



**Diarrhoeal disease in children under five years of age
in an urban community in Viet Nam**

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Abstract

Antimicrobial resistance (AMR) in infectious disease is a major global health issue. AMR is a particular problem in low to middle-income countries (LMICs), where infectious diseases, such as diarrhoea, remain common and are commonly treated haphazardly with antimicrobials. The aim of my thesis was to provide a comprehensive study of diarrhoeal disease in children under the age of five years in the community in Ho Chi Minh City (HCMC), Viet Nam. Additionally, I aimed to fill in a knowledge gap regarding antimicrobial usage for diarrhoea, the extent of AMR organisms in healthy children within the population, and investigate a potential solution for limiting AMR in the community. Active surveillance for diarrhoea in a prospective longitudinal cohort of 748 children followed for 24 months found a high incidence of diarrhoeal disease in the urban community (71.87/100 child-years of observation [95%CI: 65.68 -78.44]). Faecal samples were screened using conventional microbiology and multiplex molecular methods and found that the aetiology of this disease is highly variable. Pathogenic bacteria and viruses were detected at a high prevalence, but the most common were *Salmonella* and norovirus, respectively. Antimicrobials were commonly used for diarrhoeal treatment in hospital. Additionally, a mixed-methods approach in the community found an ease of access to antimicrobials for diarrhoeal management in the community through local pharmacies. However, despite antimicrobials being widely available in the community, antimicrobial

usage was almost 10 times greater in the hospitals. Widespread antimicrobial access may induce the selection of antimicrobial resistant organisms (AROs) and antimicrobial resistance genes (ARGs). An assessment of the prevalence of and risk factors for carrying AROs and ARGs demonstrated a high prevalence of carriage antimicrobial resistant *Escherichia coli* (*E. coli*) and associated resistance genes to fluoroquinolones and third generation cephalosporins. Both these groups are commonly used to treat diarrhoea in this setting. Children of higher body weight and height z-score were more likely to carry fluoroquinolones resistance genes and younger children were more likely to carry either third generation cephalosporin resistance genes individually or in combination with fluoroquinolones resistance genes. Lastly, hypothesising that improved breastfeeding practices may reduce diarrhoeal disease and impact antimicrobial usage and consequently AMR, I investigated risk factors for suboptimal breastfeeding using data from a birth cohort conducted in both an urban and a semi-rural area. The study indicated that having a Caesarean section and neonatal complications prevented mothers from breastfeeding their infants during the hospital stay. Work from this thesis will contribute to the efforts of tackling AMR in a LMIC, specifically in Viet Nam where there is extensive exposure to antimicrobials and sustained exposure to enteric pathogens.

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Declaration

I, Le Thi Quynh Nhi declare that the majority of the work presented in this thesis is my own and was conducted by myself under the supervision of Professor Dr Stephen Baker at the Oxford University Clinical Research Unit (OUCRU) Viet Nam. I was responsible for establishing and conducting the described projects (except the birth cohort in Chapter 6). Additionally, I conducted the majority of the data management and data analysis presented in this thesis. Other co-authors in published papers related to this thesis were researchers at OUCRU and staff at collaborating hospitals; their roles are outlined in the following table. This thesis has not been submitted for a degree or other qualification to this or any other university

Table 1. Contributions from OUCRU and collaborating hospital staff in my thesis

Chapter	Contributors	Role
Chapter 3 (Diarrhoeal cohort)	Dr. Corinne Thompson (OUCRU)	Designed the study
	Study nurses at HVH and HTD	Recruited participants and conducted the questionnaire and sample taken
	Ms. Tran Do Hoang Nhu (OUCRU)	Conducted sample processing and storage
	Ms. Ha Thanh Tuyen Mr. Voong Vinh Phat	Conducted the conventional microbiology and pathogen detection using Luminex
	Dr Phung Khanh Lam	Provided statistical advice

	(OUCRU) Dr Ronald Geskus (OUCRU)	for the calculation of incidences
Chapter 4	Research assistants (HCMC UMP)	Conducted community interviews and mapping
	Dr Phung Khanh Lam (OUCRU)	Provided statistical advice for analysis plan development and data analysis
Chapter 5	Ms. Ha Thanh Tuyen (OUCRU) Mr. Pham Duc Trung (OUCRU)	Conducted <i>E. coli</i> culture and PCR for resistance genes
	Dr Phung Khanh Lam (OUCRU)	Provided statistical advice for analysis plan development and data analysis
Chapter 6 (Birth cohort)	Dengue group (OUCRU)	Designed and conducted the study
	Dr Phung Khanh Lam (OUCRU)	Provided statistical advice for analysis plan development and data analysis

