studies from Vietnam are extremely sparse. This work is the first investigating risk factors of NoV infections in young children in Vietnam. In conclusion, young age, residential crowding, bottled water, and recent contact with a symptomatic individual are key risk factors for symptomatic NoV infections in HCMC. As most children did not attend day-care, potential preventative measures for NoV in HCMC should be targeted at improving local hygiene standards to prevent person-to-person transmission within the home.

6. THE DYNAMICS AND EVOLUTION OF NOROVIRUS GII.4 VARIANTS CIRCULATING IN HCMC

6.1. Abstract

NoV is a major cause of endemic and global epidemic gastroenteritis, yet the epidemiological significance of NoV in developing countries is poorly characterised. The aim of the work in this chapter was to investigate the spatiotemporal distribution of NoV genotypes in paediatric diarrhoeal patients and asymptomatic children in HCMC, Vietnam. NoV identified from the hospital surveillance (Chapter 4) was genotyped and mapped against the corresponding residential locations and the date of isolation. Viruses within the GII, particularly in the GII.4 lineage, were most commonly detected in both symptomatic and asymptomatic individuals. These GII.4 strains could be separated into two major variants, the GII.4-2006b (Minerva) and GII.4-2010 (New Orleans), of which the latter is a novel strain that was identified for the first time in Asia in this study. Additional analyses demonstrated no spatiotemporal structure among the endemic GII viruses; yet a significant spatiotemporal signal was detected corresponding with the novel introduction of GII.4-2010 variant. In conclusion, these data presented in this chapter showed that NoV GII.4 variants are highly endemic in HCMC and underlined a rapid NoV strain replacement occurring in early 2010 in HCMC.

6.2. Introduction

NoV disease is characterised by acute watery diarrhoea with vomiting and a low-grade fever [145]. NoV has an exceptionally low infectious dose [261], can survive on surfaces for prolonged time periods [393], and frequently causes explosive gastroenteritis epidemics [209, 214, 232-235]. Currently, the complete amino acid sequences of the major capsid protein (VP1) are used to segregate NoV into five genogroups (GI – GV) [156]. Viruses in GI and GII are responsible for the vast majority of human infections and can be divided into 8 GI and 23 GII capsid genotypes, and 14 GI and 29 GII polymerase genotypes [156, 160]. For routine NoV detection, genogrouping and genotyping, partial coding sequences of the RdRp (region A and B) [162, 163, 167-169] and VP1 (region C, D and E) [158, 161, 170, 171] can be used since obtaining the complete NoV genome sequences is impractical in many settings.

The NoV disease landscape is driven by complex interactions between various factors, including population immunity, the environmental factors, and strains circulating [394, 395]. The acknowledged interpretation of global NoV epidemiology is that strain replacement occurs every 2 – 3 years [173, 182, 184, 193, 237]. These replacements are typically caused by strains of a single GII.4 lineage, which has been responsible for the majority of NoV outbreaks worldwide since being first identified in the US in mid-1990s [182, 248]; each replacement is derived from emergence of a novel GII.4 variant, believed to escape population immunity through antigenic variation [148, 182, 184-189].

The majority of NoV studies have been performed in developed countries and disease outbreaks are continually monitored through several disease surveillance networks [396, 397]. Conversely, little is known about the transmission, molecular

diversity or the spatiotemporal dynamics of NoV infections in areas with differing public health infrastructure and demographics. NoV was first reported in HCMC in 1999 [319], and recent work (Chapter 4 and 5) has demonstrated that NoV is endemic throughout the year, in contrast to the winter outbreaks observed in temperate locations [260, 398].

The aim of this chapter was to investigate the molecular epidemiology and NoV strain diversity in HCMC, in particular the molecular and spatiotemporal distribution of NoV genotypes in young hospitalised children in HCMC, Vietnam.

6.3. Results

6.3.1. The temporal distribution of NoV genogroups and genotypes

During the multi-centre hospital-based surveillance (Chapter 4), there were 2,028 individuals enrolled with accompanied socio-demographic data; yet stool samples were collected from 2,054 individuals, because 26 individuals were self-withdrawn from the study. These 26 individuals were excluded from the risk factors analysis (Chapter 5) due to unavailable social and behavioural data, but were included in NoV detection and genetic analyses (this chapter). Among the collected 2,054 samples, NoV were detected in 315 samples (15.3 %) (detection method as described in section 2.4.3), of which 298 (94.6 %) were from symptomatic cases and 17 (5.4 %) were from asymptomatic controls. All identified NoV was genotyped following the method described in section 2.4.5.3. The genotyping data showed that a vast majority of NoV identified from the study belonged to GII (304; 96.5 %), with viruses in GI accounting for 3.5 % of the participants (Table 6-1). Diverse genotypes were detected in GI (GI.3, GI.4 and GI.5) and GII (GII.2, GII.3, GII.4, GII.6, GII.7,

GII.9, GII.12, GII.13 and GII.4U). The GII.4U represents unassigned variant within the GII.4, most closely related to the lineage containing strains OB200615 and X76716 according to RIVM-NoroNet typing tool [160].

Among NoV GII strains (N = 304), GII.4 was the most prevalent genotype, representing 81.3 % (243/304) of all GII viruses. These GII.4 strains could be differentiated into two major variants, GII.4-2006b (Minerva) (87.7 %; 213/243) and GII.4-2010 (New Orleans) (12.3 %; 30/243). All GII.4 strains from asymptomatic children (N = 4) belonged to the GII.4-2010 variant. NoV GII.4-2006b was predominantly identified in diarrhoeal cases (89.1 %; 213/239), followed by GII.4-2010 (10.9 %; 26/239). GII.3 was the second most common genotype in both cases (10 %; 29/291) and controls (17.6 %; 3/13).

Table 6-1. The distribution of NoV genogroups and genotypes in 298 NoV-positive cases and 17 NoV-positive controls in HCMC, N (%).

Genogroup/Genotype	Cases N = 298	Controls N = 17	Total
GI	**7 (2.4)**	**4 (23.5)**	**11 (3.5)**
I.3	6 (2.0)	1 (5.9)	7 (2.2)
I.4	0 (0)	1 (5.9)	1 (0.3)
I.5	1 (0.3)	2 (11.8)	3 (1.0)
GII	**291 (97.6)**	**13 (76.5)**	**304 (96.5)**
II.2	1 (0.3)	3 (17.6)	4 (1.3)
II.3	29 (10.0)	3 (17.6)	32 (10.5)
II.4	243 (83.5)	4 (23.5)	247 (81.3)
II.4-2006b	*213 (87.7)*	*0 (0)*	*213 (86.2)*
II.4-2010	*26 (10.7)*	*4 (100)*	*30 (12.1)*
II.4U	*4 (1.6)*	*0 (0)*	*4 (1.6)*
II.6	8 (2.7)	0 (0)	8 (2.6)
II.7	2 (0.7)	1 (5.9)	3 (1.0)
II.9	1 (0.3)	0 (0)	1 (0.3)
II.12	1 (0.3)	1 (5.9)	2 (0.6)
II.13	6 (2.1)	1 (5.9)	7 (2.3)
Total	**298 (94.6)**	**17 (5.4)**	**315**

The two major variants, GII.4-2006b and GII.4-2010, were the focus of subsequent analyses bearing their overall dominance and perceived epidemiological relevance in NoV-induced gastroenteritis in young children in HCMC. The monthly temporal distribution of GII viruses, with particular attention paid to two GII.4 variants (Figure 6-1), showed that GII.4 strains were present every month throughout the case enrolment period (i.e. May 2009 to April 2010), with GII.4-2006b being the only GII.4 variant identified between May and November 2009. Notably, a peak of GII.4-2006b infection was noted during September and October 2009. Since the first identification of GII.4-2010 variant in December 2009, the prevalence of this newly emergent became increasingly profound in the following months. Concurrently but disproportionally with the increasing proportion of GII.4-2010 variants, the frequency of GII.4-2006b viruses decreased from 7.36 % (17/231) in December 2009 to 1.73 % (4/231) in February 2010, and was not detected after March 2010. After April 2010, the frequency of NoV detection remained relatively low due to the overall small number of asymptomatic controls enrolled from May to December 2010.

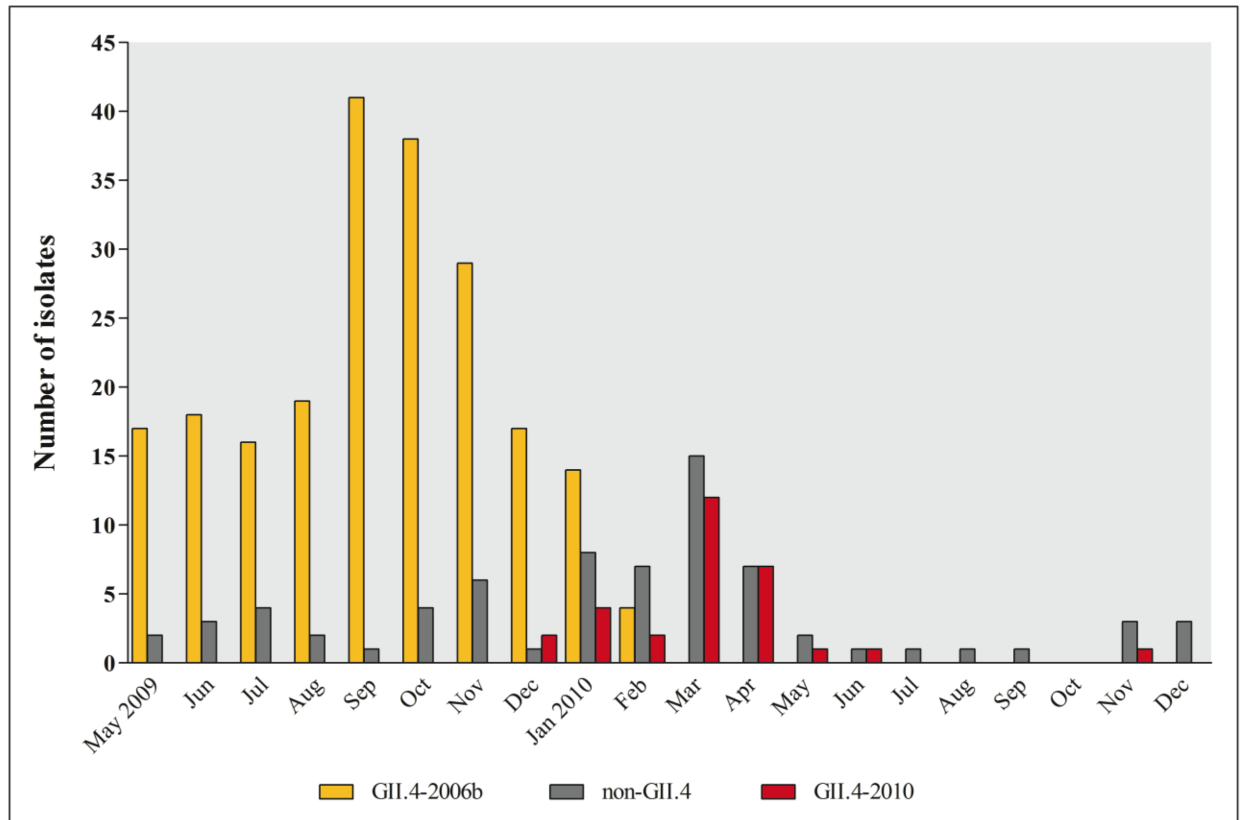


Figure 6-1. The monthly temporal distribution of NoV variants in HCMC over the period of investigation.

Graph showing the distribution of GII.4-2006b and GII.4-2010 variants against other NoV strains (total numbers identified) detected in symptomatic and asymptomatic children over the study period (GenBank accession number HE716437 to HE716751). Both monoinfections and mixed infections were included in the total pathogen count.

6.3.2. Phylogenetic analyses of NoV sequences

Phylogenetic analyses were performed on the NoV GI and GII sequences, comparing the relatedness between strains isolated from the cases and the controls (as described in section 2.4.5.5). The strains within the two genogroups separated completely into two lineages, with strains of the same genotype clustering irrespective of whether they were isolated from a symptomatic or asymptomatic individual (Figure 6-2). The mean uncorrected genetic distances among the strains within the GII and between variants in GII.4 genocluster (pairwise distance of maximum composite likelihood calculation) were 0.147 and 0.016 substitutions per site, respectively.

To obtain a phylogenetic tree with better resolution for establishing the relatedness between strains isolated from NoV-positive cases and NoV-positive controls, a sub-sampled maximum likelihood phylogenetic tree was constructed on GII sequences (N = 109 strains) by excluding GI strains and identical GII sequences as described in section 2.4.5.5 (Figure 6-3). Again, the collected strains, irrespective of the source of individuals isolated, separated precisely into genotype specific lineages, which can be clearly seen on the phylogenetic tree in Figure 6-3.

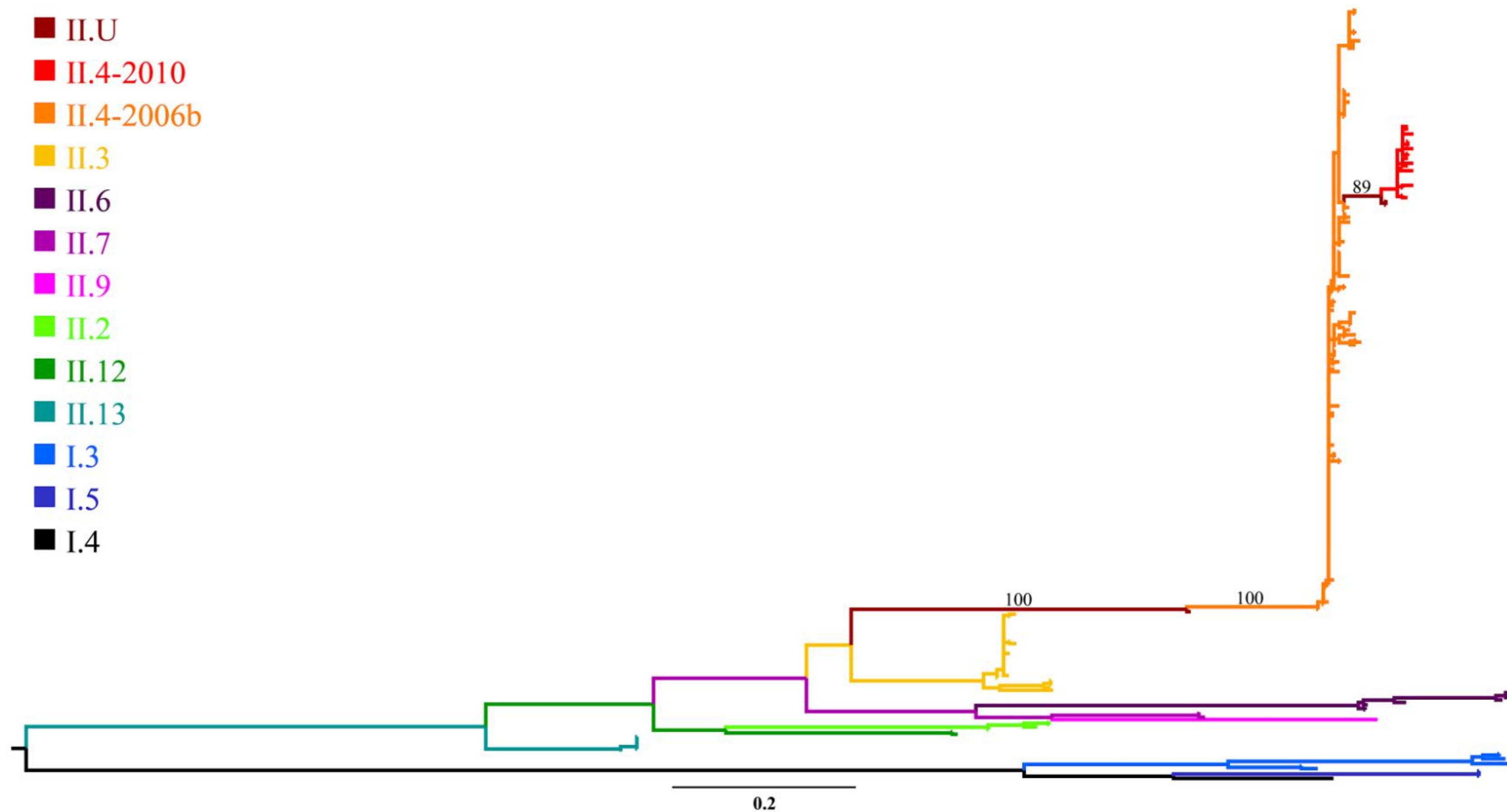


Figure 6-2. Phylogenetic tree of 315 NoV strains from HCMC.

Maximum likelihood phylogenetic tree constructed from amplification fragments of NoV GI and GII, as described in section 2.4.5.5. All horizontal branch lengths are drawn to the scale of a nucleotide substitution per site. Tree is mid-point rooted with branches coloured according to viral genogroups or genotypes/variants. Only bootstrap values of > 85 % are shown.

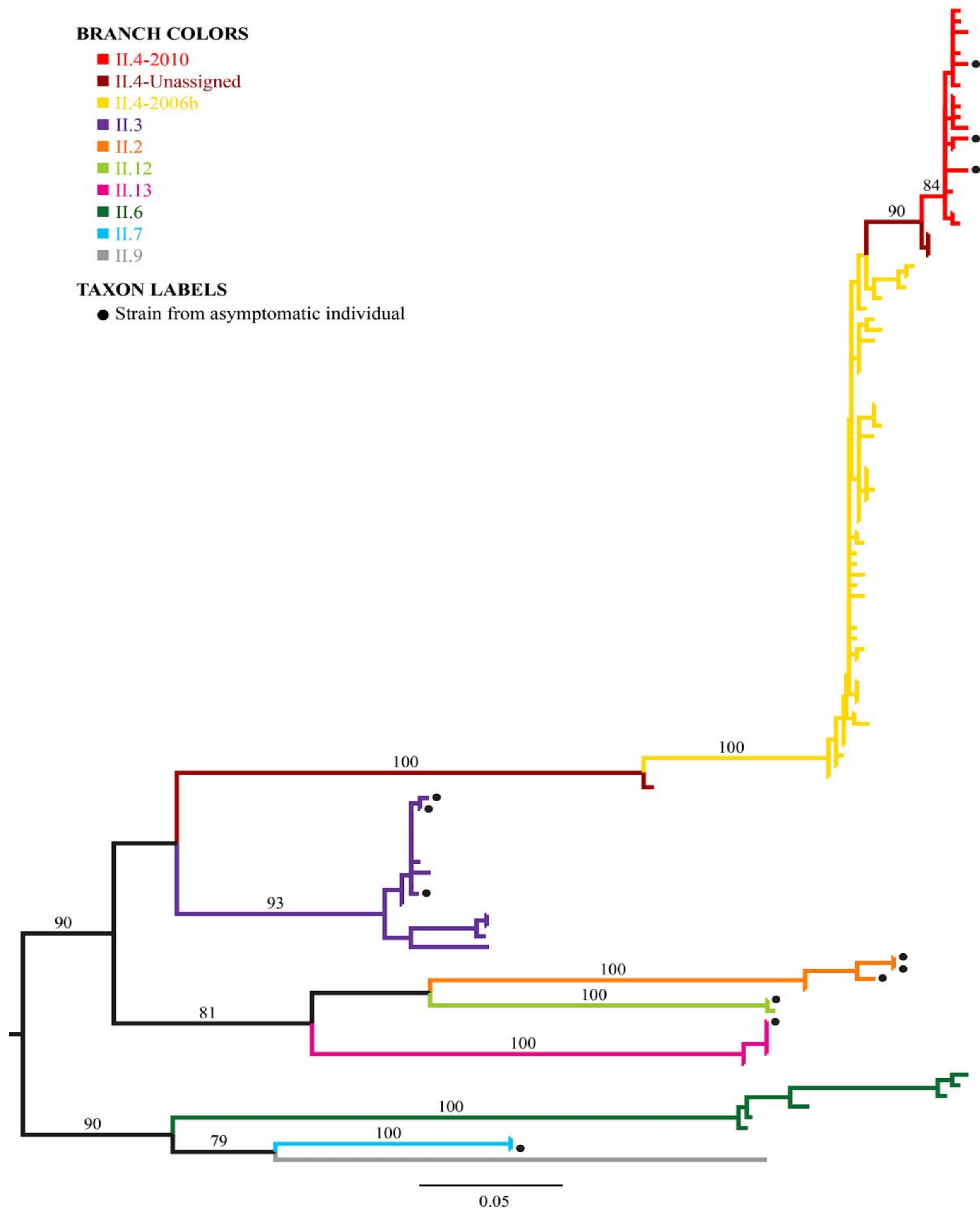


Figure 6-3. Phylogenetic tree of 109 NoV GII strains from HCMC.