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Abbreviations

AMR	Antimicrobial Resistance
ARG	AMR Genes
ARO	AMR Organism
BHI	Brain Heart Infusion
CI	Confidence Intervals
CLSI	Clinical and Laboratory Standards Institute
CRF	Case Report Form
DALYs	Disability-Adjusted Life Years
DNA	Deoxyribose Nucleic Acid
<i>E. coli</i>	<i>Escherichia coli</i>
EAEC	Enteraggregative <i>E. coli</i>
EHEC	Enterohemorrhagic <i>E. coli</i>
EIEC	Enteroevasive <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
ESBL-E	ESBL Enterobacteriaceae
ESBLs	Extended-spectrum β -lactamases
ETEC	Enterotoxigenic <i>E. coli</i>
GADM	Global Administrative Divisions Map
GBD	Global Burden of Disease Study
GEMS	Global Enteric Multi-Center Study
GPP	Gastrointestinal Pathogen Panel
GPS	Global Positioning System

HCMC	Ho Chi Minh City
HCMC UMP	Ho Chi Minh City University of Medicine and Pharmacy
HTD	Hospital for Tropical Diseases
HVH	Hung Vuong Obstetric Hospital
IRB	Institutional Review Board
ICF	Informed Consent Form
LMIC	Low and Middle Income Country
MAL-ED	Interactions of Malnutrition and Enteric Infections: Consequences for Child Health and Development
MCA	MacConkey Agar
MICs	Minimum Inhibitory Concentrations
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
OR	Odds Ratios
ORS	Oral Rehydration Solution
OUCRU	Oxford University Clinical Research Unit
OxTREC	Oxford Tropical Research Ethics Committee
PCR	Polymerase Chain Reaction
PMQR	Plasmid Mediated Quinolone Resistant
SES	Social Economic Status
SMS	Short Message Service (telephone message)
STEC	Shiga toxin-producing <i>E. coli</i>
ST-ETEC	Enterotoxigenic <i>E. coli</i>
ST-ETEC	Enterotoxigenic <i>E. coli</i>

WHO	World Health Organization
XLD	Xylose-Lysine-Deoxycholate Agar
ZN	Ziehl–Neelsen

Publications during DPhil candidature

1. **Nhi LTQ**, Alwis R de, Lam PK, Hoa NN, Nhan NM, Oanh LTT, Nam DT, Tuyen DT, Vuong VT, Vi LL, Tien BTT, Tuyet HTD, Nha LH, Thwaites GE, Dung DV, Baker S. Quantifying antimicrobial access and usage for paediatric diarrhoeal disease in an urban community setting in Asia. *Journal of Antimicrobial Chemotherapy* (Online). (Epub ahead of print):1–12. 2018 July
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3. Duong VT, Tuyen HT, Minh PV, Campbell JI, Phuc HL, Nhu TDH, Tu LTP, Chau TTH, **Nhi LTQ**, Hung NT, Ngoc NM, Huong NTTH, Vi LL, Thompson CN, Thwaites GE, Alwis R de, Baker S. No clinical benefit of empirical antimicrobial therapy for pediatric diarrhoea in a high usage, high resistance setting. *Clinical Infectious Diseases* (Online). 66(4):504–11. 2017
4. **Nhi LTQ**; Tuyen HT; Trung PD; Nhu TDH; Duy PT; Hao CT; Nhan NTT; Vi LL; Tuyet HTD; Tien BTT; Chau NVV; Lam PK; Thwaites G; Baker S. Excess body weight and age associated with the carriage of fluoroquinolone and third generation cephalosporin resistance genes in commensal *Escherichia coli* from a cohort of urban Vietnamese children. Accepted manuscript at *Journal of Medical Microbiology*. 2018 July
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1. Introduction

Located in Southeast Asia, Viet Nam is an industrializing country with a population of more than 90 million people in 2017, which ranks the country the fifteenth most populous nation worldwide. The population is estimated to be more than 100 million by 2020¹.

HCMC is the largest city and the major financial centre in the south of Viet Nam and is a typical urban and modernizing Asia megacity. With an estimated population of more than 8 million people in 2017, HCMC has a high burden of infectious disease combined with large populations of AMR pathogens.

Improving knowledge on how antimicrobial exposure affects the persistence of AMR bacteria is essential. Little is known about the burden of paediatric diarrhoeal disease in the community as well as the usage of antimicrobials for this common disease among children under the age of five years in Viet Nam, in which antimicrobial exposure may have an effect on the persistence of AMR. My thesis therefore focuses on AMR and diarrhoeal disease **in the community** with high antimicrobial exposure in HCMC, Viet Nam. I will investigate the burden and age-specific incidence of diarrhoeal disease in children under the age of five years in the community in HCMC; the management of diarrhoea in the community, including antimicrobial usage for treatment; the problem of AROs and ARGs among healthy children; and lastly explore a hypothesis of a plausible solution aiming to reduce the disease burden in the community.

This work aims to contribute to the effort of understanding AMR in a LMIC where there is extensive exposure to infectious pathogens and antimicrobials. In this introductory chapter, I will provide an overview discussion on the following issues: diarrhoeal disease,

antimicrobials and AMR, roles of breastfeeding in gastrointestinal diseases, and my research questions and objectives.

1.1 Diarrhoeal disease

1.1.1 Global burden of diarrhoeal disease

Diarrhoeal disease in young children remains a major global health issue due to its role in mortality and morbidity²⁻⁴. Diarrhoeal disease is responsible for 700,000 deaths annually⁵, which accounted for 10% of deaths globally in 2010⁶, and accounted for more than 1.7 billion disease episodes in 2010⁵. Diarrhoea is still among the top leading causes of death, and, in particular, it is the second most common cause of mortality among children aged 1 to 59 months⁴. In addition, disease burden among children in some developing regions still remains high, particularly in Africa and Asia^{4,7}. Moreover, AMR among enteric bacteria is increasing, resulting in problems treating the infections⁸⁻¹⁰. The burden of diarrhoeal disease does not only impact mortality but also reduces growth, cognitive development, and vaccine efficacy¹¹.

Generally, the burden of diarrhoeal disease and mortality rate attributable to diarrhoea has decreased in recent years; however, this trend is largely dependent on location¹². By the early 2000s, children aged under five years in LMICs had a median of 3.2 diarrhoeal episodes annually¹³. In 2010, children in LMICs suffered an average of 2.9 diarrhoeal episodes per year¹². In Sub-Saharan Africa, diarrhoea incidence over this same period was two to three episode annually¹⁴. The peak age of diarrhoea during infancy is from 6 to 11 months of age¹² and most diarrhoea deaths occur in the first two years of life¹⁵.

Encouragingly, paediatric diarrhoea deaths decreased to 499,000 in 2015¹⁶, and there was a

54% reduction in deaths due to diarrhoea in under-fives between 2000-2013¹⁷, which accounted for 8.5 % of total causes of deaths in children in 2015¹⁸.

1.1.2 Definitions

Diarrhoeal disease is a common disease among both adults and children. It can cause death by depleting bodily fluids and consequently cause profound dehydration. Diarrhoeal disease is typically defined as the passage of loose stool or watery stool at least three times within 24 hours¹⁹ or the passage of bloody or mucus stool at least once within 24 hours²⁰. Diarrhoeal diseases are classified into four clinical types: acute watery, acute bloody, persistent, and diarrhoea with severe malnutrition¹⁹. Diarrhoea severity is determined by the presence of blood in stool (commonly known as dysentery), prolonged persistent diarrhoea, and the coexistence of malnutrition¹⁹.

1.1.3 Diarrhoeal pathogens

Currently, there are limitations in the microbiological and molecular diagnosis of diarrhoea, consequently it is estimated that 30–40% of hospitalized diarrhoeal episodes do not have an associated pathogen²¹. However, multiple pathogens can cause diarrhoea and the etiological agents are broad (viruses, bacteria, and parasites) and their prevalence varies by geographical region and patient age. It is worth noting that certain enteric pathogens can cause mild or asymptomatic infections and asymptomatic carriage may be prolonged among children. Therefore, the identification of pathogens from a diarrhoeal stool specimen does not indicate the cause of illness in all cases²². In developed regions, diarrhoea is most commonly associated with viral pathogens, while bacterial and parasitic pathogens are more commonly identified in developing regions²³. Recent studies show that

bacterial and viral pathogens are responsible for most episodes of diarrhoeal disease in LMICs, specifically in locations with poor sanitation ²⁴.

The leading causes of diarrhoea deaths among children aged under five years, estimated from the Global Burden of Disease Study (GBD), were rotavirus, *Cryptosporidium*, and *Shigella*^{15,16}. The number of deaths in this age group due to these pathogens in 2015 were 146,500, 60,400, and 54,900, respectively²⁵. Other common pathogens include adenoviruses with 46,000 deaths, which accounted for 10% of deaths due to diarrhoea in children less than five years old in 2015²⁵. In contrast, pathogens that are responsible for the majority of cases of diarrhoea in developed countries are (in the USA in 2002) *Salmonella*, followed by *Campylobacter*, *Shigella*, *E. coli* O157:H7, and *Cryptosporidium*²⁶.