Tree constructed from 109 NoV GII strains collected during this study and based on the GII amplification fragment trimmed to 378bp (see section 2.4.5.5). All horizontal branch lengths are drawn to the scale of a nucleotide substitution per site. Tree is mid-point rooted with branches coloured according to viral genotypes/variants. Strains from asymptomatic individuals are labelled with a circle. Bootstrap values of > 75 % are shown.

Further analyses were performed on sequences of the two GII.4 variants from HCMC, comparing them with 269 representative global sequences (GenBank) and 10 NoV GII.4 sequences from the previous work in Vietnam (Chapter 3) (Figure 6-4) (method described in section 2.4.5.6). Using a Bayesian phylogeny approach and time-stamped sequences, the NoV evolutionary rate was calculated as 8.072×10^{-3} substitutions per site per year. The GII.4-2006b sequences from NoV originating in Vietnam, including those identified from Chapter 3 and this chapter, fell in the same clade as global GII.4-2006b viruses with clustering unrelated to either their corresponding time or place of isolation. The GII.4-2006b lineage could be further differentiated into two sub-lineages, with strains identified in HCMC present in both, highlighting the co-circulation of divergent GII.4-2006b viruses in the local population. More sequences from this study were found in the upper sub-lineage while more Vietnamese strains from previous studies fell in the lower sub-lineage. All GII.4-2010 sequences belonged in a single lineage, separated from the GII.4-2006b clade. Strains within the GII.4-2010 lineage could be partially differentiated by isolation location, with Vietnamese and Belgian sub-lineages stemming from the New Orleans GII.4-2010 (US) variant.

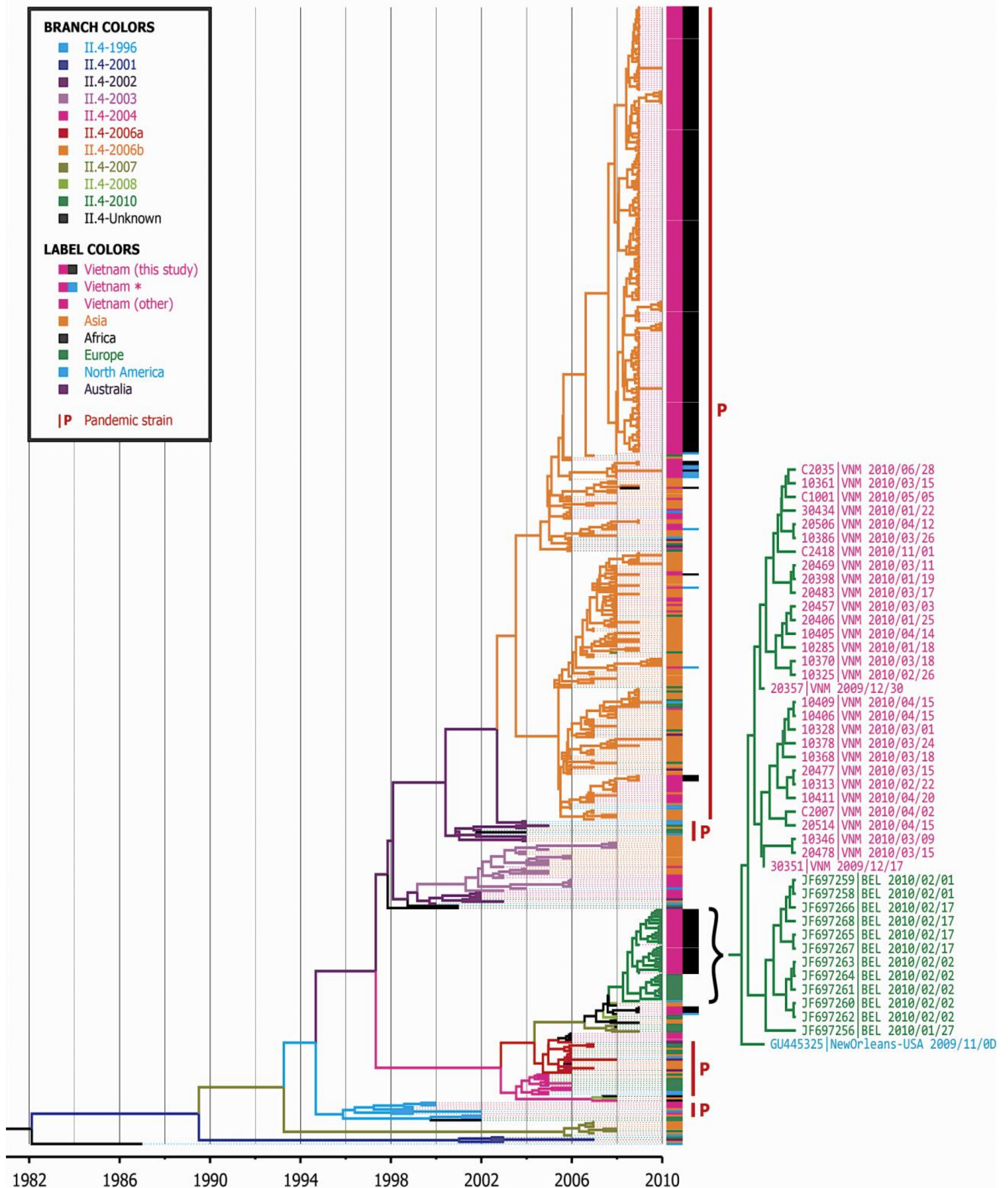


Figure 6-4. Phylogenetic tree of GII.4 NoV strains from HCMC and 526 representative global GII.4 sequences.

Maximum likelihood phylogenetic tree of 526 global and HCMC GII.4 NoV strains constructed from the amplification region trimmed to 378bp (as described in section 2.4.5.6). All horizontal branch lengths are drawn to the scale of a nucleotide substitution per site per year. Branch tips are coloured according to the genotype and colour-coded by their continent of isolation. Vietnamese strains included are from this study, previous studies (other) and from southern Vietnam (Chapter 3) denoted by (*). The GII.4-2010 lineage is magnified to highlight the strains originating from Vietnam, Belgium and US.

6.3.3. Spatiotemporal clustering of NoV in HCMC

The residences of all NoV-infected children identified from the study were mapped in Google Earth (Figure 6-5), as described in method section 2.4.6.1. Data from the monthly temporal distribution (Figure 6-1) suggested a NoV strain replacement phenomenon occurred during the period of investigation. Thus, a spatiotemporal analysis was performed to examine the genetic-spatiotemporal structure of NoV GII strains. Evidence of an association was detected between isolation time and genotype within GII sequences ($P < 0.0001$; Mantel test) and GII.4 sequences ($P < 0.0001$; Mantel test) (method described in section 2.4.6.2). Yet, there was no similar association between geographical distance and genetic distance ($P > 0.05$; Mantel test), or between isolation date and geographical distance ($P > 0.05$; Mantel test). These data indicate a lack of a local transmission signal of NoV in HCMC. Conversely, evidence from a SaTScan analysis (method described in section 2.4.6.3) supported our original hypothesis, detecting a cluster of six NoV GII.4-1010 (over other NoV GIIs (0.59 expected)) in a 3.8 km radius in northeastern of the City (relative risk = 12.65; $P = 0.0003$) (Figure 6-6), indicating that the initial dynamics of GII.4-2010 upon their introduction into HCMC were highly localised.

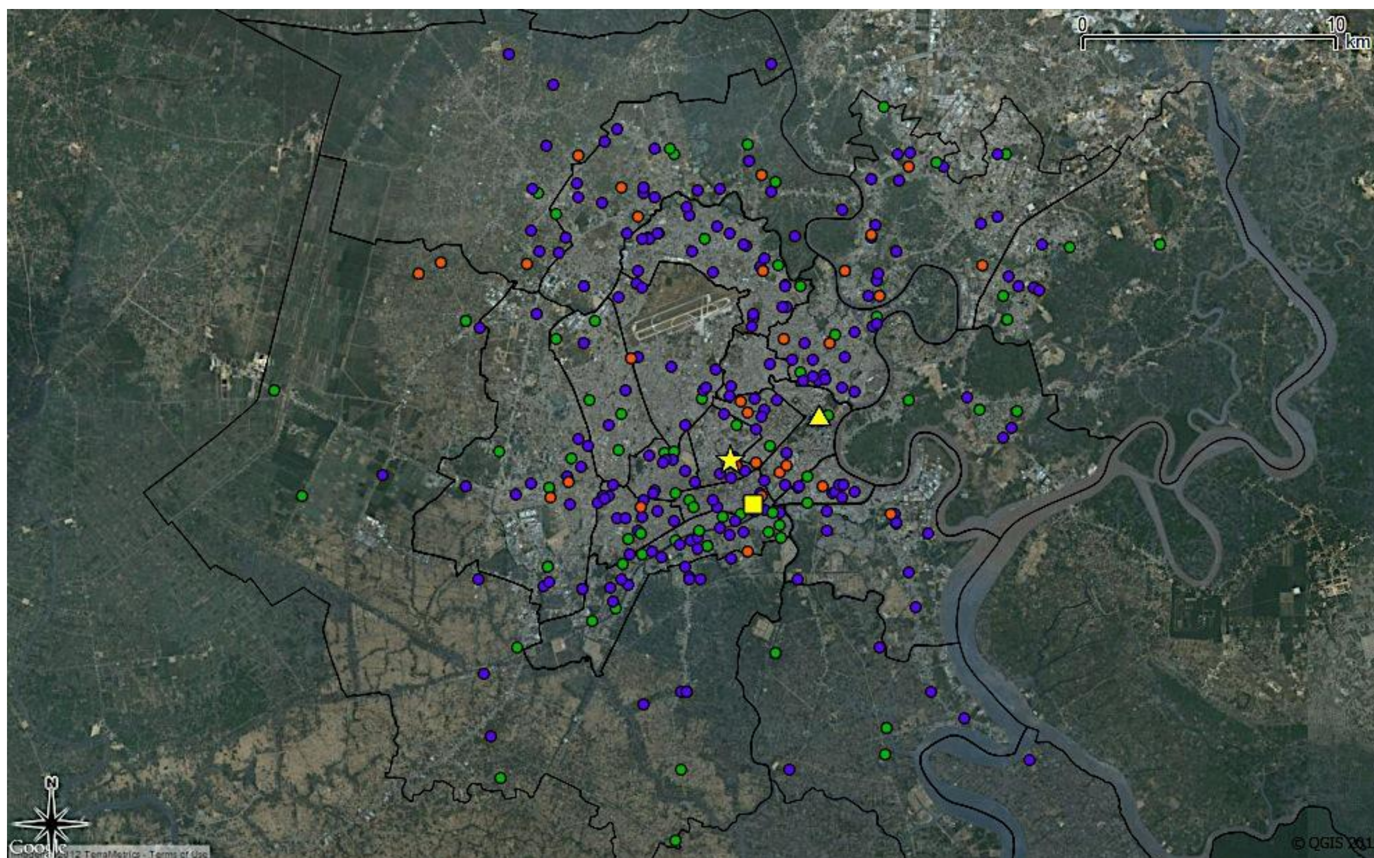


Figure 6-5. An illustration example of Google Earth map of participants.

A visualization of the residences of NoV-infected children in HCMC who were enrolled over the study period. Blue dots represent NoV GII.4-2006b; orange represents GII.4-2010 variant; green represents others NoV (non-GII.4-2006b and 2010 variants). The yellow square indicates the location of HTD, the star indicates CH1 and the triangle indicates CH2.

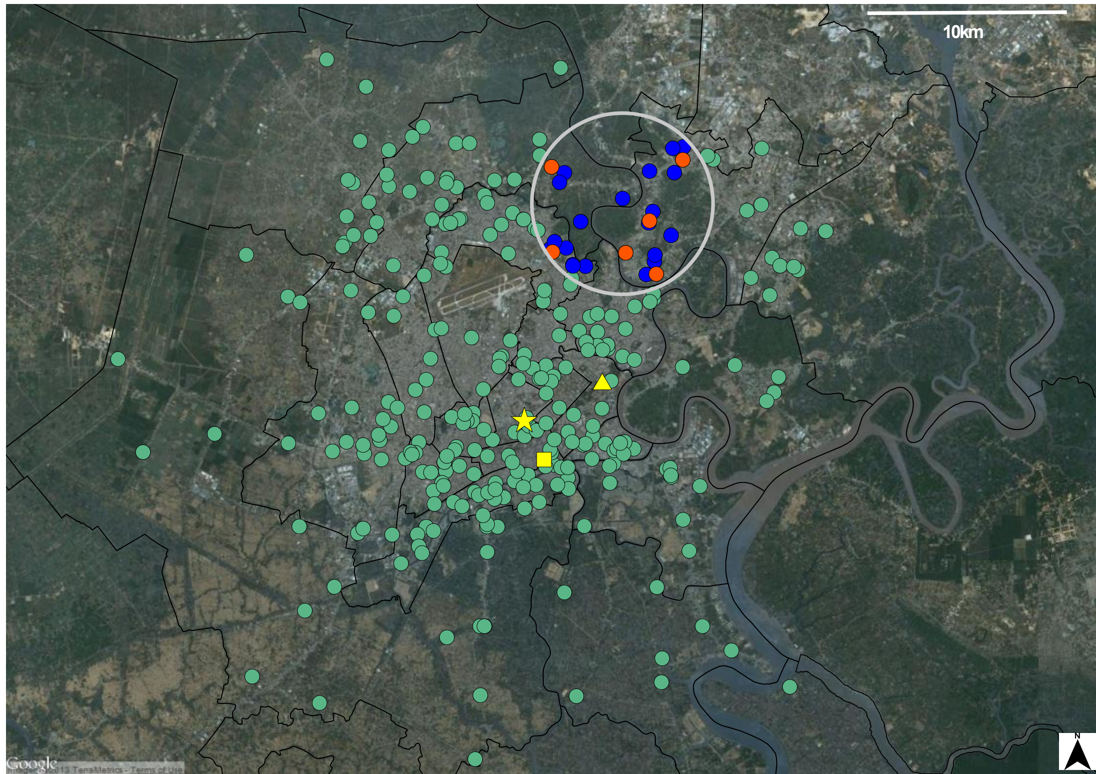


Figure 6-6. The spatiotemporal clustering of NoV GII.4-2010 strains.

When compared to all other GII strains, GII.4-2010 strains were found to cluster in northeastern of HCMC during March-April 2010. This significant cluster with radius 3.8 km, shown by the circle, contained 6 NoV GII.4-2010 strains. Orange dots represent NoV GII.4-2010 strains located within the cluster, blue dots represent the non-GII.4-2010 strains within the radius of the cluster, and the green dots represent all other NoV strains (GII.4-2010 and non-GII.4-2010) found over the study period that were not found to cluster. The yellow square indicates the location of HTD, the star indicates CH1 and the triangle indicates CH2. The thin black lines represent district boundaries within HCMC.

6.4. Discussion

Inadequate data on endemic NoV disease burden in developing countries, such as Vietnam, limit our knowledge of the viral distribution, transmission chains and local microevolution. Data on NoV genotype distribution across a range of geographical locations through time is essential for understanding the global NoV epidemiology. This is particularly important with respect to the on-going development and clinical trials of NoV vaccines [218, 264-267], which should be developed and assessed in consideration of global and regional circulating strain diversity and their ability to induce cross-protection. In this chapter, I aimed to assess the local molecular epidemiology of NoV by examining the genetic, spatial and temporal dynamics of NoV infections in young children in HCMC. The resulting data showed the co-circulation of a diverse array of NoV genotypes and reported a rapid strain replacement following the emergence of a novel variant.

It has been established that NoV GII viruses are responsible for the vast proportion of human NoV infections worldwide, and strains belonging to the GII.4 lineage play a particularly prominent role in paediatric NoV infections [173, 182, 399]. Data from the work presented herein demonstrated the presence and co-circulation of various NoV GII strains, with an overall predominance of GII.4 strains over the period of investigation. This observation concurs with previous findings from Vietnam [250-252], and other locations across Asia [259, 400-402]. The endemic circulation of GII.4-2006b detected in southern Vietnam (chapter 3 and this chapter) since its first detection in 2005 in HCMC corroborated the persistence of this particular genotype in the local population [250]. The reason for the dominance and persistence of GII.4-2006b is unknown but may be due to an, as yet, unmeasured

cumulative ecological fitness [184], permitting these GII.4 variants to persist in the community long-term [250], and displace other less-fit GII.4 genotypes.

The GII.4-2010 variant was detected here in December 2009. This variant was first identified in October 2009 in New Orleans (US) [396], which was also reported in Belgium [403] and then internationally [404-408]. NoV GII.4-2010 variant is suggestively the first highly diffusing variant to emerge after the pandemic variant GII.4-2006b (Minerva) [186, 396]. Results from phylogenetic analyses of GII.4 strains comparing with additionally representative global strains demonstrated that the GII.4-2010 strains from the US, Belgium and Vietnam were phylogenetically closely related, predicting the introduction of this strain into Vietnam in late 2009. Furthermore, the estimated evolutionary rate (8.072×10^{-3} substitutions per site per year) calculated in this study is higher than previously reported (between 3.9×10^{-3} and to 5.3×10^{-3} substitutions per site per year) [184, 191, 192]. Factors that may contribute to this discrepancy include differences in the region of sequence selected for analysis (partial 5' capsid herein versus complete capsid in previous studies), the method of evolutionary inference (linear regression, strict or relaxed clock, uncorrelated lognormal or exponential model), or the measured unit of time.

A spatiotemporal signal of GII.4-2010 was detected for several months after it was introduced into HCMC, but there was no similar spatiotemporal correlation between GII.4-2006b and non-GII.4 in HCMC. A potential explanation for the absence (GII.4-2006b and non-GII.4) and presence (GII.4-2010) of spatial signals in the NoV sequences and metadata, is that GII.4-2006b and non-GII.4 genotypes were in a state of equilibrium when GII.4-2010 was introduced, and that GII.4-2010 exhibited outbreak dynamics and exponential growth upon introduction. The replacement of GII.4-2006b viruses following the introduction of GII.4-2010 has

also been observed in Belgium [403], and other NoV strain replacement has been observed throughout various NoV pandemics between 1995 and 2006. These replacements include, GII.4-1996 (US 95/96 variant) in 1996 [248, 409, 410], GII.4-2002 (Farmington Hills) in 2002 [399, 411], GII.4-2004 (Hunter) in 2004 [412-414], and GII.4-2006 comprising of GII.4-2006a (Laurens) and GII.4-2006b (Minerva) in 2006 [415, 416]. Novel epidemic GII.4 variants emerge every 2 - 3 years and it appears that novel GII.4 strains are capable of replacing existing/ancestral GII.4 variants but not local circulating endemic strains (such as GII.3, GII.6, GII.2) [173, 182, 184, 188, 193, 237]. However, there is no current consensus on the underlying mechanism of strain replacement. Theoretically, strain replacement phenomenon in GII.4 lineage may be induced by antigenic drift and herd immunity escape [148, 182, 184-189]. This hypothesis has been strengthened by recent data on mapping blockade epitopes in GII.4 VLPs, which showed that variations in the major neutralising epitopes facilitate evasion of herd immunity against GII.4-2006b (Minerva), enabling the emergence of GII.4-2010 (New Orleans) from its potentially ancestral variant GII.4-2006b [186-188].