The Global Enteric Multi-Centre Study (GEMS), a case-controlled study of 22,568 children in Africa and Asia, determined that the most common diarrhoea aetiologies in these regions were rotavirus, *Cryptosporidium*, *Shigella* spp., and heat-stable toxin producing enterotoxigenic *E. coli* (ST-ETEC)¹⁵. They found that the most common cause of diarrhoea among children under two years was rotavirus, while *Shigella* was the primary cause among 2–5 year olds¹⁵. Depending on the geographical area, other pathogens included *Vibrio cholerae*, *Campylobacter jejuni*, adenovirus 40/41, and *Aeromonas* spp.¹⁵.

The Interactions of Malnutrition and Enteric Infections: Consequences for Child Health and Development (MAL-ED) study, a birth cohort of 2,145 children in South America, Africa, and Asia conducted over 24 months, found that the highest attributable causes of diarrhoea

in the first year of life were norovirus GII, rotavirus, *Campylobacter* spp., astrovirus, and *Cryptosporidium* spp.²⁷. In the second year of life, the most commonly identified pathogens were *Campylobacter* spp., norovirus GII, rotavirus, astrovirus, and *Shigella* spp.²⁷.

1.1.3.1 Viral diarrhoeal disease

More than half of all gastroenteritis cases globally are caused by viruses²⁸. Rotavirus and norovirus are together attributable for an estimated 40% of severe diarrhoea in children in LMICs²⁹. Rotavirus accounts for a large proportion of hospitalized cases of diarrhoea in LMICs (25%)³⁰. Up to 2013 rotavirus was the leading cause of diarrhoeal death in <5 year olds globally²¹, and in 2017 it was among the top three causes of diarrhoea deaths¹⁶.

Rotavirus is the leading cause of diarrhoea in Africa and Asia in the age groups of 0–11 and 12–23 months; it is the second most common cause of diarrhoea in the age group of 24–59 months¹⁵. Other prevalent viruses include norovirus, adenovirus, astrovirus, caliciviruses, and sapovirus^{31,32}.

1.1.3.2 Bacterial diarrhoeal disease

Bacterial infections are likely to contribute significantly to diarrhoeal diseases in developing areas²². The most common bacterial pathogens causing diarrhoea in young children are *Shigella* spp., non-typhoidal *Salmonella* spp., *Campylobacter* spp., and diarrhoeagenic *E. coli*²⁸. Bacteria cause clinical diarrhoeal disease by two primary mechanisms including toxin production and invasion of intestinal mucosa²². The production of toxins can induce vomiting and abdominal cramps within a few hours after infection. In addition, in non-inflammatory infections, the secretion of toxins after adhering to enteric epithelium can cause symptoms of diarrhoea, without blood, mucous, or

associated fever. Furthermore, in inflammatory infections, invasion of the intestinal mucosa can cause the passage of stool with blood and mucous, and can be associated with fever, lower abdominal pain, and tenemus³³.

1.1.3.2.1 Enterobacteriaceae family

The Enterobacteriaceae are a bacterial family comprised of Gram-negative bacilli of 176 species that are associated with common diseases in humans³⁴. This family of bacteria largely inhabit the gastrointestinal tract of animals, and contain organisms such as *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*, and also non-coliform organisms such as *Potex* and *Edwardsiella*. Pathogens belonging to this bacterial family are commonly transmitted faecal-orally³⁵. These bacteria are globally ubiquitous and can be commonly found in soil, water, and the environment. Broad-spectrum antimicrobials used to treat infections caused by Enterobacteriaceae include cephalosporins and fluoroquinolones. However, there is increasing resistance of these organisms to these antimicrobials³⁶.

1.1.3.2.2 *Shigella*

Shigella is a genus of Gram-negative bacteria belonging to Enterobacteriaceae, and is comprised of four species: *S. flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii*³⁷. Shigellosis, the disease caused by infection with *Shigella*, was among the most common causes of diarrhoea in the early 2000s with >163 million infections per year globally³⁸. Historically, the most important cause of shigellosis was *S. flexneri*, which is dominant in developing countries^{39,40}, while *S. sonnei* predominated in developed countries^{41–43}. However, recent changes have occurred in the epidemiology of *Shigella* resulting in *S. sonnei* becoming disproportionately distributed as the primary agent of shigellosis in many locations^{40,44,45}. *S.*

dysenteriae serogroup 1 expresses the highly virulent Shiga-toxin and was the main cause of severe shigellosis epidemics in developing countries³³. *Shigella* transmission occurs through direct contact with an infected person or through exposure to contaminated food and drink⁴⁶. Infections with *Shigella* start with a two to three day incubation period³³. Common symptoms include abdominal pain, fever, malaise, and anorexia. Diarrhoea is usually characterised by watery, progressing to bloody, mucous stool with tenesmus. These symptoms can persist for up to 14 days.