This study has some limitations that need to be considered; the source and/or route of transmission or examine the genotype distribution were not tracked after the period of investigation. Therefore, it cannot be determined if the shift in the distribution of the GII.4 variants over time is due to an emergent virus becoming fixed in the population, or a local outbreak in the northeastern part of the city. Short temporal investigation also limits the determination of the magnitude to which the local NoV dynamics observed in HCMC reflect or follow the global evolutionary trend, i.e. whether the GII.4-2010 variant described in this population was replaced following the emergence of the newest variant GII.4-2012 (Sydney) as observed internationally [417-419]. Moreover, the status of population-level immunity to NoV

in HCMC is unknown, so it is unclear if exposure to NoV GII.4-2006b provides protection against the GII.4-2010 variants. These finding highlight a broader scientific issue concerning outstanding questions on immune cross-protection in the space of NoV variants [187, 188]. Furthermore, the analysis was not performed on whole genome sequences and focused on a fragment of the genome, which may restrict the phylogenetic interpretation. Full genome sequences would greatly improve the utility of NoV epidemiological datasets, specifically to study the evolution of novel GII.4-2010 variants, aiding the detection of genomic sites that may induce potential antigenic variation. Finally, our hospital-based study design may be influenced by healthcare-seeking behaviour, and may not be necessarily representative of the NoV in the local community.

6.5. Conclusion

This chapter extends the knowledge of endemic NoV infections in developing countries, demonstrating the co-circulation of heterogeneous NoV strains in HCMC over the period of investigation. The identification of GII.4-2010 (New Orleans) described in this chapter is the first report of this variant detected in Asia. Additionally, the replacement of a GII.4-2006b variant by an emergent GII.4-2010 strain was proposed. In conclusion, NoV GII.4 infections in young children in HCMC demonstrate a spatiotemporal phylogenetic relationship, driven by the emergence of the novel GII.4-2010 (New Orleans) variant.

7. GENERAL DISCUSSION

Diarrhoeal diseases are a common cause of hospitalisation in young children in low and middle-income countries, and Vietnam is no exception. However, the disease has had inadequate attention from the country public health community, which in conjunction with an overall lack of routine diarrhoeal aetiological diagnosis, has led to an under-appreciation of this omnipresent disease and an under-investigation of the epidemiology of the various aetiological agents. The purpose of this project was to define some aspects of diarrhoeal disease aetiology and epidemiology in children in HCMC, Vietnam. The pilot study (Chapter 3) was performed as a baseline investigation to provide initial data for the focus of this project. This study provided a crucial comparison in the relative importance of four different enteric viruses causing acute childhood diarrhoea in two distinct locations and identified NoV as an emergent pathogen in Vietnam. The multi-centre study (Chapter 4) was then designed to investigate the aetiology and epidemiology of NoV in more detail and to provide longitudinal data defining the clinical and socio-economic characteristics of diarrhoeal disease in children younger than five years of age in this location. Analyses regarding social and spatiotemporal phylodynamics of NoV (Chapter 5 and 6) provided the first insight into the epidemiology, risk factors and spatiotemporal-phylogeny of NoV in the south of Vietnam.

In the light of rapid NoV evolution, the observation of the replacement of the variant GII.4-2006b (Minerva) following the emergence of descendent variant GII.4-2010 (New Orleans) provides very important information for the NoV epidemic mechanism, particularly in developing countries where the level of investigation on the disease does not correlate with the magnitude of disease burden, and the spatiotemporal phylodynamics of a majority of viral infections are poorly

characterised. Unfortunately, it is undetermined whether the NoV GII.4-2010 (New Orleans) variant that emerged in the end of 2009 and the beginning of 2010 in HCMC (Chapter 6) is in circulation within this setting after the study period, and whether the variant has the capacity to cause an outbreak in this location or whether this variant has diffused to other locations across the country.

The generalizability of results on NoV evolutionary analyses (Chapter 6) was, inevitably, hampered by the short partial coding sequences, such that limits the prediction for the next emergence of endemic or pandemic NoV variants. Application of next-generation sequencing (such as 454 or Illumina sequencing methods) to obtain full genome sequences in combination with mathematical modelling would help predict the epitope evolution in emergence of novel NoV strains and/or the next global outbreak of NoV-associated gastroenteritis. In particular, in collaboration with the Viral Genomics group at the Wellcome Trust Sanger Institute (Cambridge, UK), complete genome sequencing has been currently performed for all identified NoV from the hospital surveillance (Chapter 4), in addition to an ad-hoc collection of NoV from diarrhoeal patients. This project recruited paediatric patients, who were admitted to the hospital due to primarily respiratory illnesses (no diarrhoea presented), had diarrhoea after 48 hours of hospital admission; thus suggesting that NoV-induced illnesses in these individuals were nosocomial NoV infections or acute NoV-induced diarrhoea after a long incubation period. This project, when completed, will represent the largest dataset of complete NoV genomes in one location in three different sets of populations: acute diarrhoea NoV-positive patients, diarrhoea-free NoV-positive children, and potentially prolonged-incubated or hospital-acquired NoV infections.

A differential distribution of enteric viruses and NoV was observed between urban (HCMC) versus rural (DT) locations (Chapter 3), such that greater proportion of NoV was identified in HCMC than in DT, suggesting different epidemiological risk factors of NoV potentially related to urbanisation. Results from the work in Chapter 5 demonstrated a high prevalence of endemic NoV infections in hospitalised children in HCMC with major risk factors (young age, household crowding and contact to symptomatic individual) intrinsic to this 'young' and densely populated city. Given the highly infectious nature the virus and resistance to commonly used disinfectants, vaccination against NoV should have a considerable impact on the disease control and prevention. However, the vaccine development community encounters formidable obstacles, primarily due to a lack of efficient and productive tissue culture and animal model systems for human NoV. The NoV VLP, non-replicating virus unit expressed in VEEV (Venezuelan equine encephalitis virus) replicon or baculovirus systems [420, 421], has been suggested and currently used as a vaccine candidate [422]. Clinical trials of Norwalk virus (GI.1) VLPs delivered orally [267, 423] and intranasally [218, 266] demonstrated varied antibodies responses following challenge infections; alternatively, a parenterally administered VLP vaccine, which is thought to improve the frequencies and magnitude of seroresponses, is also undergoing clinical trial (ClinicalTrials.gov, NCT01168401) [422]. However, there are important uncertainties regarding (1) the capacity of vaccine to provide cross-reactive protection against heterologous strains (heterotypic immunity, especially to the most common genotype GII.4), which may be further aggravated by the great diversity of NoV genotypes circulating and the rapid genetic evolution; (2) the duration of vaccine-induced protection; (3) possible reduced vaccine efficacy when delivered to children in developing countries, compared to in

developed countries, as seen in the case of RoV vaccines; (4) the relative role and consequences of host-genetic factors (such as HBGAs) on the magnitude of immune responses following vaccination; and (5) the vaccine price when it's commercially available, whether it is affordable in poor and resource-limited areas where the need is greatest. Tackling these issues clearly requires intensive international collaborations (multi-national surveillance), extensive multi-centre (in both developing and developed areas) clinical trials, and massive financial investments, which practically would not happen in the next 3 – 5 years. For the time being, the control and prevention of NoV illnesses in Vietnam are suggested to rely heavily on public health interventions with particular attention paid to personal hygiene and increase in public awareness of the disease, primarily to prevent person-to-person transmission.

Despite the availability of two licensed vaccines against RoV (Rotarix™ and RotaTeq™), RoV vaccination has not been widely implemented in the national immunisation programs for children in many developing countries in Africa and Asia, where a brunt of the disease morbidity and mortality occurs [121]. The vaccination costs have in part limited the uptake of licensed vaccines in the resource-limited countries including Vietnam, although it has been shown that RoV vaccination is indeed cost effective in areas of high disease burden [29, 121, 127, 134-140]. The limited use of licensed vaccines in Vietnam may also be attributed to the reduced vaccines efficacy in developing countries compared to developed regions [124, 125, 128-131, 424]. Particularly, the vaccine efficacy against severe RoV gastroenteritis during the first year of life was 51 % (decreasing to 46 % in the second year of life) in Asia [129, 131], compared with an efficacy of 96 % observed in the US and Finland [124, 424]. Apart from the longitudinal sentinel surveillance of

RoV burden and genotype distribution in Vietnamese children from 1998 – 2003 [312], there is a paucity in national estimate of the RoV disease burden and circulating genotypes in young children. Reassessment of RoV morbidity and mortality in childhood gastroenteritis, strain circulation, and (homotypic and heterotypic) efficacy of potential RoV vaccines would undoubtedly benefit the health authorities to consider the inclusion and the type of RoV vaccine to be used in nation-wide, particularly when a promising Vietnamese RoV vaccine candidate (Rotavin-M1) is under advanced phase of clinical trial [316, 317].

The data presented in this thesis found that enteric viruses, particularly RoV and NoV, are highly dynamic in the southern Vietnam, and exhibit variations in geographical distribution and endemicity. Notably, multiple RoV and NoV genotypes were found to be co-circulating in different areas. Homologous recombination in NoV, interspecies transmission and reassortment in RoV from human and/or animal sources are widely acknowledged as the main source of genetic variations for these pathogens [55, 425, 426]. The wide diversity in enteric viral pathogens, the rate of mixed infections, asymptomatic infections, and the close proximity of humans to livestock and other animals in Vietnam suggest possible cross-species transmission and constant exposure to multiple viral pathogens within the local population. In other words, zoonotic infections in the study location may represent an epidemiologic niche, and their role in disease dynamics and viral evolution could have been overlooked.

This study did not identify NoV strains recombined from human and animal sources. However, it is possible that cross-species transmission of NoV does occur in nature, yet at very low frequencies, and may induce very mild symptoms, leaving such cases undocumented in surveillance of acute gastroenteritis [172, 425]. Viruses

within the genus *Norovirus* are capable of infecting a variety of non-human mammalian animals (see section 1.2.2.2). Three porcine NoV genotypes, GII.11, GII.18 and GII.19, have been described closely related to human GII strains of the GII lineage, the genogroup that is responsible for preponderance of human NoV infections [159, 178, 181]. Most recently, viral shedding and replication were observed in four experimental gnotobiotic pigs following oral inoculation with human NoV GII.g/GII.12 (St George) virus, isolated from human cases in a gastroenteritis outbreak in Ohio (US) in January 2010 [427]. This evidence, in accordance with prior data on zoonotic risks as reviewed by Scipioni *et al.* [425], strengthens the hypothesis of possible interspecies transmission and emergence of recombinant animal/human viruses among genetically compatible NoV [425].

Data on zoonotic aspects of RoV, on the other hands, have been increasingly and globally reported, as summarised in Cook *et al.* and Martella *et al.* [55, 426]. In Vietnam, although there have been a limited number of studies on RoV diseases, the existence of animal-related RoV strains causing acute diarrhoea in children has been reported, including porcine-related strains (G5P[6], G4P[6], G9P[6] and G9P[19]), bovine-related virus (G10P[8]) [61, 350, 428, 429], and a G12 genotype (Chapter 3) which was suggested to have a porcine origin [430, 431]. These cumulative evidence, thus, add granularity to the importance of potential animal reservoirs for human RoV, the prevalence of zoonotic reassortment RoV strains and their impacts on genetic diversity and evolution, and as a consequence, vaccine efficacy should be re-evaluated.

Preliminary genotyping data demonstrated the circulation of multiple RoV genotypes in diarrhoeal cases, while viruses detected from diarrhoea-free controls were exclusive to RoV P[8] with a G1 or an untyped G. Among all typeable strains,

RoV G1P[8] was the predominant genotype followed by G3P[8]. Interestingly, no G12 genotype was detected in this yearlong hospital surveillance, questioning the relative epidemiologic fitness of this particular reassortant in the local population. Notably, animal-related and novel/rare RoV strains were identified, including G9P[19], G26P[19] and G3P[19]. These preliminary findings provide essential data for the consideration of RoV vaccine introduction, and warrant further investigations into RoV dynamics.

The determination of the novel genotype RoV G26P[19] is probably the most interesting finding presented. At the moment, RoV G26 genotype has never been reported in human RoV infections; however, there is one report on a RoV G26 isolated from a piglet in 2010 in Japan [432], suggesting a porcine origin of this G26 virus. The detection of RoV G26P[19] in 4 acute gastroenteritis patients from the hospital surveillance (Chapter 4) represents the first human RoV G26 infections in the literature, further reinforcing the zoonotic potential of RoV and pigs as an animal reservoir for human RoV. Given a cross-sectional design of the hospital surveillance, the detection of G26 in 4 patients at different time points and at distinct locations in HCMC suggests that there would be more G26 strains circulating in the community, and that these G26 cases probably represented sporadic infections rather than a local outbreak.

To characterise the origin of G26 strains, the whole genome sequencing of one representative G26 strain was performed, in collaboration with the Enteric viruses lab (Murdoch Children Research Institute, Melbourne, Australia). The genome constellation of the selected strain was as G26-P[19]-I5-R1-C1-M1-A8-N1-T1-E1-H1, showing both human-derived segments (VP1, VP2, VP3, NSP2, NSP3, NSP4 and NSP5), and porcine-related segments (VP7, VP4, VP6, and NSP1).

Therefore, I suggest that this G26P[19] strain is derived from porcine and human reassortment event, probably following a direct interspecies transmission in a human or pig host, or in an intermediate unknown host. Overall, the identification of this porcine-origin G26P[19] further aggravates the concern of zoonotic capacity of RoV, with constant unpredictable and uncontrollable emergence of novel genotypes from (human/animal or human/human) reassortment and cross-species transmission. Consequently, the degree of vaccine-induced protection against such emergent is surmised to be low, thus pressurising continuous efforts in strain monitoring, and vaccine research and development. Sequencing complete genomes of the other 3 RoV G26 strains (and potentially other novel RoV genotypes), in addition to determination of local versus global RoV evolution through phylogenetic analyses of strains identified from Chapter 3 and 4 and global strains, will be my next study project to contribute to the understanding of zoonotic RoV infections in Vietnam.

Due to time and cost limitations, the prevalence of pathogenic *E.coli* in the diarrhoeal patients and diarrhoea-free children in the hospital surveillance (Chapter 4) was not determined, leaving avenues for future research. As mentioned in Chapter 1 of this thesis (section 1.1.2), diarrhoeagenic *E.coli* are among the common bacterial pathogens causing diarrhoea in young children, in addition to the study targeted bacteria (*Shigella*, non-typhoidal *Salmonella* and *Campylobacter* species). However, diarrhoeagenic *E.coli* cannot be distinguished from commensal *E.coli* by classical microbiology culture and identification method used in this thesis. Indeed, the detection of this group of *E.coli* pathogens, including enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent [433], often requires specialised and intensive diagnostic methods that are largely unavailable in resource-limited settings, leading to its under-recognition in most

studies in developing countries [434, 435]. Development and validation of detection method to screen and confirm five major pathogenic *E.coli* in stool samples will be a next phase of this research.

Finally, data from the work presented in this thesis demonstrated that viral diarrhoeal infections, RoV and NoV infections, in young children in HCMC are endemic and highly dynamic. Given the high burden of acute viral gastroenteritis, it is suggested that vaccination against RoV and NoV would be a cost-effective intervention in this population and, therefore, should be considered to be included in the childhood immunisation schedule. However, provided the presence of novel strains of potentially zoonotic origin (RoV) and rapid emergence of new variants (NoV), vaccine efficacy must be carefully evaluated.

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9. APPENDICES

9.1. Patient Information Form (PIS)

9.1.1. For patient enrolment

Site number [__]__|__] Study ID Number: [__|__|__|__|__]

INFORMATION SHEET

OxTREC No: 0109
Protocol No: CTU09AVDEC08. PIS V4.0, 06Mar09

A Descriptive and Molecular Epidemiological Study of Children with Diarrhoeal Disease in three Hospitals in Ho Chi Minh City, Viet Nam.

Your child is being invited to take part in a research study of diarrhoeal disease in children. We want you to know that:

- Taking part in research is entirely voluntary
- You may choose not to take part or your child may withdraw from the study at any time. In either case, your child will not lose any benefits to which he/she is otherwise entitled.
- Some people have personal, religious or ethical beliefs that may limit the kinds of medical or research treatments they would want to receive. If you have such beliefs, please discuss them with your doctors or research team before you agree to the study.

Please read this information sheet carefully or have someone read it for you. You will be given a copy of this form to keep.

Before you decide for your child to take part, please take as much time as you need to ask any questions and discuss this study with anyone at this hospital, or with family, friends or your personal physician or other health professional. If you have any questions about the study please ask the doctor or nurse on the ward for more details.

Introduction to the study

Diarrhea is a common disease among children under 5 years old. The aim of this study is to study the prevalence of diarrheal disease and its causes in Ho Chi Minh City. The information from this study will help us to better understand the distribution of the disease and to identify the most prominent pathogens causing diarrheal disease within the City.

What will the Study Involve?

A questionnaire asking you some simple questions, which are related to your child's illness.

We will take your address to map the location of your residence, this information will not be passed on to another party and will not be linked to your name or other details (see confidentiality below).
The Doctor will make some clinical observations and record them.

What will we do with the stool specimen?
The stool specimens will be used for identification of pathogenic causes of diarrhoea in your child as a normal care procedure. Samples will be stored and will be used only for future research relating to this study and will not be used for studies regarding human genetics.

Confidentiality
We will keep the information obtained from you and/or your child as private. Your child's name will not be on his/her test results. We will use a coded study identification number.

Risks
There is no risk involved in being enrolled in this study.

Cost
There will be no cost to you.

How will this study benefit your child?
It will aid other research in the future as we will know which are the most problematic causes of diarrheal disease in the city. Additionally, possible intervention would be recommended to prevent the disease occurrence in highlighted areas of the city.

Refusal to participate
You may refuse to participate in the study. If you do not wish to be in the study, the decision will not interfere in any way with your child receiving proper medical care and attention.
Any questions please direct to
Dr Stephen Baker (OUCRU) (or Ms. Phan Vu Tra My)
Email: sbaker@oucru.org
Phone: +84 8 39239210

9.1.2. For control enrolment

Site number [__]__|__]   Control ID Number: [_**C** _|__|__|__|__]

INFORMATION SHEET FOR CONTROL GROUP

OxTREC No: 0109
Protocol No: CTU09AVDEC08, PIS Control Group, V2.0, 12Jan10

A Study of Children with Diarrhoeal Disease in Ho Chi Minh City, Viet Nam.

Your child is being invited to take part in a research study of diarrhoeal disease in children. There will be 300 children invited to participate in this part of the study. Your child's participation in this study is completely your decision. Please read this information sheet carefully or have someone read it for you before you decide if you want your child to be in the study. You will be given a copy of this form to keep.

Introduction to the study

Diarrhea is a common disease among children under 5 years old. This study will look at what happens to children who have diarrhoeal disease and what type of problems they have. We are inviting your child to be in the study to help compare children who are healthy but may have some of the bacteria which causes diarrhoeal disease to the children who do get sick.

What will the Study Involve?

If you agree for your child to be in the study, our study staff will ask you a list of questions about your child's daily habits and your household. We will ask for your address and contact details. We will also collect a sample of your child's stool the next time they go to the bathroom. This sample will be tested for the germs which cause diarrhoeal disease. If you agree we will store this sample to test it in the future if new tests become available that can tell us more about the disease. The tests will be done at the Oxford University Clinical Research Unit in Ho Chi Minh City.

What are the risks of being in the study and who will know that my child is in the study?

We do not expect that your child will have any problems if they participate in the study. All of the information collected will be kept secret and will only be seen by the study staff. Any information about your child will be marked with a number and will not include your child's name.

Can I choose if my child is in the study?

Being in this study is completely your decision. Your child will receive the same care from the hospital staff whether or not you decide to participate. If you do decide for your child to

be in the study you can change your mind at any time and stop the study. The research team or the Hospital Research Ethics Board can also decide to stop the study.

Is there any cost if my child is in the study?

There is no extra cost if your child is in the study. The study tests will be paid by the research staff. You will pay for your child's regular care as usual.

Who can I talk to about this study?

If you have any questions about the study you may contact Dr. Stephen Baker or Phan Vu Tra My at 08 39239210. If you have any questions about being in a research study and your rights you may contact the Clinical Research Office at the Hospital for Tropical Diseases at 08 3924 1983.

9.2. Informed Consent Form (ICF) to participation

9.2.1. For patient

Site number [__]__|__]   Study ID Number: [__|__|__|__|__]

CONSENT FORM

OxTREC No: 0109
Protocol No: CTU09AVDEC08. Consent Form V4.0, 06Mar09

A Descriptive and Molecular Epidemiological Study of Children with Diarrhoeal Disease in three Hospitals in Ho Chi Minh City, Viet Nam.

I have read the information sheet and been fully informed to the purpose of the study and I agree that my child can participate. I additionally agree to complete the questionnaire and understand that my address and details will be recorded in a confidential database and will not be used for any other purposes.

Consent from parent or guardian:

Patient's name: ______________________

Name of relative: _____________________

Relationship with patient: _____________________

Signature: _________________________

Date: _________________________

Name of Physician: ___________________

Signature: _________________________

Date: _________________________

9.2.2. For control

Site number [__]__|__]   Control ID Number: [_**C** _|__|__|__|__]

CONSENT FORM FOR THE CONTROL GROUP

OxTREC No: 0109
Protocol No: CTU09AVDEC08, ICF Control Group, V2.0, 12Jan10

A Study of Children with Diarrhoeal Disease in Ho Chi Minh City, Viet Nam.

Consent to fill in the questionnaire in the study from the parent or guardian

I have read the information sheet and been fully informed to the purpose of the study. I have had the opportunity to ask questions about being in the study and they have been answered to my satisfaction. I agree to complete the questionnaire and understand that my address and details will be recorded in a confidential database and will not be used for any other purposes.

Patient's name: ________________________

Name of person giving consent: _______________________

Relationship with patient: _______________________

Signature: ___________________________ Date: ___________________________

Consent to sample storing from the parent or guardian

I agree that the stool sample may be taken and stored for future testing to study diarrhoeal disease.

Patient's name: ________________________

Name of person giving consent: _______________________

Relationship with patient: _______________________

Signature: ___________________________ Date: ___________________________

Name of person obtaining consent: ___________________________

Signature: ___________________________ Date: ___________________________

9.3. Questionnaire

9.3.1. For patient

Site number [__|__|__] Study ID Number: [__|__|__|__|__|__]

CONFIDENTIAL QUESTIONNAIRE FOR PARENT OR GUARDIAN

OxTREC number: 0109
Protocol number: CTU09AVDEC08. Questionnaire V4.0, 06Mar09

A Descriptive and Molecular Epidemiological Study of Children with Diarrhoeal Disease in three Hospitals in Ho Chi Minh City, Viet Nam.

INCLUSION CRITERIA

Child is under 5 years of age [__]
Has been admitted with diarrhoeal disease [__]
Permanently lives in Ho Chi Minh City [__]
Informed consent is given [__]
Child has had no pre-treatment with antimicrobials [__]

QUESTIONS REGARDING YOUR CHILD

1. What is your child's name?

[____]

2. How old is your child?

[__|__] . [__] Months

3. Which one of these best describes how your child is / was fed as a baby during the first year of life?

☐ Breast Milk only
☐ Milk Formula only
☐ Milk Formula with Probiotic supplement only
☐ A combination of Breast Milk and Milk Formula
☐ A combination of Breast Milk and Milk Formula with Probiotic supplement
☐ Other (Specify):_______________________

4. Does the child have Probiotic regularly?

☐ Yes, the child has Probiotic regularly. Specify the Probiotic:_________________
☐ Yes, the child did have but no longer have it. Specify the Probiotic:____________
☐ Use Probiotic as a treatment for the current diarrhoea
☐ No, the child does not have any Probiotic
☐ Not sure

5. What sex is your child?

☐ Male
☐ Female

6. How long has your child been ill?
[___] Days

7. How frequent is the diarrhoea?

[___] Episodes per day

8. Has your child had a similar illness with in the last year?

☐ Yes
☐ No

9. Is there any person that the child comes in contact with at home or school with similar symptoms?

☐ Yes
☐ No

10. Has your child had any treatment with antibiotics within the last four weeks before this episode of illness?

☐ Yes (go to Question 11)
☐ No (go to Question 12)

11. If Yes, which antibiotic and for how many days?

[________________________________] Antibiotic
[___] Days

12. Which one of these things best describes where your child normally stays during the day?

☐ Daycare
☐ Nursery school
☐ Stay at home
☐ Stays at the house of friend or relative
☐ Other (Specify):________________________

QUESTIONS REGARDING THE HOUSEHOLD OF THE CHILD

13. Do you live with the child?

☐ Yes
☐ No

14. Where does the child live?

[____] House number
[____] Street
[____] Ward / Hamlet
[____] Commune / District

15. How many rooms does the house that the child lives in have? (including bedrooms and sitting room, excluding bathroom and kitchen)

[___] Rooms

16. Which one of these kinds of toilets best describes the type of toilet in the house where the child lives?

- [ ] Water Closet (Inside)
- [ ] Water Closet (Outside)
- [ ] Standing toilet without flush
- [ ] Hole in the ground
- [ ] Bucket
- [ ] Other (Specify):________________________

17. How many toilets are in the house where the child lives?

[___] Toilets

18. How many motorbikes belong to people living in the same house where the child lives?

[___] Motorbikes

19. Do you have a mobile phone?

- [ ] Yes
- [ ] No

20. Does the house where the child lives have a fridge?

- [ ] Yes
- [ ] No

21. Where are the majority of meats and vegetables that the child eats bought? Please check <u>one</u>.

- [ ] Market
- [ ] Supermarket
- [ ] Both market and supermarket
- [ ] Other (Specify):________________________

22. How many adults normally live in the same household as the child, i.e. spend most nights sleeping in the same house as the child?

[____] Adults

23. How many children under the age of 15, including other people's children, currently live in the same household as the child, i.e. spend most nights sleeping in the same house as the child?

[___] Children

24. Which <u>one</u> of these best describes the house that the child lives in?

- [ ] Detached, brick
- [ ] Attached, brick
- [ ] Apartment
- [ ] Metal sheets
- [ ] Boat
- [ ] Other (Specify):________________________

25. How many levels does the house that the child lives in have? (ground floor counted as 0)

[____] Levels

26. Where does the water for hand-washing, cooking and other daily activities come from in the house where the child lives?

☐ From government pipeline-water supply
☐ From a well
☐ Stored rain water
☐ Bought from the governmental water-truck dispenser
☐ Other (Specify): ______________________

27. Where does the drinking water come from in the house where the child lives?

☐ From government pipeline-water supply
☐ From a well
☐ Buy bottled water from a shop
☐ Stored rain water
☐ Bought from the governmental water-truck dispenser
☐ Other (Specify): ______________________

28. Is the drinking water normally boiled in the house where the child lives?

☐ Yes
☐ No

29. Does the child wash their hands or have help washing their hands after they have been to the toilet?
☐ Yes
☐ No
☐ Sometimes
☐ Don`t know
☐ Not applicable because the child is too small

30. Are there any animals in the house where the child lives?

☐ No
☐ Yes, dog
☐ Yes, cat
☐ Yes, other (Specify)_________________

31. What is the approximate monthly income of the child's parents?

☐ Less than 3,000,000 VND
☐ Between 3,000,000 and 5,000,000 VND
☐ Between 5,000,000 and 10,000,000 VND
☐ Between 10,000,000 and 15,000,000 VND
☐ More than 15,000,000 VND

32. What is your normal occupation?

☐ Stay at home/homemaker
☐ Government/state employee

- [ ] Private employee (describe)_____________________________
- [ ] Self-employed (describe)_____________________________
- [ ] Unskilled labor
- [ ] Student
- [ ] Unemployed
- [ ] Other (Specify)_________________________________

9.3.2. For control

Site number [__]__|__]   Control ID Number:[_**C**_|__|__|__|__]

CONFIDENTIAL QUESTIONNAIRE FOR PARENT OR GUARDIAN

OxTREC No: 0109
Protocol No: CTU09AVDEC08, Questionnaire Control Group, V2.0, 12Jan10

A Study of Children with Diarrhoeal Disease in Ho Chi Minh City, Viet Nam.

INCLUSION CRITERIA FOR CONTROL GROUP

Child is under 5 years of age	[__]
Permanently lives in Ho Chi Minh City	[__]
No diarrhoea or respiratory illnesses	[__]
Not currently undertaking any antimicrobials	[__]
Informed consent is signed by parent/guardian	[__]

QUESTIONS REGARDING YOUR CHILD

1. How old is your child?

[__|__] . [__] Months

2. Which one of these best describes how your child is / was fed as a baby during the first year of life?

☐ Breast Milk only
☐ Milk Formula only
☐ Milk Formula with Probiotic supplement only
☐ A combination of Breast Milk and Milk Formula
☐ A combination of Breast Milk and Milk Formula with Probiotic supplement
☐ Other (Specify):________________________

3. Does the child have Probiotic regularly?

☐ Yes, the child has Probiotic regularly. Specify the Probiotic:__________________
☐ Yes, the child did have but no longer have it. Specify the Probiotic:_____________
☐ No, the child does not have any Probiotic
☐ Not sure

4. What sex is your child?

☐ Male
☐ Female

5. Which one of these things best describes where your child normally stays during the day?

☐ Daycare
☐ Nursery school
☐ Stay at home
☐ Stays at the house of friend or relative
☐ Other (Specify):________________________

6. Did the child have any diarrhoeal episode during the last 4 weeks?

☐ Yes
☐ No
☐ Don't remember

7. Is there any person that the child has regular contact with at home or school that has diarrhoea?

☐ Yes, person at home. Specify the relation:________________________________
☐ Yes, person at school. Specify the relation:_______________________________
☐ No
☐ Don't know

8. Who spend most of the time taking care of the child?

☐ Mother
☐ Father
☐ Grandparent
☐ Relative
☐ Baby-sitter
☐ Other (Specify):_______________________

QUESTIONS REGARDING THE HOUSEHOLD OF THE CHILD

9. Do you live with the child?

☐ Yes
☐ No

10. Where does the child live?

[____] House number
[____] Street
[____] Ward / Hamlet
[____] Commune / District

11. How many rooms does the house that the child lives in have? (including bedrooms and sitting room, excluding bathroom and kitchen)

[___] Rooms

12. Which one of these kinds of toilets best describes the type of toilet in the house where the child lives?

☐ Water Closet (Inside)
☐ Water Closet (Outside)
☐ Standing toilet without flush
☐ Hole in the ground
☐ Bucket
☐ Other (Specify):_______________________

13. How many toilets are in the house where the child lives?

[___] Toilets

14. How many motorbikes belong to people living in the same house where the child lives?

[___] Motorbikes

15. Do you have a mobile phone?

☐ Yes
☐ No

16. Does the house where the child lives have a fridge?

☐ Yes
☐ No

17. Where are the majority of meats and vegetables that the child eats bought? Please check <u>one</u>.

☐ Market
☐ Supermarket
☐ Both market and supermarket
☐ Not applicable as the child's current diet is milk
☐ Other (Specify):________________________

18. How many adults normally live in the same household as the child, i.e. spend most nights sleeping in the same house as the child?

[____] Adults

19. How many children under the age of 15, including other people's children, currently live in the same household as the child, i.e. spend most nights sleeping in the same house as the child?

[___] Children

20. Which <u>one</u> of these best describes the house that the child lives in?

☐ Detached, brick
☐ Attached, brick
☐ Apartment
☐ Metal sheets
☐ Boat
☐ Other (Specify):________________________

21. How many levels does the house that the child lives in have? (ground floor counted as 0)

[____] Levels

22. Where does the water for hand-washing, cooking and other daily activities come from in the house where the child lives?

☐ From government pipeline-water supply
☐ From a well
☐ Stored rain water

☐ Bought from the governmental water-truck dispenser
☐ Other (Specify): ______________________

23. Where does the drinking water come from in the house where the child lives?

☐ From government pipeline-water supply
☐ From a well
☐ Buy bottled water from a shop
☐ Stored rain water
☐ Bought from the governmental water-truck dispenser
☐ Other (Specify): ______________________

24. Is the drinking water normally boiled in the house where the child lives?

☐ Yes
☐ No

25. Does the child wash their hands or have help washing their hands after they have been to the toilet?

☐ Yes
☐ No
☐ Sometimes
☐ Don`t know
☐ Not applicable because the child is too small

26. Are there any animals in the house where the child lives?

☐ No
☐ Yes, dog
☐ Yes, cat
☐ Yes, other (Specify)_________________

27. What is the approximate monthly income of the child's parents?

☐ Less than 3,000,000 VND
☐ Between 3,000,000 and 5,000,000 VND
☐ Between 5,000,000 and 10,000,000 VND
☐ Between 10,000,000 and 15,000,000 VND
☐ More than 15,000,000 VND

28. What is the child's mother's occupation?

☐ Stay at home/homemaker
☐ Government/state employee
☐ Private employee (describe)___________________________
☐ Self-employed (describe)___________________________
☐ Unskilled labor
☐ Student
☐ Unemployed
☐ Other (Specify)______________________________

29. What is the child's father's occupation?

☐ Stay at home/homemaker
☐ Government/state employee

- ☐ Private employee (describe)____________________________
- ☐ Self-employed (describe)____________________________
- ☐ Unskilled labor
- ☐ Student
- ☐ Unemployed
- ☐ Other (Specify)_______________________________

9.4. Clinical Report Form (CRF) for diarrhoeal patients

Site number [__]__|__]   Study ID Number: [__|__|__|__|__]

CASE REPORT FORM

OxTREC number: 0109
Protocol number: CTU09AVDEC08. CRF V4.0, 06Mar09

A Descriptive and Molecular Epidemiological Study of Children with Diarrhoeal Disease in three Hospitals in Ho Chi Minh City, Viet Nam.

INCLUSION CRITERIA

Child is under 5 years of age [__]
Has been admitted with diarrhoeal disease [__]
Permanently lives in Ho Chi Minh City [__]
Informed consent is given [__]
Child has had no pre-treatment with antimicrobials [__]

Name [____]

Diagnosis at admission [__________________________________]

Admission Date (ddmmyy) [__|__|__|__|__|__]   **Weight** [__|__] Kg

Sex Male [__] Female [__]

Fever Yes [__] No [__] Temperature [__|__]. [__] ^{o}C

Vomiting Yes [__] No [__]

Headache Yes [__] No [__] Not known [__]

Abdominal Pain Yes [__] No [__] Not known [__]

Myalgia Yes [__] No [__] Not known [__]

Diarrhoea Watery [__] Mucoid [__] Bloody [__] Watery with solids [__]

Highest number of diarrhoeal episodes per day [__] episodes on day [__]

Average number of diarrhoeal episodes per day [__]

Dehydration Severe [__] Moderate [__] No [__]

Anorexia Yes [__] No [__]

Lethargic Yes [__] No [__]

Blood Pressure[__|__|__] / [__|__|__] mmHg

Pulse [__|__|__] bpm

WBC [__|__]. [__] cells/l   **HCT** [__|__] %

Neut [__|__] % **Lympho** [__|__] % **Mono** [__|__] % **Eosin** [__|__] %

Sodium [__|__|__] mmol/l **Potassium** [__] . [__] mmol/l

Treatment (drug) for diarrhoea[____]

Dose [____]

Treatment (drug) for diarrhoea[____]

Dose [____]

Treatment (drug) for diarrhoea[____]

Dose [____]

Treatment (drug) for diarrhoea[____]

Dose [____]

Treatment (drug) for other symptom: [____]

Dose [____]

Treatment (drug) for other symptom: [____]

Dose [____]

Duration of Treatment [__|__] days **Discharge Date** (ddmmyy) [__|__|__|__|__|__]

Patient's condition when discharged [____]

Study doctor (name, date and signature): ____

9.5. Laboratory Report Form

DIARRHOEAL DISEASE

AT ADMISSION

Date analysed: ……/……../………...

Label sample Here

For lab use only: **MICROBIOLOGY LAB**

Stool specimen →
- ❒ **Smear**
- ❒ **Culturing**
- ❒ **Store in liquid nitrogen at -80^{0}C**

1. Stool smear:

Wet preparation

WBC/hpf:

RBC/hpf:

Parasites:

Other smear:

2. Media cultures:

Blood agar (BA):

MacConkey agar (MC):

XLD agar:

Campylobacter culture microaerophilic at 43^{0}C

Enrichment culture: Selenite broth →
- **XLD:**
- **MC:**

3. Short-sets:

KIA	Gas	H_2S	MOT	IND	CIT	URE	MR	LYS	ORN

ESBL testing:

CAZ		CTX	
CAZ-CLA		CTX-CLA	

4. API-20E:

Label sample Here

5. Serological test:

<u>Identification:</u>

6. Antibiotic sensitivity tests:

Ab	Amp	Aug	Azi	Ceftri	Cefta	Chloram	Cipro	Genta	Gatiflo	Nalid	Oflox	Bactrim
1												
2												
3												
MIC 1												
MIC 2												
MIC 3												

Freeze-dry position:

Date:……./….…./…………….

Authorized by (name and signature):

9.6. Publications as first author during DPhil candidature

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The Emergence of Rotavirus G12 and the Prevalence of Enteric Viruses in Hospitalized Pediatric Diarrheal Patients in Southern Vietnam

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Abstract. Diarrhea is a major cause of childhood morbidity and mortality in developing countries, and the majority of infections are of viral etiology. We aimed to compare the etiological prevalence of the major enteric viruses in an urban and a rural setting in southern Vietnam. We simultaneously screened fecal specimens from 362 children in Ho Chi Minh City and Dong Thap province that were hospitalized with acute diarrhea over a 1-month-long period for four viral gastrointestinal pathogens. Rotavirus was the most common pathogen identified, but there was a differential prevalence of rotavirus and norovirus between the urban and rural locations. Furthermore, rotavirus genotyping and phylogenetic analysis again differentiated the genotypes by the sampling location. Our data show a disproportional distribution of enteric viral pathogens in urban and rural locations, and we provide evidence of continual importation of new rotavirus strains into southern Vietnam and report the emergence of rotavirus genotype G12.

INTRODUCTION

Diarrhea is the second most common cause of childhood mortality worldwide, estimated to be responsible for 1.76 million deaths annually between 2000 and 2003 and 1.87 million deaths in children under the age of 5 years in 2004.[1–3] It is young children and infants in unindustrialized and industrializing countries that are disproportionately affected, and in such locations, diarrheal infections are often severe, frequently requiring hospitalization.[4] The etiological agents of diarrhea are numerous, including multiple viral, bacterial, and parasitic pathogens. However, it is viruses that are responsible for the vast burden of morbidity and mortality, causing up to 40% of all severe diarrhea cases in developing countries.[5] The most prevalent viruses causing endemic childhood gastroenteritis are rotavirus, norovirus, enteric adenovirus, and astrovirus.[6] Rotavirus is the dominant agent of viral diarrhea and is the suspected etiological agent in 39% and 45% of all hospital admissions related to diarrhea globally and in Asia, respectively.[7,8] In Vietnam, rotavirus is estimated to be responsible for between 44% and 67.4% of all childhood diarrheal infections requiring hospitalization.[9–11]

Rotavirus has a genome comprised of 11 segments of double-stranded RNA (dsRNA) encoding six structural proteins and six non-structural proteins.[12] It is genetic heterogeneity in two of the structural regions that encode the viral capsid proteins, VP7 and VP4, that permit the differentiation of individual rotavirus strains.[12] Sequencing of the VP7 region (glycoprotein) defines the G type, and VP4 region sequencing (protease-sensitive protein) determines the P type. To date, 19 G and 28 P genotypes have been described, 10 and 11 of which, respectively, have been isolated from humans.[12,13] Strain G1P[8] is the most frequent rotavirus A GP combination isolated from symptomatic humans worldwide.[14]

Transmission and distribution of rotavirus A is complex and influenced by multiple social, demographic, and environmental factors.[15,16] Vietnam is a typical industrializing country where the agents of infectious diseases are changing rapidly.[17] Existing data on viral gastrointestinal pathogens are available from sentinel surveillance in Ho Chi Minh City (HCMC) between 1998 and 2007.[18–24] However, because detection and identification of enteric pathogens are not performed routinely in hospitals, little is known about the prevalence of viral diarrheal pathogens and the strains that circulate in different geographic and demographic locations in Vietnam. We aimed to investigate the distribution of norovirus, enteric adenovirus, astrovirus, and rotavirus genotypes causing diarrhea in hospitalized children in distinct urban and rural locations in southern Vietnam.