It is estimated that of 163 million episodes of shigellosis globally, >99% occur in LMICs. Children aged under five years are the worst affected, comprising 69% of episodes and 60% of deaths³⁸. Correspondingly, in developed countries there are approximately 1.5 million cases of shigellosis annually³⁸. Deaths due to shigellosis have been decreasing over time with an estimated 28,000 *Shigella*-attributable deaths in 2013²¹ compared with 667,695 in 1995³⁸. Notably, the peak incidence of *Shigella* in children tends to be older than other bacterial pathogens at 12 to 47 months of age^{38,40,47}.

Similar to diarrhoeal diseases caused by other pathogens, hygiene and sanitation is recommended for the prevention of *Shigella* infections. Such measures include hand-washing with soap, safe drinking water, safe food handling and processing, controlling flies, safe human waste disposal, and breastfeeding for infants and young children⁴⁶.

Antimicrobials, such as ciprofloxacin as a first line therapy, ceftriaxone, pivmecillinam or azithromycin as a second line therapy, are recommended for bloody diarrhoea⁴⁶, while azithromycin is preferred in young children and in pregnancy³³. However, antimicrobials are not recommended for prevention as a chemoprophylaxis⁴⁶.

1.1.3.2.3 *Campylobacter*

Campylobacter is a genus of atypical Gram-negative bacteria that have a curved or rod-shaped morphology⁴⁸. This genus includes 17 species⁴⁹, and of these, the most common are *C. jejuni* and *C. coli*, which are associated with the majority (99%) of human cases^{50,51}.

Campylobacteriosis is often self-limiting but can occasionally last for several weeks.

Depending on host susceptibility and the virulence of the species, clinical symptoms of the infection can be diverse. Incubation time is between one to six days and symptoms include acute diarrhoea with loose or watery diarrhoea and/or bloody diarrhoea, in addition to cramps and fever⁵². White blood and red blood cells can commonly be observed in the stool sample⁵³. Complications of *Campylobacter* infection include bacteraemia and osteomyelitis; Guillain-Barre syndrome⁵⁴, myocarditis, and reactive arthritis are among common sequelae of *C. jejuni* infections^{51,55}.

Campylobacter infections in developing countries are underestimated and there is considerable evidence that AMR in *Campylobacter* isolates is increasing. Despite being poorly studied, *Campylobacter* is among the most common cause of bacterial gastroenteritis worldwide. In 2010, it was estimated that *Campylobacter* infections could be attributed to 7.5 million disability-adjusted life years (DALYs) per year. In comparison, the equivalent DALYs for *Shigella* and ETEC were 7.1 and 6.9 million, respectively⁵⁶. Despite a lack of epidemiological data regarding *Campylobacter* disease in Asia, a high prevalence of *Campylobacter* infection has been recorded (4.84% of 3,061 diarrhoeal patients) and an incidence of 1,512/100,000 population per year has been recorded in Japan⁵⁷.

Campylobacter tends to be a more common cause of disease among children rather than adults⁵⁸. The peak age of *Campylobacter* infections in LMICs is <1 year of age, and is more common in males than females⁵⁹. The infection is hyperendemic among children <2 years of age in LMICs and has a low mortality risk. *Campylobacter* is commonly associated with the consumption of contaminated poultry, which is the most important reservoir for infections in humans⁴⁸. To prevent *Campylobacter* infection, it is important to limit faecal-oral transmission and reduce the likely contamination of food—especially chicken—and drinking water. Most *Campylobacter* infections do not require antimicrobials. However, in cases that are severe or persistent, fluoroquinolones, macrolides, and aminoglycosides are preferred⁵⁷.