MATERIALS AND METHODS

Study sites and population. Verbal informed consent was obtained from the parents or legal guardians of minors enrolled in this study. This work was approved by the institutional ethical review boards of the Hospital for Tropical Diseases, HCMC and Dong Thap Provincial Hospital, Dong Thap. Patient recruitment was performed over one calendar month from November 1, 2008 to November 30, 2008 at two hospitals, pediatric ward B at the Hospital for Tropical Diseases (HTD) in HCMC and pediatric infections ward at Dong Thap Provincial Hospital (DTPH) in Dong Thap (DT) province. DTPH is 154 km away from HCMC and located within the Mekong Delta region of southern Vietnam; it is a rural location, with a lower population density than HCMC. We enrolled all pediatric (under the age of 15 years) patients who had been hospitalized at HTD or DTPH because of acute watery diarrhea (defined as three or more loose stools within a 24-hour period) without any additional underlying complications, such as febrile convulsions, extensive dehydration, or stool-containing blood or mucus. The age of each patient was recorded, and a stool specimen from each patient was collected in a sterile container on the day of admission and was stored at −20°C.

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RNA extraction and virus detection. Total viral RNA was extracted from 10% (in phosphate-buffered saline [PBS]) fecal specimens using the QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. RNA preparations were converted to complementary DNA (cDNA) by reverse transcription (RT), and an aliquot of RNA of each sample was stored at −80°C until required. For RT, extracted RNA was reverse-transcribed by SuperScript Reverse Transcriptase III and RNase Inhibitor (Invitrogen) combined with a random hexamer (Roche Diagnostics, United Kingdom) according to the manufacturer's instructions. The resulting cDNA were stored at −80°C.

All stool samples were screened for rotavirus A, norovirus (genogroups I and II), enteric adenovirus, and astrovirus using IDEIA™ direct antigen detection kits according to the manufacturer's instructions (Oxoid; Thermo Fisher Scientific, United Kingdom). Rotavirus outer capsid genes (VP7 and VP4) detection was performed by RT-polymerase chain reaction (PCR) in stool specimens that were positive for EIA rotavirus A. Briefly, viral cDNA was subjected to RT-PCR to amplify the VP7 and VP4 genes using primers and amplification conditions as previously described.[25] Amplification of VP7 and VP4 regions was performed individually, and PCR reactions were predicted to generate amplicons of 881 and 663 bp, respectively. PCR amplicons were visualized on 2% agarose gels under ultraviolet (UV) light after staining with 3% ethidium bromide.

Sequencing, genotype determination, and phylogenetic analysis. PCR amplicons from successful VP7 and VP4 PCR amplifications were DNA sequenced using the amplification primers. PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN, Germany), and DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, United Kingdom). Direct DNA sequencing was performed using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's recommendations. All DNA sequences were generated using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems), and the resulting DNA sequences were assembled using DNA Baser Sequence Assembler v3.0.17 (Heracle Biosoft, Pitesti, Romania).

The resulting VP4 and VP7 sequences (Genbank accession numbers: VP4, FR820957–FR821065; VP7, FR822209–FR822321) were compared with other corresponding genotype sequences using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for genotype determination. Coding sequences were manually aligned using Se-AL v2.0a11 (http://tree.bio.ed.ac.uk/software/), and additional sequences were trimmed to correspond with our sequences to maximize sequence homology. Maximum likelihood trees for each of the genotypes were inferred using RAxML v 7.0.4[26] employing the general time-reversible model of nucleotide substitution with a γ-distribution of among-site rate variation (GTR+Γ), which was determined using jModelTest.[27] One thousand bootstrap replicates were used as implemented in a rapid bootstrap algorithm available in RAxML. The resulting trees were visualized in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/), and genetic distances were estimated using HyPhy v2.0 (http://www.datam0nk3y.org/hyphy/doku.php).[28] All statistical analyses were performed in R version 2.9.0 (The R foundation for Statistical Computing); P values < 0.05 were considered statistically significant.

RESULTS

Enteric virus prevalence. A total of 362 children from the two locations (252 from HCMC and 110 from DT) with acute gastroenteritis were concurrently enrolled in the 1-month-long study. We were able to detect rotavirus A, norovirus, adenovirus, or astrovirus using EIA in 195 samples (53.9%), and eight children had more than one viral pathogen in their stool (Table 1). In the 252 samples originating from HCMC, 75 (29.8%) were positive for group A rotavirus, and 34 (13.5%) were positive for norovirus (Table 1). In the DT samples, 72 (65.5%) were positive for group A rotavirus, and 4 (3.6%) were positive for norovirus. Enteric adenovirus and astrovirus collectively comprised only a small proportion of all the acute diarrheal cases in HCMC (2.4% and 2.8%, respectively); this finding was also the case in DT (2.7% and 1.8%, respectively).

The age distribution of all the hospitalized patients with diarrhea was similar in both locations, with a mean of 15.8 months (median = 13 months, range = 2–96 months) in HCMC and a mean of 15.3 months (median = 10 months, range = 1.5–156 months) in DT. The preponderance of patients were less than 24 months of age (90.6% in HCMC and 94.9% in DT), and the most common 6-month age group was between 7 and 12 months. This peak age group was comprised of 43.6% (51/117) of the positive samples from HCMC and 48.7% (38/78) of the positive samples from DT. We found that the prevalence of viral positive samples in cases from HCMC was significantly lower in those children younger than 6 months and older than 24 months of age compared with those children in the intermediate age group ($P < 0.001$, χ^2 test). In DT, the prevalence of viral diarrhea was lower in patients older than 18 months of age compared with children aged between 0 and 18 months ($P < 0.001$, χ^2 test). Furthermore, the proportion of rotavirus infections in patients under the age of 6 months from DT was significantly higher than the corresponding age group from HCMC ($P = 0.0289$, χ^2 test). Conversely, in the group consisting of children aged from 19 to 24 months, the proportion of rotavirus infections was significantly greater in HCMC than DT ($P = 0.009457$, χ^2 test).

Distribution of rotavirus genotypes. All stool samples were subjected to RT-PCR for the VP4 and VP7 regions of rotavirus A. None of the samples that were negative for rotavirus using EIA were positive by PCR, and 133 of 157 samples of the positive EIA samples gave a PCR amplicon for one or both of the loci (PCR compared with EIA: sensitivity, 90%; specificity, 100%). All positive VP4 and VP7 PCR amplicons were

Table 1

Enzyme immunoassay detection of four viral pathogens in stool samples from children with acute diarrhea in Ho Chi Minh City and Dong Thap

Viral assay	Number of positive samples in Ho Chi Minh City (%)*	Number of positive samples in Dong Thap (%)†
Group A rotavirus‡	75 (29.8)	72 (65.5)
Norovirus	34 (13.5)	4 (3.6)
Adenovirus	6 (2.4)	3 (2.7)
Astrovirus	7 (2.8)	2 (1.8)
Multiple positive	5 (4.3)	3 (2.7)
Total viruses detected	122	81
Total positive samples	117 (46.4)	78 (73.6)

* $N = 252$.
† $N = 110$.
‡ Equivocal results were found in two samples from Ho Chi Minh City.

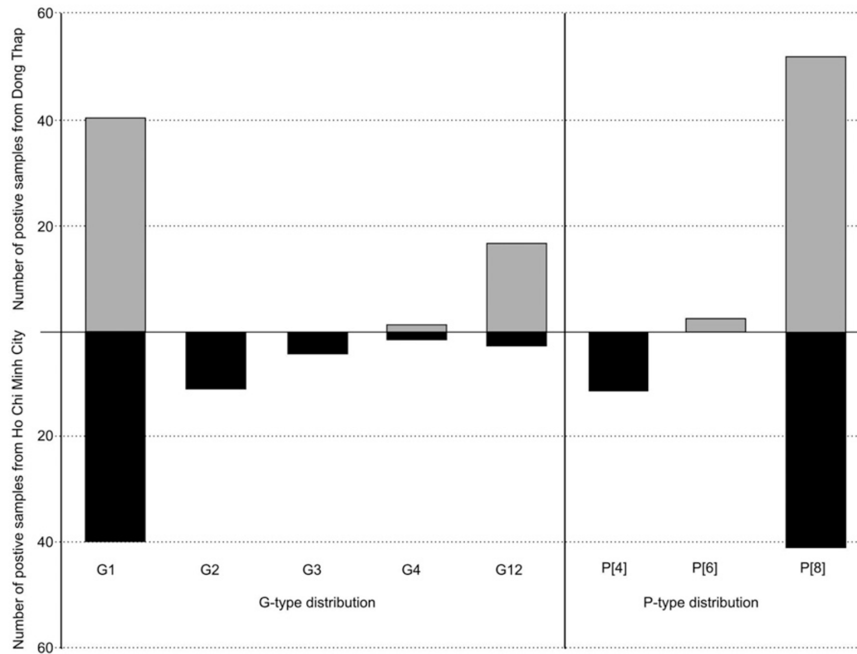

FIGURE 1. The distribution of rotavirus G and P types detected in the stools of children with diarrhea in Ho Chi Minh City and Dong Thap. The graph shows the distribution of rotavirus G types 1, 2, 3, 4, and 12 and P types P[4], P[6], and P[8] in 118 and 109 VP7 and VP4 PCR amplification-positive samples, respectively. The graph is subdivided into G (N = 60) and P types (N = 56) from DT (upper, grey) and G (N = 58) and P types (N = 53) from HCMC (lower, black).

DNA-sequenced; 118 of 133 amplicons produced a sequence consistent with the VP4 region, and 109 of 133 amplicons produced a sequence consistent with the VP7 region. We found that genotype G1 was the dominant circulating G type, comprising 82 of 118 (69.5%) of all typeable G types (Figure 1). The novel emerging genotype, G12, was the second most common G type, representing 16.1% (19/118) of all G types. Other less common G genotypes, including G2, G3, and G4, were also detected in small proportions in the remainder of the positive samples (Figure 1). Genotype P[8] was the most prevalent typeable P genotype, accounting for 88.1% (96/109) of all P amplicons, followed by P[4] (10.1%, 11/109) and P[6] (1.8%, 2/109).

The most common GP combination was G1P[8], comprising 78.5% (73/93) of all samples that were positive for both amplification targets. Other globally diffuse GP genotypes, including G2P[4] and G3P[8], were also detected but again, in a limited number of samples (10.8%, 10/93; 2.2%, 2/93, respectively) (Figure 1). The overall distribution of G and P types differed substantially between the two locations. Genotypes G2 and G3 were present in several rotavirus positive samples in HCMC, but neither was identified in positive amplifications from DT. Genotype G12 was identified in both locations but was more prevalent in DT than in HCMC, representing 28.3% and 3.4% of all G types in these locations, respectively (P = 0.0016, χ^2 test). Furthermore, P[4] was identified in 20.8% of the P-type samples in HCMC but was not detected in DT, and P[6] was detected only in samples originating from DT.

Phylogenetic analysis of rotavirus sequences. We performed phylogenetic analyses on the G1, G12, and P[8] sequences produced from the VP7 and VP4 amplifications, comparing them with additional global sequences available in public databases. The G1 rotavirus sequences from DT and HCMC exhibited extensive genetic diversity [maximum genetic distance (uncorrected) = 0.0774] and could be differentiated into three distinct lineages (Figure 2). We could identify a significant phylogenetic association between the DT and HCMC G1 sequences, signifying the circulation of closely related rotavirus strains in these two locations. Comparing these G1 data with previous rotavirus sequence data originating in Vietnam, one lineage showed a close phylogenetic relationship to rotaviruses also isolated in HCMC, indicating local persistence of this particular lineage (Figure 2).[29] The distribution of the P[8]-type sequences was comparable with the distribution observed throughout the G1 sequences, exhibiting extensive genetic diversity (maximum genetic distance [uncorrected] = 0.159) (Figure 3). The P[8] sequences could be differentiated into four phylogenetically distinct clusters that seem to be closely related to sequences from other regions around Asia (Figure 3). Sequences from the G12 lineage, which were primarily detected in samples from DT, were closely related to Thai and Indian sequences (Figure 4). However, less overall diversity was detected among the G12 genotype sequences (maximum genetic distance [uncorrected] = 0.0130), with only one major lineage identified (Figure 4), signifying potential recent introduction of this variant.

DISCUSSION

Enteric viruses are a predominant cause of acute childhood gastroenteritis in Vietnam.[9,11,20,21,30–33] Our study was designed to examine the prevalence and distribution of four enteric viruses causing hospitalization in children attending two defined healthcare centers in one rural and one urban location in southern Vietnam. We found a substantial proportion of diarrhea to be caused by viral pathogens and an overall dominance of group A rotavirus. Our data suggest that, although this study represents a 1-month long snapshot of acute childhood

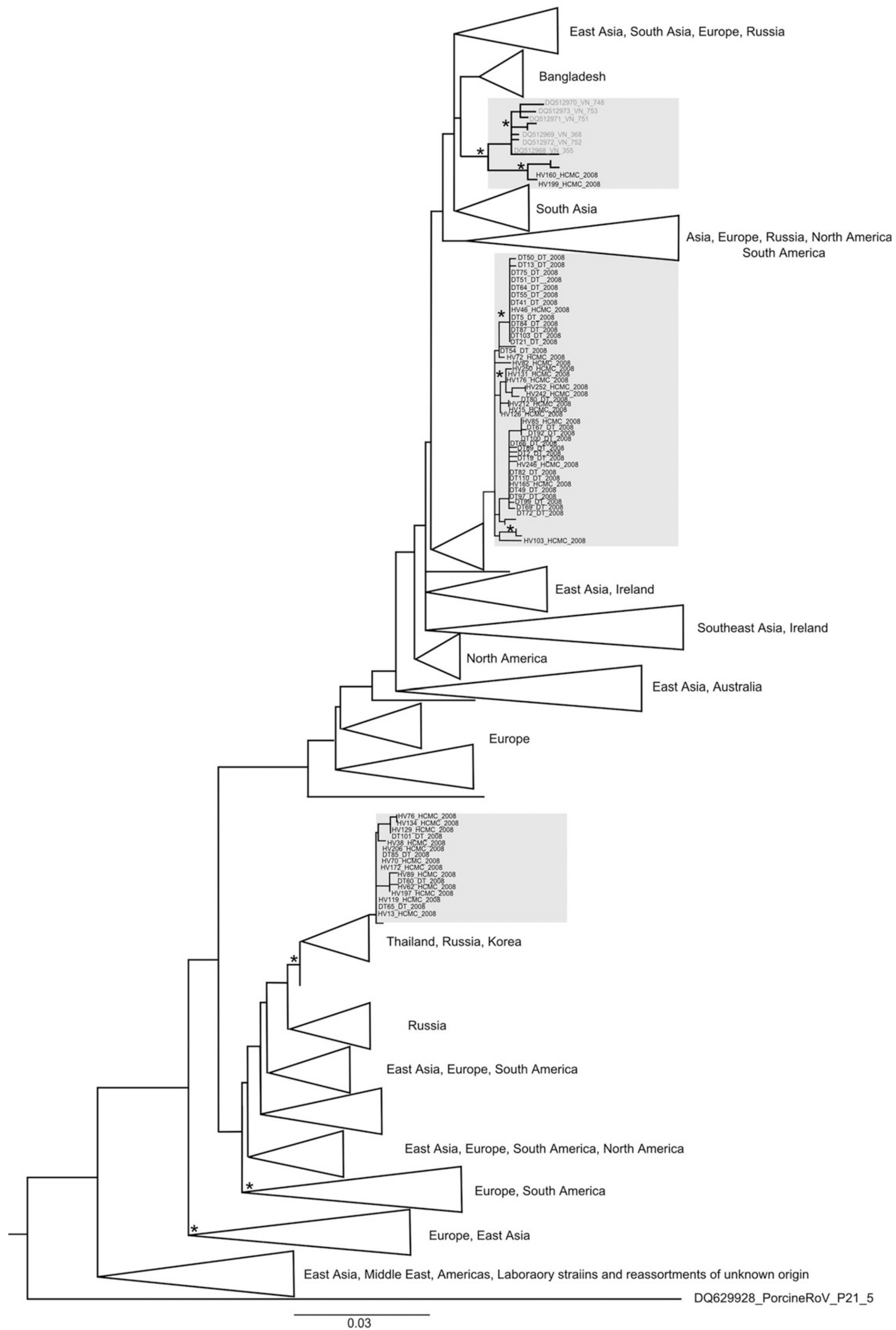

FIGURE 2. Phylogenetic tree of 81 rotavirus G1 sequences from Ho Chi Minh City and Dong Thap combined with representative global rotavirus G1 sequences. Maximum likelihood phylogenetic tree was constructed from G1 sequences from the amplification and sequencing of the VP7 gene. Sequences generated from this study are indicated in black. HCMC indicates samples from Ho Chi Minh City, and DT indicates samples from Dong Thap. Vietnamese isolates from previous studies are highlighted in grey. The tree is midpoint-rooted, with all horizontal branch lengths drawn to the scale of a nucleotide substitution per site. Bootstrap values > 85% are indicated by asterisks, and triangles represent compressed regions of the tree.

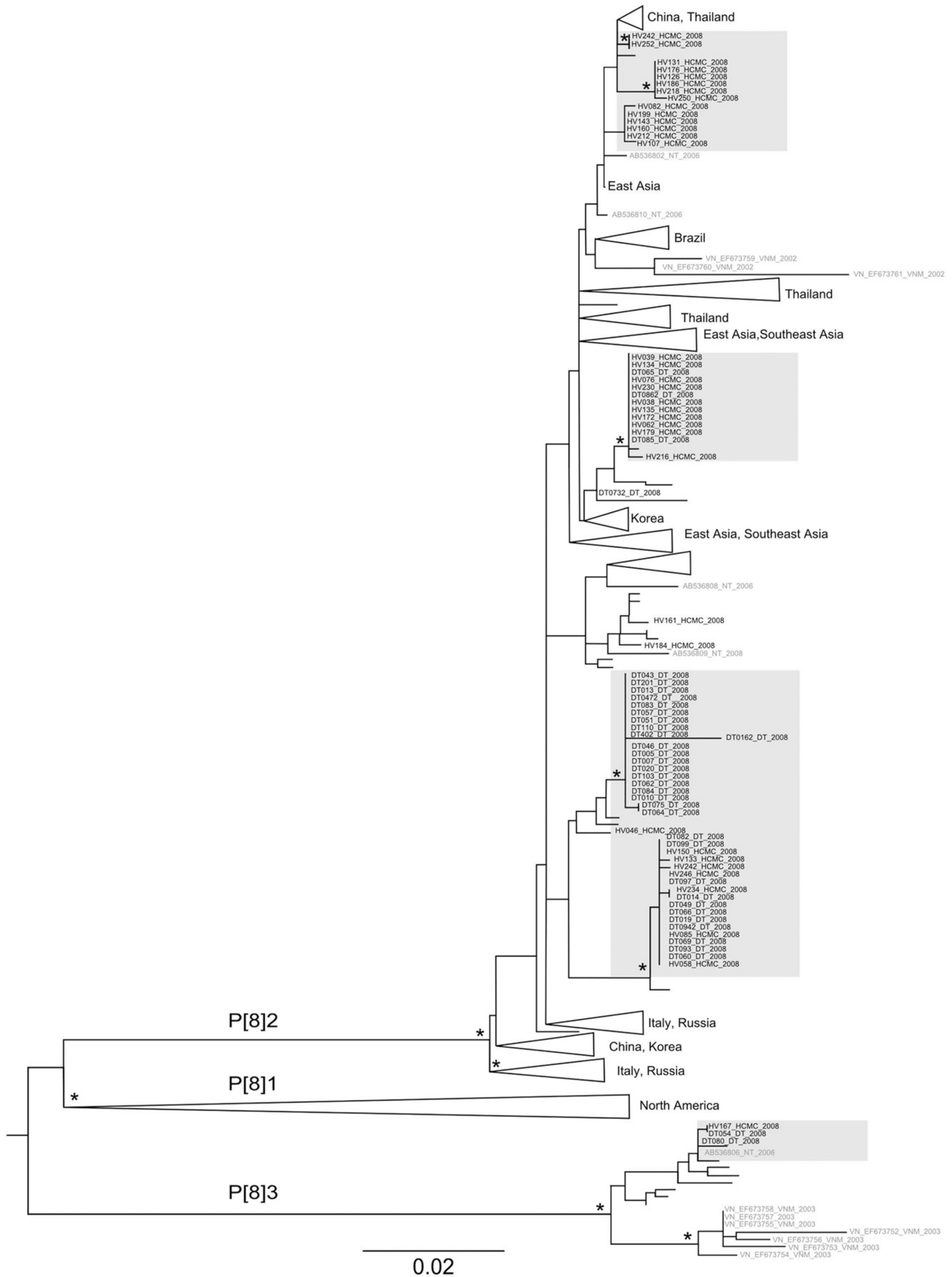

FIGURE 3. Phylogenetic tree of 96 rotavirus P[8] sequences from Ho Chi Minh City and Dong Thap combined with representative global rotavirus P[8] sequences. Maximum likelihood phylogenetic tree [VP4 gene] constructed from P[8] sequences and representative global sequences of rotavirus P[8] type. Tree rooting, bootstrap values, branch lengths, and font correspond to those factors presented in Figure 2.

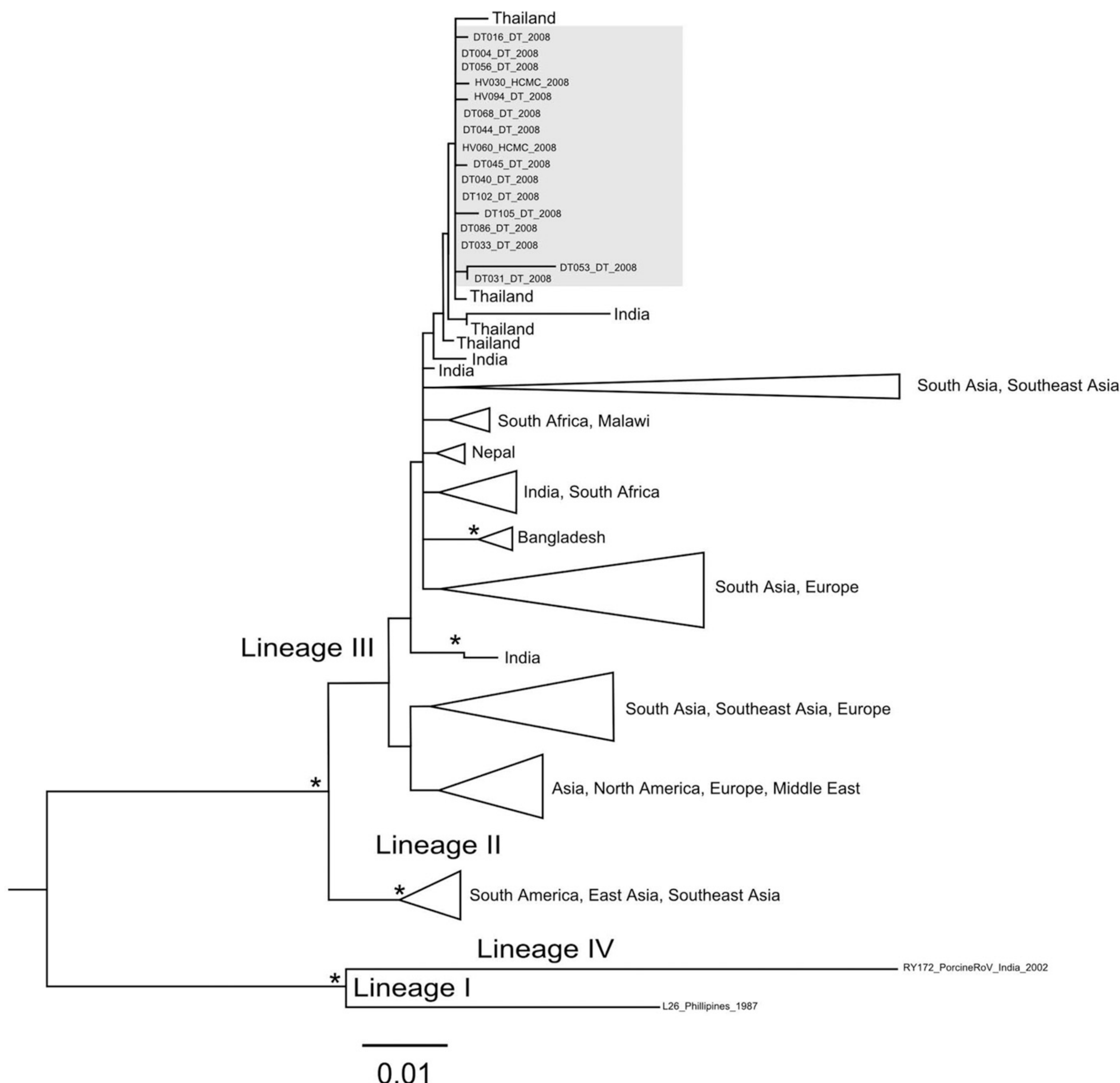

FIGURE 4. Phylogenetic tree of 19 rotavirus G12 sequences from Ho Chi Minh City and Dong Thap combined with representative global rotavirus G12 sequences. Maximum likelihood phylogenetic tree [VP7 gene] constructed from G12 sequences and representative global sequences of rotavirus G12. Tree rooting, bootstrap values, branch lengths, and font correspond to those factors presented in Figure 2.

diarrhea, viral pathogens are predominant etiological agents of acute childhood gastroenteritis in this location, a view that concurs with previous studies.[9,11,20,30] We additionally show a variable distribution of enteric viruses in children hospitalized with acute watery diarrhea in distinct urban and rural locations in southern Vietnam, suggesting the circulation of different pathogens and corresponding differential infection risks.

Our findings show that group A rotaviruses were the predominant viral cause of diarrheal disease in children in both sampling locations. Within the group A rotavirus-positive samples, we identified extensive genetic diversity despite the limited temporal distribution and relatively small number of samples. Such dramatic diversity within the G1 group is consistent with multiple introductions of the G1 genotype to the region. Furthermore, only one of three G1 lineages showed a relationship to rotavirus sequences from HCMC in 2002–2005, again supporting on-going strain introduction with limited *in situ* evolution.[29] Of all typeable P strains, P[8] predominated, with a comparable prevalence with North America, Australia, and Europe but higher prevalence than the prevalence previously reported in Vietnam.[9–11,14,20] Again, phylogenetic analysis indicates that the extensive heterogeneity observed within the P[8] sequences is likely caused by multiple strain introduction rather than clonal expansion.

We identified a differential distribution of viral diarrheal pathogens between the urban and rural locations. We note a

significantly higher prevalence of rotavirus genotype G12 in the rural location compared with the urban location. This study is the first to report a rotavirus genotype G12 in Vietnam, and since the primary detection in the Philippines in 1987, it has become increasingly prevalent worldwide.[34] Our high detection rate (28.3% of all G types in DT) of this variant highlights the capacity of this genotype to spread and become fixed in a local population, and it has direct implications for rotavirus vaccination, because protective immunity of two available rotavirus vaccines (RotaRix and RotaTeq) against G12 genotype is currently undetermined. However, a high evolutionary rate of the VP7 gene (1.66×10^{-3} substitutions/site per year) suggests that the introduction of either vaccine may impose a selective pressure on circulating strains and accelerate evolutionary rates, facilitating the emergence and rapid spread of variants.[35] Such factors emphasize the need for on-going modification and development of rotavirus vaccines and continued surveillance for genotype circulation.

Secondary data supporting a differential distribution of enteric viruses between urban and rural settings is the distribution of norovirus in the two locations. Norovirus was the second most commonly identified virus in the patients' stool samples, which is similar to global data.[36] From the positive stool samples in HCMC, norovirus constituted 29% of positive sample compared with only 5.1% in DT. We suggest different epidemiological risk factors related to this organism in these locations, which necessitates additional investigation. Norovirus is highly contagious and is related to isolated outbreaks in developed countries.[37] HCMC is more densely populated and has undergone a greater level of development with respect to the surrounding province, such as DT. Transmission and the corresponding exposure to this particular pathogen are likely to follow such a developmental change. In parallel to rotavirus infections, the majority of patients in which we detected norovirus in the stool samples was in the 7–12 months age group. This age group is the key age group for children with diarrheal infections and poses the greatest number of epidemiological questions. Decreased rates of all viral pathogens detected in the stool samples outside this key age group may be related to risk, exposure, healthcare-seeking behavior, maternal immunity, and natural postinfection immunity. Additional studies on the effect maternal antibody or immunity from previous exposure to norovirus should help guide vaccine development and usage within this age group.

Our study design does have several caveats; first, although the age distribution of enrolled patients was similar in both locations, such sampling was open to selection bias. However, we aimed to understand the distribution viral diarrheal pathogen and not determine risk factors; therefore, a limited period of sampling may bias the distribution of the various agents. Second, it is known that 5% of control healthy patients (defined as inpatients for non-infectious causes without diarrhea in the previous 2 weeks) between the ages of 3 months and 5 years can carry viral pathogens in their stool specimens, and additional sampling is necessary to understand the transmission dynamics and asymptomatic carriage of enteric viruses.[32] We did find some dual viral infections (4.1%), but we were unable to elucidate the impact in pathogenesis and their clinical significance, which also may be an issue when other bacterial and parasitic agents are considered.

In conclusion, this report is the first report of rotavirus G12 in Vietnam, and we show a differential distribution of the major enteric viral pathogens and rotavirus genotypes causing childhood acute gastroenteritis in two distinct locations in southern Vietnam. We highlight the need for longitudinal research of enteric viruses in this location and continued monitoring of circulating rotavirus strains for effective prevention and vaccination strategies.

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Endemic Norovirus Infections in Children, Ho Chi Minh City, Vietnam, 2009–2010

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We performed a case–controlled investigation to identify risk factors for norovirus infections among children in Vietnam. Of samples from 1,419 children who had diarrhea and 609 who were asymptomatic, 20.6% and 2.8%, respectively, were norovirus positive. Risk factors included residential crowding and symptomatic contacts, indicating person-to-person transmission of norovirus.

Norovirus (NoV) is a leading cause of acute gastroenteritis in children <5 years of age (*1*). The epidemiology of NoV in industrialized countries has been intensively investigated, yet the contribution of this pathogen to the effects of diarrheal disease in low- and middle–income countries is not well characterized (*1,2*). Gaining insight into the epidemiology of NoV infections of children in such countries is essential for disease control, particularly considering that several vaccine candidates are in advanced-stage clinical trials (*3*). To address the lack of data on risk factors for endemic NoV infections in low-income countries, we conducted a prospective case–control study among hospitalized children in a major urban location in southern Vietnam.

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The Study

This study was conducted in 3 hospitals (Children's Hospital 1, Children's Hospital 2, and the Hospital for Tropical Diseases) in Ho Chi Minh City, Vietnam, during May 2009–December 2010. Written informed consent from a parent or legal guardian was mandatory for participation. Children <5 years of age who resided in Ho Chi Minh City, who had acute diarrhea on admission (≥3 loose stools or ≥1 bloody loose stool within a 24-hour period), and were given no antimicrobial drug treatment 3 days before hospitalization, were invited to participate during May 2009–April 2010. To collect control data, during March–December 2010, we enrolled children who were attending outpatient and inpatient clinics in the nutrition or gastroenterology departments for routine health checks or conditions unrelated to gastroenteritis. Children in this control group met the same demographic criteria, did not have diarrhea, and had not received antimicrobial drugs during the preceding 3 weeks.

Stool specimens were collected from case-patients on the day of admission (n = 1,419) and from control participants while they were attending the clinic (n = 609). All stool samples were cultured by using classic microbiologic methods to detect *Shigella, Salmonella*, *Campylobacter,* and *Yersinia* spp. and were microscopically examined for *Entamoeba, Cryptosporidium,* and *Giardia* spp. Methods are described in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/6/11-1862-Techapp1.pdf). Conventional reverse transcription PCR was performed on RNA extracted from stool samples to detect rotavirus (*4*) and NoV genogroups I (GI) and II (GII) (*5*), followed by direct sequencing of the amplicons for genotyping.

After rotavirus (46.6%; 661/1,419), NoV was the second most common pathogen detected in symptomatic case-patients (20.6%; 293/1,419); diarrheal bacteria and parasites were cumulatively found in 14.5% (online Technical Appendix Table). The prevalence of NoV was higher than in a pooled international estimate (*1*) and than in previous studies performed in Ho Chi Minh City (*6*–*8*), yet was lower than that found in a study conducted in northern Vietnam (*9*). The frequency of NoV detected in control participants was 2.8% (17/609), similar to a pooled international estimate (*1*). The majority of NoV-positive case-patients experienced nonbloody, nonmucoid watery diarrhea, vomiting, and fever. These symptoms were comparable to those in previous studies of diarrheal infections in children in Vietnam (*7,9*).

NoV was detected throughout the study period (online Technical Appendix Figure). There was a positive linear correlation between NoV infections and monthly rainfall (R = 0.550, p = 0.029), but no similar correlation with temperature (range 22.1°C–37.8°C) (R = 0.308, p = 0.330). This association of NoV infections with the tropical rainy

Table 1. Baseline characteristics of NoV-positive and NoV-negative case-patients and control participants, Vietnam, 2009–2010*

Characteristic	Case-patients		Controls	
	NoV positive, n = 241	NoV negative, n = 1,126	NoV positive, n = 15	NoV negative, n = 592
Male sex	147 (61.0)	724 (64.3)	8 (53.3)	314 (53.0)
Mean age, mo (range)	13.3 (2–45)	15.8 (1–59)	15.8 (2.3–52)	16.8 (0–60)
Age groups, mo				
≤6	24 (10.0)	165 (14.7)	3 (20.0)	98 (16.6)
7–12	102 (42.3)	375 (33.3)	4 (26.7)	208 (35.1)
13–18	76 (31.5)	245 (21.8)	3 (20.0)	113 (19.1)
19–24	25 (10.4)	147 (13.1)	3 (20.0)	56 (9.5)
24–60	14 (5.8)	194 (17.2)	2 (13.4)	117 (19.8)
Poor Z score†	18 (7.5)	73 (6.5)	1 (6.7)	75 (12.7)
Breastfed	187 (77.6)	790 (70.2)	11 (73.3)	452 (76.4)
Daily activity				
Day care/nursery school	30 (12.5)	194 (16.4)	4 (26.7)	89 (15.2)
Home	211 (87.6)	938 (83.6)	11 (73.3)	498 (84.8)

*Values are no. case (%) unless otherwise specified. Case-patients indicate patients who had diarrhea; controls indicate asymptomatic (diarrhea-free) children. Study dates span May 2009–December 2010. NoV, norovirus; WHO, World Health Organization.
†Weight-for-age Z score calculated based on WHO Child Growth Standards guidelines (www.who.int/childgrowth/standards/technical_report/en/); Z score below −2 was considered to indicate that a child was malnourished.

season may reflect differential transmission between different climatic regions because NoV infections are typically associated with the winter season in industrialized countries in temperate regions (*10*).

GII NoV was detected in 239 (99.1%) of 241 and 11 (73.3%) of 15 NoV-positive stool samples from the symptomatic and asymptomatic enrollees, respectively. The remaining children were infected with NoV GI (GI.3, GI.4, GI.5); 1 enrolled case-patient was infected with 2 genotypes: NoV GI.3 and GII.4. Of the GII strains, GII.4 was the most prevalent genotype, comprising 201 (84.1%) of the 239 samples. The next most prevalent was GII.3: 24 (10.0%) were identified in the symptomatic and asymptomatic groups. Other GII genotypes (GII.2, GII.6, GII.7, GII.9, GII.12, and GII.13) were found in <3% of NoV-positive samples.

Socioeconomic and behavioral data were obtained from all enrollees by using a questionnaire and analyzed by using Stata Version v9.2 (StataCorp LP, www.stata.com) (Table 1). We used χ^2 and Fisher exact tests to compare proportions between groups and Mann-Whitney U tests for nonparametric data. Univariate analyses were performed to assess factors associated with symptomatic NoV infections. Factors found to be significantly associated with infection in the univariate analysis, in addition to a-priori factors of age, sex, and income level, were then included in a multivariate logistic regression model to simultaneously control for confounding effects. Two-sided p values <0.05 were considered significant throughout (Table 2).

NoV infections are commonly associated with outbreaks in enclosed environments (*2*), yet we found attendance in daycare centers and nursery schools was not common; the majority of children remained at home during the day. However, several factors were significantly and independently associated with symptomatic NoV infections. Demographic risk factors included younger age (in months) (adjusted odds ratio [aOR] 0.96, 95% CI 0.94–0.98, p<0.001) and household crowding (≥3 children in the house) (aOR 1.70, 95% CI 1.0–2.9, p = 0.052). Living in a household where food was regularly purchased from outdoor markets added a significant risk (aOR 4.99, 95% CI 3.1–7.9, p<0.001). Unpredictably, we found that consuming bottled water, rather than pipeline water (aOR 2.18, 95% CI 1.4–3.4, p< 0.001), was a risk factor and did not correlate with household income. However, those drinking municipal water also reported boiling or filtering water before consumption, and those drinking bottled water did not. This association suggests that bottled water in this location may be of poor quality. A further unexpected finding was the protective nature of outdoor toilets (aOR 0.22, 95% CI 0.1–0.4, p<0.001), which may be a result of the sterilizing capabilities of sunlight or of containing fecal contamination outside the residence, possibly protecting children during the period of infancy before they can use toilets. We found that the greatest risk factor for symptomatic NoV infections (aOR 26.14, 95% CI 10.4–65.9, p<0.001) was contact with a person who recently had a diarrheal infection. This finding is consistent with previous investigations showing that person-to-person transmission is predominant during sporadic outbreaks (*11–14*).

This study has several limitations. First, passive case detection limits generalizability because health care–seeking behavior may depend on disease severity and income in this setting. Second, the control participants may not be entirely representative of the population from which the case-patients arose because a large proportion of the control participants were visiting the hospital for nutritional advice, which may have an effect on diarrheal disease risk (*15*). Yet, a limited sensitivity analysis comparing NoV-positive case-patients to NoV-negative control participants and NoV-negative case-patients to NoV-negative control participants demonstrated

Table 2. Univariate and multivariate analysis of risk factors for symptomatic NoV infections, Vietnam, 2009–2010*

Risk factor	NoV-positive case-patients	NoV-negative control participants	OR	95% CI	aOR	95%CI
Mean age, mo (range)	13.3 (2–45)	16.8 (0–60)	**0.97**	**0.96–0.99**	**0.96**	**0.94–0.98**
Male sex (%)	147 (61.0)	314 (53.0)	**1.38**	**1.0–1.9**	1.38	0.9–2.0
Poor Z-score	18 (7.5)	75 (12.7)	**0.56**	**0.3–0.9**	0.61	0.3–1.1
Low income†	150 (62.2)	335 (56.6)	1.26	0.9–1.7	0.89	0.6–1.3
≥5 adults in hh	72 (29.9)	158 (26.7)	1.17	0.8–1.6	NI	NI
≥3 children in hh	36 (14.9)	58 (9.8)	**1.62**	**1.0–2.5**	**1.70**	**1.0–2.9**
Refrigerator in hh	187 (77.6)	506 (85.5)	**0.59**	**0.4–0.9**	0.73	0.5–1.2
Consumes market food	201 (84.1)	345 (58.4)	**3.77**	**2.6–5.5**	**4.99**	**3.1–7.9**
Household water source						
Pipeline‡	132 (54.8)	347 (58.6)	1.00	NA	NI	NI
Well	96 (39.8)	220 (37.2)	1.15	0.8–1.6	NI	NI
Other§	13 (5.4)	25 (4.2)	1.37	0.7–2.8	NI	NI
Drinking water source	NI	NI	NI	NI	NI	NI
Pipeline‡	116 (48.1)	334 (56.4)	1.00	NA	1.00	NA
Bottled water	69 (28.6)	122 (20.6)	**1.63**	**1.1–2.3**	**2.18**	**1.4–3.4**
Well	42 (17.4)	109 (18.4)	1.11	0.7–1.7	0.94	0.6–1.5
Other§	14 (5.8)	1.49	0.8–2.9	0.25	1.45	0.6–3.2
Toilet type						
Indoor‡	213 (90.6)	446 (75.9)	1.00	NA	1.00	NA
Outdoor	22 (9.4)	142 (24.2)	**0.32**	**0.2–0.5**	**0.22**	**0.1–0.4**
Hand washing¶	130 (85.0)	291 (89.5)	0.66	0.4–1.2	NI	NI
Attends day care/nursery school	30 (12.5)	89 (15.2)	0.80	0.5–1.2	NI	NI
Contact with symptomatic persons	38 (16.5)	8 (1.4)	**14.23**	**6.5–31.0**	**26.14**	**10.4–65.9**
Rural residence#	36 (14.9)	75 (12.7)	1.21	0.8–1.9	NI	NI

*Values are no. case (%) unless otherwise specified. Values in **boldface** indicate statistical significance at p≤0.05. NoV, norovirus; OR, odds ratio; aOR, adjusted OR; NA, not applicable; NI, not included in multivariable analysis; hh, household.
†Classified as making less than the Gross National Income ($232/mo) according to World Bank (http://data.worldbank.org/indicator/NY.GNP.PCAP.CD).
‡Reference group.
§Rain water, water from a truck provided by the government, or other water source.
¶Washing of children's hands, either by an adult or the child, after the child uses the toilet.
#Binh Chanh, Can Gio, Cu Chi, Hoc Mon, and Nha Be districts.

several differences in risk factors, suggesting that the identified risk factors are associated with NoV rather than health care–seeking behavior (online Technical Appendix Table).