1.1.3.2.4 *Salmonella*

Salmonella is a large genus of Gram-negative bacteria belonging to the Enterobacteriaceae family; these organisms include more than 2,000 serotypes. Most clinical *Salmonella* infections were caused by *Salmonella enterica* in which the *Salmonella enterica* subspecies *Enterica* (I) contributes to the majority (99%) of cases⁶⁰. Clinical onset includes nausea, vomiting, and watery diarrhoea, in addition to fever (70% of cases)²². Invasive salmonellosis can cause enteric fever (including typhoid and paratyphoid fever) and non-typhoidal *Salmonella* infections (NTS)⁶¹. Enteric fever is usually associated with *Salmonella* serotype Typhi, causing typhoid fever, or serotype Paratyphi A, B or C, causing paratyphoid fever. Typhoid fever, which is typical with fever lasting for more than 3 weeks and is not uncommonly present with diarrhoea, is most often identified among school-age children and younger²². Globally, typhoid fever is estimated to cause 25 million illnesses annually⁶¹. Other NTS serovars that typically cause self-limiting diarrhoea can also cause

systemic infection, usually called invasive NTS (iNTS). iNTS infections are estimated to cause 93.8 million cases of gastroenteritis worldwide each year⁶². Both enteric fever and invasive NTS disease are major public health problems among children in developing countries, especially in Africa⁶¹. Risk factors for *Salmonella* infections can be genetic (the HLA-DRB1*0405 gene causes a 5-fold decrease in typhoid fever risk)⁶¹, sociocultural, environmental (low levels of water and sanitation facilities and low elevations), and behavioural^{63,64}.

1.1.3.2.5 *E. coli*

E. coli is a large genus of Gram-negative bacteria, and are present in all mammals⁶⁵. *E. coli* is a member of the normal gastrointestinal microbiota, but can also be a common cause of intestinal and extra-intestinal infections. *E. coli* is a lifelong colonizer of the human gastrointestinal tract, and more than 1,000 different species of bacteria have been described in the human gut⁶⁵. Although *E. coli* are ubiquitous in the human gastrointestinal tract, they can also be opportunistic pathogens and cause community and hospital acquired urinary tract infections, bloodstream infections, neonatal meningitis, and foodborne infections⁸. Pathogenic variants of *E. coli* associated with diarrhoeal disease are called diarrhoeagenic *E. coli*, and include Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroevasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC), and Enteroaggregative *E. coli* (EAEC).

E. coli is abundant in human faeces and is widely used as a marker organism to study AMR^{66–69}. *E. coli* develop resistance in response to antimicrobials and they can exchange

ARG with other pathogenic bacteria^{70,71}. Therefore, commensal *E. coli* play important roles as reservoirs for transmissible ARG^{72–74}.

1.1.4 Diarrhoeal treatment and prevention

Diarrhoea is usually a self-limiting disease; however, the most severe form of diarrhoea can cause death through fluid loss and dehydration²⁴. The WHO recommends the use of oral rehydration solution (ORS) and zinc supplementation for at least 10 days for the treatment of diarrhoea¹⁹. ORS and other fluids can be used at home to prevent dehydration, which itself is caused by the loss of water and electrolytes (sodium, chloride, potassium, and bicarbonate) through loose stool. Clinically, the severity of diarrhoeal disease and treatment therapy depends on the extent of dehydration, bloody diarrhoea, persistent diarrhoea, malnutrition, and serious non-intestinal complications. It is not recommended to use antimicrobials routinely for diarrhoea except for suspected cholera or acute bloody diarrhoea (dysentery)¹⁹. For acute bloody diarrhoea, ciprofloxacin is recommended for a period of three days, or alternative antimicrobials for which *Shigella* remain susceptible may also be used. Other drugs are commonly used in practice to treat diarrhoea, but generally are not indicated in paediatric diarrhoea. These include¹⁹ “antidiarrheal drugs”; adsorbents (e.g. kaolin, attapulgate, smectite, activated charcoal, and cholestyramine); antimotility drugs (e.g. loperamide hydrochloride, diphenoxylate with atropine, tincture of opium, camphorated tincture of opium, paregoric, codeine); Bismuth subsalicylate; combinations of drugs (adsorbents, antimicrobials, antimotility drugs or other agents); antiemetics (e.g. prochlorperazine, chlorpromazine); cardiac stimulants (e.g. adrenaline, nicotinamide); blood or plasma; steroids; and purgatives.

Regarding diarrhoea prevention, public health interventions related to nutrition and hygiene are recommended⁷⁵. These interventions include nutritional rehabilitation, zinc supplementation, exclusive breastfeeding, and WASH (Water, Sanitation and Hygiene) strategies⁷⁵. A large body of evidence has shown that improvement in these factors could reduce both fatality cases due to diarrhoea²⁴ and morbidity attributable to diarrhoea^{76,77}. Improvements in hygiene, sanitation and nutrition have been well documented in developed countries. In Viet Nam, significant improvements have been implemented in drinking water sources and sanitation facilities, including household toilets⁷⁸.