Conclusions

This epidemiologic investigation showed that 20.6% of hospitalized children with acute diarrhea in Ho Chi Minh City tested positive for NoV, compared with 2.8% of diarrhea-free control participants. We conclude that young age, residential crowding, use of bottled water, and recent contact with a symptomatic individual are key risk factors for symptomatic NoV infection in this location. Because most children did not attend day care, potential preventative measures for NoV infection in Ho Chi Minh City should be focused on improving local hygiene standards to prevent person-to-person transmission within the home.

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The dynamics of GII.4 Norovirus in Ho Chi Minh City, Vietnam

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ABSTRACT

Norovirus (NoV) is a major cause of epidemic gastroenteritis in industrialized countries, yet the epidemiological significance of NoV in industrializing countries remains poorly understood. The spatiotemporal distribution of NoV genotypes identified in 2054 enrolled children was investigated between May 2009 and December 2010, in Ho Chi Minh City (HCMC), Vietnam. A total of 315 NoV extracted from stool samples were genotyped and GPS mapped to their source. Genogroup II NoV, particularly GII.4, were predominant, and the GII.4 strains could be subgrouped into GII.4-2006b (Minerva) and GII.4-2010 (New Orleans) variants. There was no spatiotemporal structure among the endemic GII strains; yet a significant spatiotemporal signal corresponding with the novel introduction of GII.4-2010 variant was detected. These data show that NoV GII.4 variants are highly endemic in HCMC and describe a scenario of rapid NoV strain replacement occurring in HCMC in early 2010.

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1. Introduction

Norovirus (NoV) is a non-enveloped positive-sense single-stranded RNA virus belonging to the taxonomic family *Caliciviridae* (Green et al., 2000; Jiang et al., 1990). NoV accounts for a significant proportion of the global burden of viral gastroenteritis (Glass et al., 2000, 2009; Patel et al., 2009, 2008), and up to 50% of all-cause outbreaks of diarrhea (Patel et al., 2009). The disease typically presents as acute watery diarrhea with vomiting and a low-grade fever (Patel et al., 2009), and is usually self-limiting, lasting between one and three days, but can be aggressive, severe and protracted in young children, the elderly and the immunocompromised (Estes et al., 2006). NoV has an exceptionally low infectious dose (10–100 viral particles (Patel et al., 2009)) and can survive on surfaces for prolonged time periods (Weber et al., 2010); as a result, NoV frequently causes explosive gastroenteritis epidemics (Estes et al., 2006; Patel et al., 2009, 2008).

The 7.5 kb genome of NoV has three open reading frames (ORFs), encoding the RNase-dependent RNA polymerase (RdRp, ORF1), the major capsid protein (VP1, ORF2) and the minor capsid protein (VP2, ORF3) (Bertolotti-Ciarlet et al., 2003; Jiang et al., 1993). The current classification divides NoV into five genogroups (GI – GV) on the basis of sequence identity within the major capsid protein (VP1). Genogroups GI, GII and GIV are associated with infections in humans (Zheng et al., 2006). Molecular characterization of coding sequences within RdRp (region A and B) (Ando et al., 1995, 2000; Fankhauser et al., 2002; Jiang et al., 1999; Vinje and Koopmans, 1996) and ORF2 (region C, D and E) (Kageyama et al., 2004; Kojima et al., 2002; Noel et al., 1997; Vinje et al., 2004) is targeted for NoV detection, genogrouping and genotyping. Genogroups I and II are the most common cause of human infections (Donaldson et al., 2010), and can be differentiated into 8 GI

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and 23 GII capsid genotypes, and 14 GI and 29 GII polymerase genotypes (Kroneman et al., 2011; Zheng et al., 2006).

The epidemiology of NoV is complex and is influenced by a multitude of factors, including population immunity, the environment, and seasonality (Donaldson et al., 2010; Marshall and Bruggink, 2011), making molecular epidemiology challenging. The acknowledged interpretation of global NoV epidemiology, particularly GII.4 genotype, is that strain replacement occurs every two to three years (Bull et al., 2010; Bull and White, 2011; CDC, 2010; Donaldson et al., 2008; Siebenga et al., 2009). Over the last two decades, these replacements were typically caused by strains of a single lineage of a GII.4 genotype, which have been responsible for the majority of NoV outbreaks worldwide since being first identified in the USA in the mid 1990s (Bull and White, 2011; Noel et al., 1999). At least four major global NoV replacements have been described since 1995, each due to a novel GII.4 variant (Bull et al., 2006; Noel et al., 1999; Siebenga et al., 2010; Tu et al., 2008), believed to have escaped immunity in the population through antigenic variation (Lindesmith et al., 2012b, 2012c, 2011).

The majority of NoV studies are performed in industrialized countries and disease outbreaks are continually monitored through several disease surveillance networks (Vega et al., 2011; Verhoef et al., 2009). However, little is known about the transmission, molecular diversity or spatiotemporal dynamics of NoV infections in areas with differing public health infrastructure and demographics. Vietnam is an industrializing country with densely populated urban centers and a changing spectrum of infectious diseases as a presumed consequence of rapid economic development and urbanization (Vinh et al., 2009). NoV was first reported in Ho Chi Minh City (HCMC) in 1999 (Hansman et al., 2004), and our recent work has demonstrated that NoV is endemic throughout the year, in contrast to the winter outbreaks observed in temperate locations (Lopman et al., 2009; Mounts et al., 2000). To understand the epidemiology and NoV strain diversity in HCMC, we investigated the molecular and spatiotemporal distribution of NoV genotypes in young hospitalized children between May 2009 and December 2010 in HCMC, Vietnam.

2. Material and methods

2.1. Study setting and design

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the ethical review boards of Children's Hospital 1 (HCMC), Children's Hospital 2 (HCMC), the Hospital for Tropical Diseases (HCMC), and the Oxford Tropical Research Ethics Committee (OxTREC Approval No. 0109) (Oxford). The parents or legal guardians of the enrolled children were required to provide written informed consent for sample collection and residential mapping.

A stool specimen was collected within 24 h of enrollment from each recruited individual (1443 diarrheal patients and 611 asymptomatic controls). These participants ($N = 2054$) were children of 0–60 months of age and residents of HCMC, Vietnam, over the study period from May 2009 to December 2010. Diarrheal patients were children with acute diarrheal disease ($\geqslant 3$ loose stools or at least one bloody loose stool within 24 h period (WHO, 2005)) who were admitted to the three study sites and had not received treatment with antimicrobials in the three days prior to hospital admission. Asymptomatic controls were diarrhea-free children attending Children's Hospital 1 or Children's Hospital 2 for nutritional health checks or for other gastrointestinal issues unrelated to diarrhea or gastroenteritis without any history of diarrhea, respiratory illness or treatment with antimicrobials within 7 days of study enrollment.

2.2. Norovirus detection

Total viral RNA was extracted and reverse transcribed into cDNA as previously described (Tra My et al., 2011). Norovirus genogroup I (GI) and II (GII) were detected in separate reactions by conventional Reverse Transcriptase Polymerase Chain Reaction (RT PCR) using consensus primers, G1SKF/G1SKR (Kojima et al., 2002) and COG2F/G2SKR (Kageyama et al., 2003; Kojima et al., 2002) for GI and GII, respectively. These PCR primers amplify a region between position 5342 and 5671 (330 bp) in the genome of NoV GI (Norwalk/68, GenBank accession No. M87661) containing an overlap of 17 bp of 3′ end ORF1 and 313 bp of 5′ end ORF2, and between position 5003 and 5389 (387 bp) in the genome of NoV GII (Lordsdale/93, GenBank accession No. X86557) containing an overlap of 83 bp of 3′ end ORF1 and 304 bp of 5′ end ORF2.

2.3. Norovirus genotyping

NoV positive PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany), and subjected to direct sequencing using the amplification primers. DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, United Kingdom) and direct sequencing was performed using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and generated with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, USA). DNA sequences were assembled using DNA Baser Sequence Assembler v3.0.17 (Heracle Biosoft, Pitesti, Romania). NoV genotypes were assigned based on ORF2 sequences using the online Norovirus Automated Genotyping Tool, as directed (Kroneman et al., 2011).

2.4. Construction of NoV phylogenies

DNA sequences were uploaded into GenBank (HE716437 to HE716751) and used for local phylogenetic construction. Manual alignment of all sequences was performed in Se-AL (http://tree.bio.ed.ac.uk/software/figtree/) prior to phylogenetic reconstruction. Maximum likelihood (ML) trees were inferred using RAxML (Stamatakis et al., 2008), employing the general-time reversible model of nucleotide substitution with a gamma distribution of among-site rate variation ($GTR + \Gamma$) and 1000 bootstrap replicates. Resulting trees were visualized in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) and mean pairwise genetic distances were estimated in MEGA 5 (Tamura et al., 2011).

Two hundred and sixty-nine global GII.4 strains encompassing the global diversity of GII.4 variants were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/); 43 of these strains originated from previous studies conducted in Vietnam (Hansman et al., 2004; Nguyen et al., 2008, 2007a, 2007b; Tamura et al., 2010; Trang et al., 2012). In addition, available archived samples that were Enzyme-Immuno Assay (EIA) positive for NoV (GI and GII) from our previous work in 2008 from southern Vietnam (Tra My et al., 2011) were also selected for analysis and genotyped using the same methodology.

The time of isolation for each of the NoV strains was retrieved from GenBank or the publication associated with the sequence, and the year of isolation was used to calculate evolutionary rate. All sequences were aligned in Se-AL and trimmed to 378 bp to correspond with the sequences identified in this study to maximize sequence homology for phylogenetic reconstruction. Phylogenetic reconstructions of relationships among the GII.4 variants identified in this study and global GII.4 sequences were inferred using the Bayesian Markov chain Monte Carlo (MCMC) method as implemented in BEAST (Drummond and Rambaut, 2007). A GTR substitution model with gamma-distributed rate variation and a

relaxed uncorrelated lognormal clock model with a constant population size were employed. The MCMC analysis was run for 50 million generations (with a burn-in of 5 million) and analyzed using Tracer (http://tree.bio.ed.ac.uk/software/tracer/) to ensure that all parameters had converged. Maximum clade credibility trees were annotated using TreeAnnotator v1.6.1 (BEAST) and visualized in FigTree v1.3.1. The weighted average evolutionary rates across branches were assessed in Tracer.

2.5. Mapping of corresponding residential addresses

The location of each enrollee's residence was recorded using an eTrex Legend GPS device (Garmin, United Kingdom) and verified by an additional member of the study team. Latitude and longitude of each residence (recorded in decimal degrees) were entered along with patient metadata in Microsoft Excel (Microsoft, Redmond, USA). Location data were converted to KML format and locations were visualized and validated in Google Earth version 5 (http://www.google.com/earth/index.html) (Supplementary Figure).

2.6. Spatiotemporal analyses

Mantel tests were performed to assess potential correlations between genetic, temporal, and spatial distances of GII strains and variants within the GII.4 clade, using the *ade4* package in R (R_Development_Core_Team, 2011) (ww.ats.ucla.edu/stat/r/faq/mantal_test.htm). A Bernoulli model was used to examine spatiotemporal clusters of GII.4-2010, using all non-GII.4-2010 to represent the background distribution of the NoV population using SaTScan v9.1.1 software (http://www.satscan.org/). For the current analysis, the upper limit for cluster detection was specified as 10% of the study population over 10% of the study duration. The significance of the detected clusters was assessed by a likelihood ratio test, with a *P*-value obtained by 999 Monte Carlo simulations generated under the null hypothesis of a random spatiotemporal distribution.

3. Results

3.1. The temporal distribution of NoV genogroups and genotypes

Over the period of study, 315 NoV positive stool samples from 2054 individuals were identified (15.3%). Genotyping of these 315 strains demonstrated that the predominant NoV genogroup was GII (304; 96.5%), with NoV GI identified in only 3.5% of enrollees (Table 1). An array of GI (GI.3, GI.4 and GI.5) and GII (GII.2, GII.3, GII.4, GII.6, GII.7, GII.9, GII.12, GII.13 and GII.4U (genotype 4 with unassigned variants, outgroup of the lineage containing strains OB200615 and X76716 according to RIVM-NoroNet)) genotypes were additionally detected. Among the 304 NoV GII strains, GII.4 was the most frequently identified (247; 81.3%) and could be separated into two major variants, GII.4-2006b (Minerva) (86.2%; 213/247) and GII.4-2010 (New Orleans) (12.1%; 30/247). The GII.4-2006b and GII.4-2010 variants were the focus of subsequent analyses as a consequence of their overall dominance and their perceived epidemiological relevance in HCMC.

The genotyping data were combined with isolation dates to illustrate the distribution of GII.4 variants over the period of sample collection (Fig. 1). NoV GII.4 strains were detected every month from May 2009 to April 2010, with GII.4-2006b being the sole GII.4 variant identified between May and November 2009. However, this trend was not uniform as there was a substantial increase in GII.4-2006b infections during September and October 2009. The GII.4-2010 variant was first detected in December 2009 and became increasingly prevalent in the following months. Concurrently, the

Table 1
The distribution of NoV genogroups and genotypes identified in enrollees over the sample collection period in HCMC, Vietnam.

Genogroup/Genotype	Total *N* (%)
GI	**11 (3.5)**
I.3	7 (2.2)
I.4	1 (0.3)
I.5	3 (1.0)
GII	**304 (96.5)**
II.2	4 (1.3)
II.3	32 (10.2)
II.4	247 (78.4)
II.4-2006b	*213 (67.6)*
II.4-2010	*30 (9.5)*
II.4U	*4 (1.3)*
II.6	8 (2.5)
II.7	3 (1.0)
II.9	1 (0.3)
II.12	2 (0.6)
II.13	7 (2.2)
Total	315

proportion of GII.4-2006b variants decreased from 7.36% (17/231) in December 2009 to 1.73% (4/231) in February 2010, and was not detected after March 2010. However, trends in the distribution of NoV variants were difficult to assess during the period after April 2010 due to the limited number of enrollees recruited in this period.

3.2. Phylogenetic analyses of NoV sequences

Phylogenetic analyses were performed on all GI and GII NoV sequences, and the mean uncorrected genetic distances among the strains within the GII and between variants within the GII.4 genotype (pairwise distance of maximum composite likelihood calculation) were 0.147 and 0.016 substitutions/site, respectively. Based on this primary phylogenetic analysis, the GI strains were excluded and the GII strains were subsampled by removing identical GII sequences to reconstruct a maximum likelihood phylogenetic tree summarizing the genetic diversity present in HCMC ($N = 109$) (Fig. 2).

Sequences of the two GII.4 variants from HCMC ($N = 247$) were compared with 269 global sequences and 10 selected GII.4 sequences isolated in 2008 in southern Vietnam (Tra My et al., 2011) (Fig. 3). Using the Bayesian MCMC method and time-stamped sequences, the evolutionary rate of NoV GII.4 was estimated to be 8.072×10^{-3} substitutions/site/year (95% Highest Probability Density (HPD): 6.195×10^{-3}, 1.012×10^{-2}). The GII.4-2006b sequences from NoV originating in Vietnam fell in the same clade as global GII.4-2006b viruses, with clustering unrelated to the time or place of isolation. This GII.4-2006b lineage could be further divided into two sub-lineages; strains from HCMC could be found in both, confirming co-circulation of divergent GII.4-2006b viruses. Notably, the upper sub-lineage contained more sequences from this study while more Vietnamese strains from previous studies fell in the lower sub-lineage. The GII.4-2010 strains clustered in a single lineage, separate from the GII.4-2006b lineage. The GII.4-2010 lineage could be differentiated partially by location, with Vietnamese and Belgian sub-lineages stemming from the New Orleans GII.4-2010 variant.

3.3. Spatiotemporal clustering of NoV in HCMC

The temporal data suggested that a NoV strain replacement occurred during the period of investigation. There was a significant association between the genetic distance of strains within GII and their date of isolation ($p < 0.0001$; Mantel test), this association

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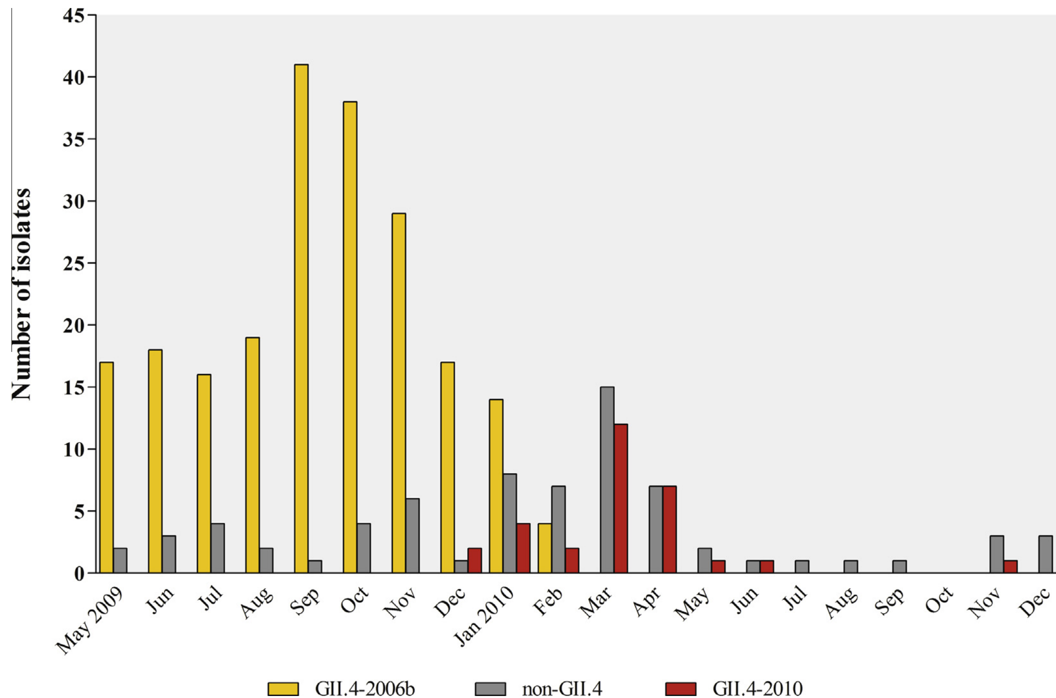

Fig. 1. The temporal distribution of NoV GII.4 variants in HCMC over the period of study, from May 2009 to December 2010. Graph showing the distribution of GII.4-2006b and GII.4-2010 variants against other NoV strains (total numbers identified) detected in symptomatic and asymptomatic children over the study period (GenBank accession number HE716437 to HE716751).

was particularly apparent between the GII.4 sequences and their isolation date ($p < 0.0001$; Mantel test). However, there was no similar association between geographical distance and genetic distance ($p = 0.197$ for GII strains; $p = 0.844$ for GII.4 sequences), or between isolation date and geographical distance ($p = 0.248$ for GII strains; $p = 0.851$ for GII.4 sequences). These data indicate a lack of a local transmission signal of NoV in HCMC. Yet, a spatio-temporal cluster detection analysis performed in SaTScan supported our original hypothesis, detecting a cluster of six GII.4-2010 NoV (over other NoV GIIs (0.59 expected)) in a 3.8 km radius in the northeast of the City (relative risk = 12.65, $p = 0.0003$) (Fig. 4), indicating that the initial dynamics of GII.4-2010 were highly localized during their introduction period into HCMC.