1.2 Antimicrobials

Antimicrobials are frequently used as treatment for diarrhoea in LMICs such as Bangladesh (34.3%), Viet Nam (36.1%), Thailand (45%)⁷⁹ and India (77.9%)⁸⁰; however, it is difficult to quantify how antimicrobials are actually used, especially in the community⁸¹. Moreover, little has been reported regarding antimicrobials used for diarrhoeal treatment in urban communities in Viet Nam. Therefore, in this thesis, I aimed to quantify the annual antimicrobial usage in the community and assess the access to antimicrobials through community pharmacies in the study area. Results from these investigations are presented in chapter 4. In this section I describe an overview of antimicrobials.

1.2.1 Definition of antimicrobials

Antimicrobials are substances of natural, semisynthetic, or synthetic origin that are used in modern medicine to selectively kill or inhibit the growth of microorganisms for the prevention or treatment of infections. Antimicrobials—from the latin meaning against (anti) little (mikros) life (bios)—includes all antibiotics. Substances that are synthetic, such

as sulphonamides and quinolones, and semisynthetic, such as methicillin and amoxicillin, are not included in the term “antibiotics”. Antibiotics (a term that was first used in 1942⁸²), such as penicillin, refers to naturally produced chemicals that are able to destroy or weaken certain microorganisms. In Viet Nam, the words “kháng sinh” or “trụ sinh” are commonly used for drugs with the efficacy to kill or inhibit bacteria, viruses, and fungi. The mechanism of antimicrobials can be divided into bactericidal (kill target organisms), bacteriostatic (inhibit or delay bacterial growth and replication), or both depending on the dose, the duration of exposure, and the state of invading bacteria. Antimicrobials kill or inhibit the growth of microorganisms by five major mechanisms: (1) inhibiting cell wall synthesis, (2) disrupting the bacterial membrane, (3) inhibiting protein synthesis, (4) inhibiting nucleic acid synthesis, and (5) inhibiting metabolic pathways⁸³. Figure 1.1 depicts these antimicrobial mechanisms. Table 1.1 shows a description of the most common classes of antibacterials based on the time they were first isolated.

Table 1.1 Antimicrobial classification based on the year of isolation.

Class	Antimicrobial agents	Producing organisms	Year of isolation
β -lactam antibiotics	Natural penicillins	Penicillium notatum	1920, 1940
	Cephalosporin C	P. chrysogenum	1945–53
		Cephalosporium acremonium	1976
		Streptomyces cattleya	1981
		Clostridium spp.	
		Chromobacterium violaceum	
Glycopeptides	Vancomycin	Amycolopsis orientalis	Mid 1950s, 1975
	Teicoplanin, avoparcin	Amycolopsis coloradensis subsp labeda	
Macrolides	Erythromycin	Streptomyces erythraeus	1952
	Spramycin	Streptomyces ambofaciens	1955
Lincosamides	Lincomycin	Streptomyces lincolnensis	1963
Streptogramins	Streptogramins A and B	Streptomyces diastaticus	1953
	Virginamycins A and B	Streptomyces	1955
		virginiae	1948
Tetracyclines	Chlortetracycline	Streptomyces aureofaciens	1948
	Oxytetracycline	Streptomyces rimosus	

Class	Antimicrobial agents	Producing organisms	Year of isolation
Phenicol	Chloramphenicol	Streptomyces venezuelae	1947
Aminoglycosides	Streptomycin	Streptomyces griseus	1943
	Neomycin	Streptomyces fradiae	1949
	Kanamycin	Streptomyces kanamyceticus	1957
	Gentamicin	Micromonospora purpurea	1963
	Tobramycin	Streptomyces tenebrarius	1967
Aminocyclitols	Spectinomycin	Streptomyces spectabilis	1961
Pleuromutilins	Tiamulin	Pleurotus spp.	
Polypeptide antibiotics	Polymixin B	Bacillus polymyxa (aerosporus)	1947
	Polymixin E (Colistin)	Bacillus polymyxa subsp colistinus	1949
	Bacitracin	Bacillus licheniformis	1943
Sulfonamides	Prontosil	Synthetic	1935
	Sulfamethoxazole		
Trimethoprim	Trimethoprim	Synthetic	1956
Quinolones	Nalidixic acid	Synthetic	1962
Fluoroquinolones	Ciprofloxacin	Synthetic	1973
	Levofloxacin		
Oxazolidones	Linezolid	Synthetic	1987, 1996