4. Discussion

There are inadequate data regarding the burden of NoV disease in industrializing countries such as Vietnam; this limits our knowledge of viral distribution, transmission chains and local microevolution. Data on NoV genotype distribution across a range of geographical locations through time is essential for understanding global NoV epidemiology. This is particularly important with respect to the ongoing development and clinical trials of NoV vaccines (Atmar et al., 2011; El-Kamary et al., 2010; Parra et al., 2012), which should be developed in consideration of global and regional strain circulation and their ability to induce cross-protection. Here, by examining the genetic, spatial and temporal dynamics of NoV in children in HCMC, we aimed to assess the local molecular epidemiology of NoV. Our data show a diverse array of NoV genotypes and the emergence of a novel variant. The emergence of the GII.4-2010 and subsequent lack of GII.4-2006b isolates suggest that a rapid strain replacement event may have occurred in the population, although the small numbers of isolates from the latter half of the study preclude strong inference on these dynamics.

Strains belonging to GII are responsible for the vast proportion of human NoV infections worldwide, and GII.4 variants play a particularly important role in pediatric NoV infections (Bull and White, 2011; Lopman et al., 2004; Siebenga et al., 2009). Here, a variety of NoV GII genotypes were found to be co-circulating, with GII.4 predominating. This observation is consistent with work originating in northern Vietnam (Trang et al., 2012) and other locations across Asia (Zeng et al., 2012), and we confirm that GII.4-2006b has continued to circulate in southern Vietnam since it was first detected in 2005 (Nguyen et al., 2008).

The GII.4-2010 variant detected here in December 2009, and first identified in October 2009 in New Orleans (USA) (Vega et al., 2011), was also reported in Belgium (Mathijs et al., 2011) and then internationally (Greening et al., 2012; McAllister et al., 2012; Nguyen and Middaugh, 2012; Puustinen et al., 2011; White et al., 2012), suggesting that this variant is the first globally disseminated strain to emerge since the pandemic GII.4-2006b (Minerva). The phylogenetic analyses demonstrated that the GII.4-2010 strains from the USA, Belgium and Vietnam were closely related, suggesting that these strains may have been introduced into Vietnam from the USA or Europe in 2009. Furthermore, the substitution rate for GII.4 (8.072×10^{-3} substitutions/site/year) estimated here is higher than previously reported (between 3.9×10^{-3} and to 5.3×10^{-3} substitutions/site/year) (Bok et al., 2009; Bull et al., 2010; Siebenga et al., 2010). This new estimate might reflect an increase in the rate of GII.4 evolution involving GII.4-2010 viruses, since Bok et al. (2009), Bull et al. (2010) and Siebenga et al. (2010) would have used a different sequence dataset (i.e. no GII.4-2010 sequences) given that their work was conducted prior to the emergence of GII.4-2010 (Bok et al., 2009; Bull et al., 2010; Siebenga et al., 2010). However, it is important to note that differences in the region of sequence selected for analysis (partial 5′ capsid herein versus complete capsid in previous studies), in addition to the differential method of evolutionary inference (linear regression, strict or relaxed clock, uncorrelated lognormal or exponential model) or the measured unit of time, do not enable an accurate evolutionary

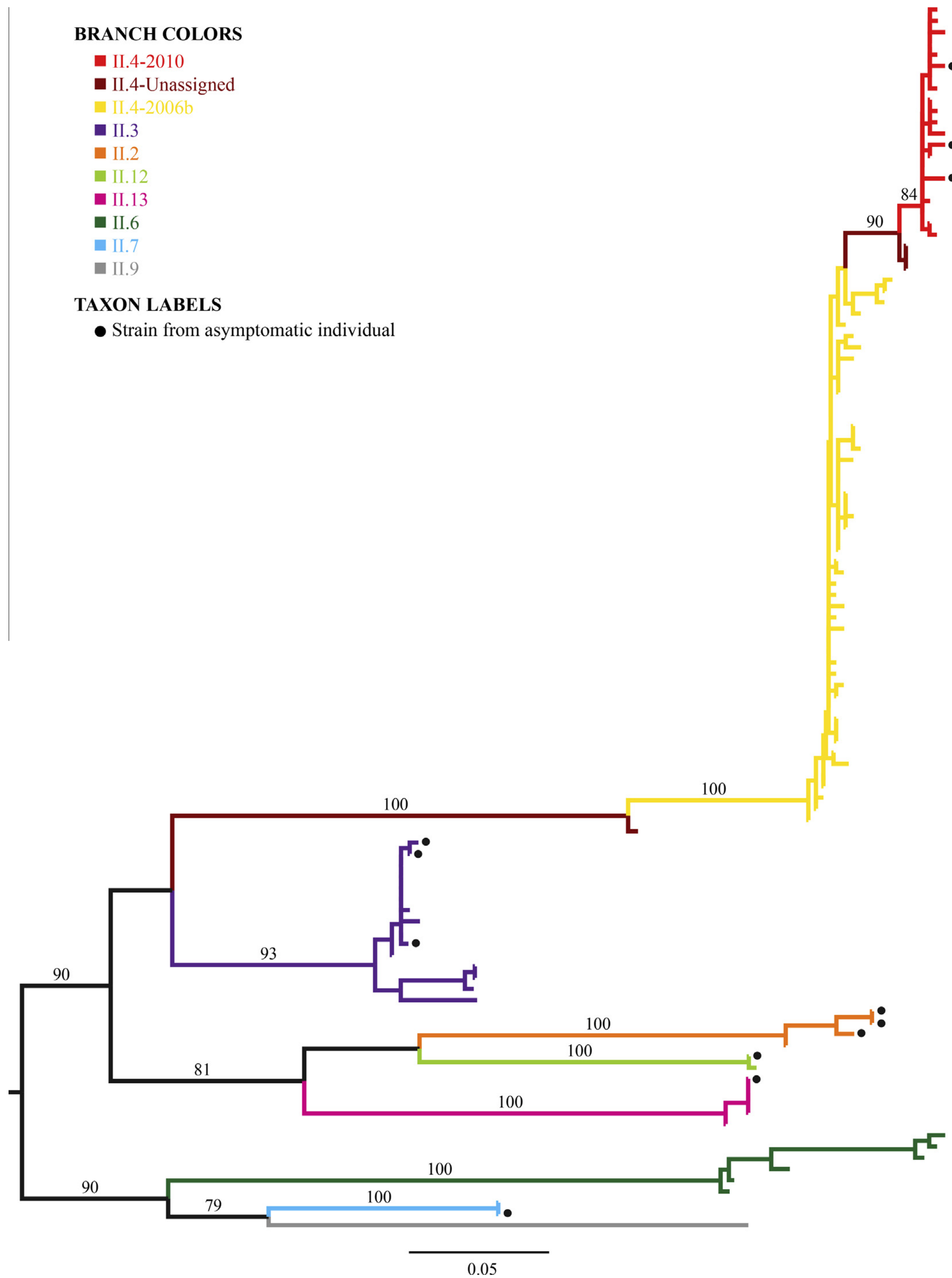

Fig. 2. Phylogenetic tree of 109 NoV GII strains from HCMC. Tree constructed from 109 NoV GII strains collected during this study and based on the GII amplification fragment trimmed to 378 bp. All horizontal branch lengths are drawn to the scale of a nucleotide substitution per site. Tree is mid-point rooted with branches colored according to viral genotypes/variants. Strains from asymptomatic individuals are labeled with a circle. Only bootstrap values of >75 are shown.

comparison between studies. Nevertheless, the phenomenon observed in this study certainly warrants further investigation.

A spatiotemporal signal for GII.4-2010 was detected for several months after it was introduced into HCMC but there was no similar spatiotemporal association for GII.4-2006b and non-GII.4. A potential explanation for the absence (GII.4-2006b and non-GII.4) and presence (GII.4-2010) of spatial signals in the NoV sequences is that GII.4-2006b and non-GII.4 genotypes were in a state of equilibrium when GII.4-2010 was introduced, and the GII.4-2010 exhibited an outbreak dynamic and exponential growth upon introduction. Similar replacement of GII.4-2006b viruses following the introduction of GII.4-2010 has been observed in Belgium (Mathijs et al., 2011), and NoV strain replacement has been observed in various NoV pandemics. These replacements include, GII.4-1997 (USA 95/96 variant), -2002 (Farmington Hills variant), -2004 (Hunter variant), -2006 comprising of GII.4-2006a (Laurens variant) and the -2006b (Minerva variant) (Bull and White, 2011; Donaldson et al., 2008; Lindesmith et al., 2011). Novel pandemic NoV GII.4 strains emerge every two to three years and it appears that novel GII.4 genotypes are capable of replacing existing GII.4

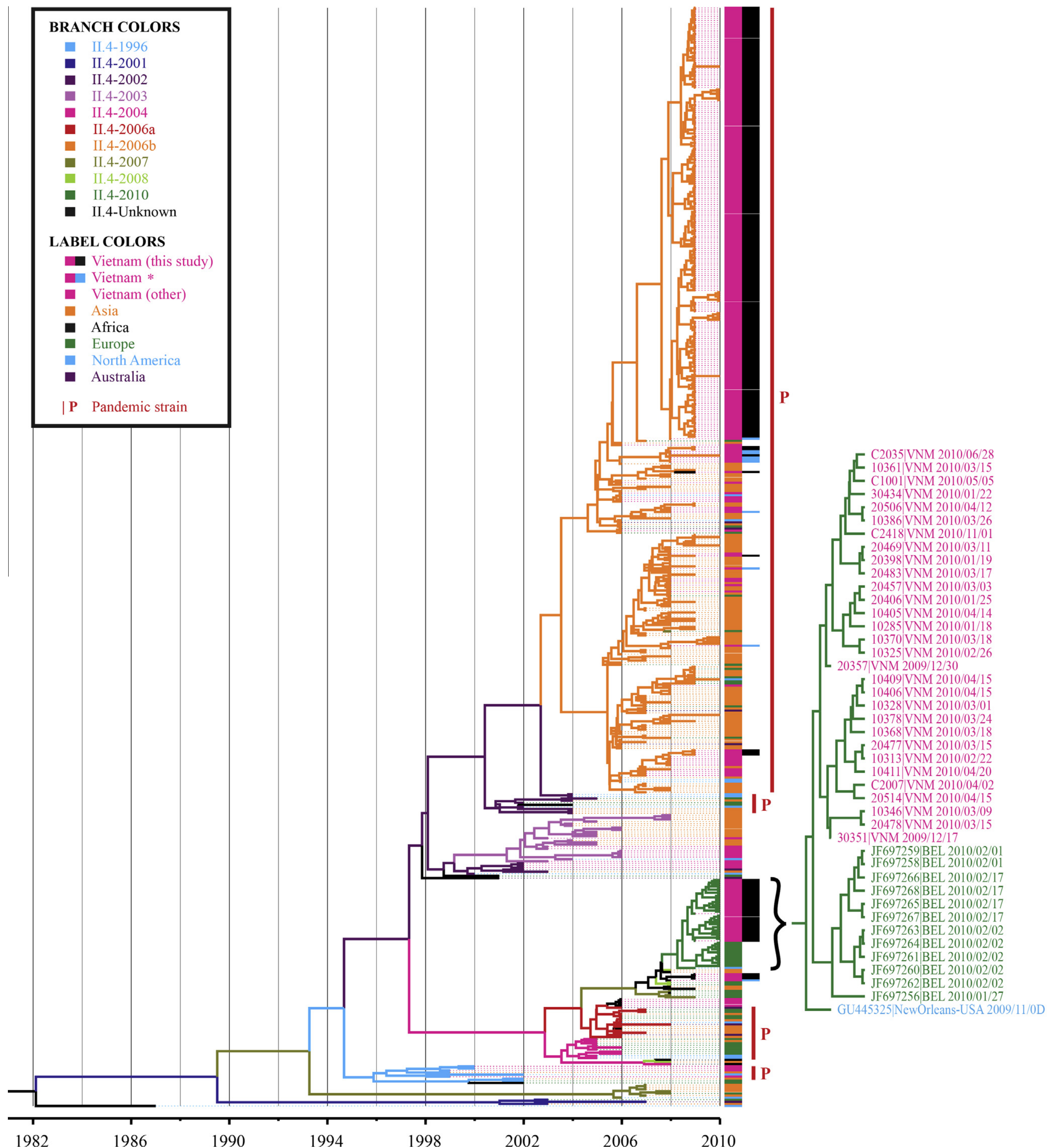

Fig. 3. Phylogenetic tree of GII.4 NoV strains from HCMC and globally representative sequences. Maximum likelihood phylogenetic tree of 526 global and HCMC GII.4 NoV strains constructed from the amplification region trimmed to 378 bp. All horizontal branch lengths are drawn to the scale of a nucleotide substitution per site per year. Branch tips are colored according to the viral genotype and color-coded by their continent of isolation. Vietnamese strains included are from this study ($N = 247$), other studies ("other"; $N = 43$) and from a 2008 study in southern Vietnam ($N = 10$) (Tra My et al., 2011) denoted by (*). The GII.4-2010 clade is magnified to highlight the strains originating from Vietnam, Belgium and America.

variants but not other endemic strains (such as GII.3, GII.6, GII.2) (Bull et al., 2010; Bull and White, 2011; CDC, 2010; Donaldson et al., 2008; Lindesmith et al., 2011; Siebenga et al., 2009). However, there is no current consensus on the underlying mechanism of GII.4 strain emergence and replacement, but may be induced by antigenic drift and herd immunity escape (Debbink et al., 2012; Donaldson et al., 2010; Lindesmith et al., 2012a, 2012c). Recent studies on mapping blockade epitopes in GII.4 VLPs (virus-like particles) have provided evidence that variation in the major neutralizing epitopes facilitate evasion of herd immunity against GII.4-2006b (Minerva), thus facilitating the emergence of GII.4-2010 (New Orleans) (Lindesmith et al., 2012b, 2012c, 2011).

Our study has some limitations; including not tracking the source and/or route of transmission or examining the genotype distribution after the period of investigation. Therefore, it is difficult to determine if the shift in the distribution of the GII.4 variants

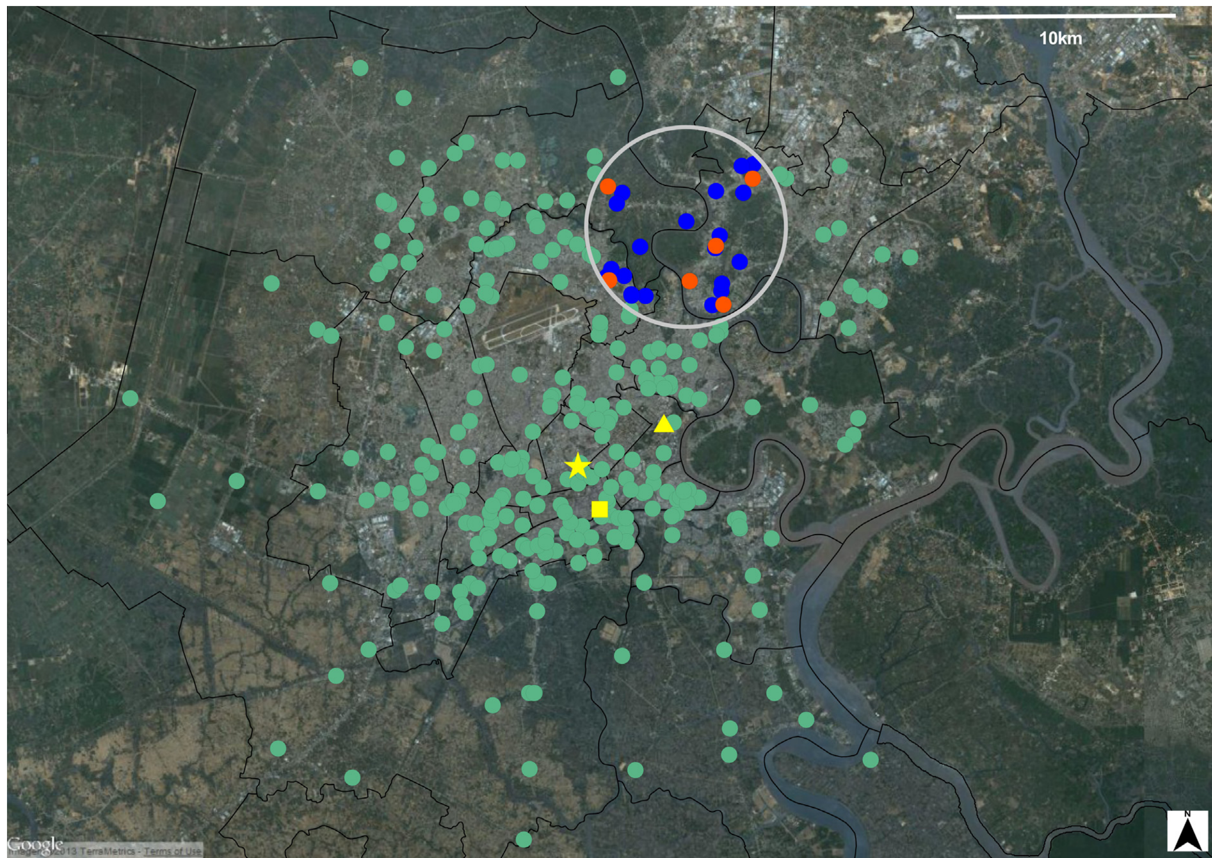

Fig. 4. The spatiotemporal clustering of NoV GII.4-2010 strains. When compared to all other GII strains, GII.4-2010 strains were found to cluster in the northeast of Ho Chi Minh City during March–April 2010. This significant cluster with radius 3.8 km, shown by the black circle, contained 6 GII.4-2010 NoV (0.59 expected, relative risk = 12.65, $p = 0.0003$). Orange dots represent NoV GII.4-2010 strains located within the cluster, blue dots represent the non-GII.4-2010 NoV within the radius of the cluster, and the green dots represent all other NoV strains (GII.4-2010 and non-GII.4-2010) found over the study period that were not found to cluster. The yellow square indicates the location of the Hospital for Tropical Diseases, the star indicates Children's Hospital 1 and the triangle indicates Children's Hospital 2. The thin black lines represent district boundaries within Ho Chi Minh City.

over time is due to an emergent virus becoming fixed in the population, or a local outbreak in the northeast of the city. The short temporal investigation also limits the determination of the magnitude to which the local NoV dynamics observed in HCMC reflect or follow the global evolutionary trend, such that we are unable to determine whether GII.4-2010 viruses continued circulating within this setting after the study period or were capable of diffusing across the country in the presence or absence of GII.4-2006b viruses. The status of population-level immunity to NoV in the population of HCMC is unknown, so we are unsure if exposure to GII.4-2006b NoV is protective against the -2010 variants. These findings highlight a broader scientific issue concerning outstanding questions on immune cross-protection in the space of NoV variants (Lindesmith et al., 2012c, 2011). Furthermore, the analysis was not performed on whole genome sequences and focused on a fragment of the genome, which may restrict the phylogenetic interpretation. Whole genome sequencing would greatly improve the utility of NoV epidemiological datasets, specifically to study the evolution of novel GII.4-2010 variants, aiding the detection of genomic sites that may induce potential antigenic variation. Finally, our hospital-based study design may be influenced by healthcare-seeking behavior, and may not be representative of the NoV in the local community.

5. Conclusions

This study expands the knowledge of NoV in industrializing countries, outlining a range of endemic NoV genotypes over a one-year period in HCMC. The analysis describes the co-circulation of heterogeneous NoV strains, and reports the identification of GII.4-2010 (New Orleans) in Asia, resulting in the replacement of a GII.4-2006b variant by the emergent GII.4-2010 strain. In conclusion, during the period of study, NoV GII-4 infections in HCMC demonstrated a spatiotemporal phylogenetic relationship, driven by the emergence of the GII.4-2010 (New Orleans) variant.

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Conflict of interest

The authors do not have a commercial or other association that might pose a conflict of interest. The funding bodies have no role in the study design, data collection and analysis, and the decision to publish. The authors wish to declare no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.04.014.

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