1.2.2 Fluoroquinolones

I observed that antimicrobials were commonly prescribed for diarrhoea cohort I was conducting in 2014. However, antimicrobials are not recommended in the routine treatment of diarrhoeal disease⁸⁴. In fact, fluoroquinolones are frequently used for bloody diarrhoea⁸⁵, even though there remains many concerns about their side effects in children⁸⁴. Currently, increasing resistance to fluoroquinolones is a problem in cases of diarrhoea caused by many different diarrhoeal pathogens³⁶. Therefore, an understanding of fluoroquinolones usage in the community and fluoroquinolones resistance in organisms colonising healthy children is essential in tackling the issue of AMR to this type of antimicrobial. In chapter 4 I describe the ease of access to fluoroquinolones in the urban area in Viet Nam. In addition, I investigate the problem of AROs and ARGs to fluoroquinolones in chapter 5.

Fluoroquinolones are a subgroup of quinolones, which are a family of synthetic broad-spectrum antimicrobials. Fluoroquinolones are very potent drugs and have effects on both Gram-negative and Gram-positive bacteria⁸⁶. Currently, there are four generations of (fluoro)quinolones, and which have been structurally modified to increase their coverage and potency when treating bacterial infections (Table 1.2)⁸⁷. The first generation of quinolones was introduced in 1962, and nalidixic acid was introduced into clinical usage in 1964 and was the first quinolone used for diarrhoea and urinary tract infections⁸⁶. The second generation of quinolones included the addition of a fluoro group to generate fluoroquinolones. This generation included ciprofloxacin and ofloxacin, which are still among the most commonly used antimicrobials today. When first used, these chemicals had increased activity against Gram-negative bacteria. The third generation of quinolones

contained additional components to the quinolone structure to increase activity against Gram-positive and anaerobic bacteria. Quinolones bind to specific targets and arrest bacterial deoxyribose nucleic acid (DNA) replication, which induces cell death.

Fluoroquinolones prevent bacterial replication by targeting functional effects of essential enzymes in bacterial DNA processes, namely the type I and type II topoisomerases.

Fluoroquinolones interact with DNA gyrases and other type II topoisomerases, and block the breaking and re-joining of DNA strands, subsequently impacting the transcription and replication of bacterial DNA.

Table 1.2 Quinolones classification with some common drugs in groups.

Quinolones generation	Agents
First generation	Nalidixic acid
	Cinoxacin
Second generation (2a)	Norfloxacin
	Pefloxacin
	Lomefloxacin
	Ciprofloxacin
	Ofloxacin Levofloxacin
Second generation (2b)	Sparfloxacin
	Grepafloxacin
Third generation (3a)	Gatifloxacin
	Trovafloxacin
	Moxifloxacin
Third generation (3b)	Gemifloxacin
Fourth generation	Garenoxacin

1.2.3 Third generation cephalosporins

Similar to fluoroquinolones, third generation cephalosporins are common antimicrobials used in paediatric diarrhoea and other infectious diseases^{19,46,85}. Importantly, increasing resistance to many empirical antimicrobials in Southeast Asia, comprised mainly of fluoroquinolones and cephalosporins, is associated with excessive use of antimicrobials in humans and animals⁸. I investigate the AROs and ARGs associated with these antimicrobials in chapter 5. Therefore, in this section I describe third generation cephalosporins.

β -lactam antimicrobials are comprised of compounds that have a β -lactam ring in their chemical structure, which include penicillins, cephalosporins, monobactams, carbapenems, and β -lactamase inhibitor combinations. β -lactam antimicrobials have a broad range of clinical indications. The most important mechanism for bacterial resistance to β -lactams is the production of β -lactamase enzymes, such as the extended-spectrum β -lactamases (ESBLs), which are a major group of enzymes associated with β -lactam globally and particularly in Asia⁸⁸.

Cephalosporins are a group of β -lactam bactericidal antimicrobials that inhibit cell wall synthesis. They are among the most widely prescribed antimicrobials because of their wide range of activity and low degree of toxicity. Like fluoroquinolones they are divided into several generations (1st to 5th) based on their antibacterial spectrum. First-generation cephalosporins are active chiefly against Gram-positive bacteria and have limited activity on Gram-negative bacteria. Second-generation cephalosporins have greater activity against Gram-negative bacteria than first-generation cephalosporins. Third-generation

cephalosporins, such as cefotaxime, ceftazidime, and ceftriaxone, have a greater spectrum of activity against Gram-negative bacteria and are more resistant to Gram-negative β -lactam enzymes than first- and second-generation cephalosporins. Fourth-generation cephalosporins, such as Cefepime (USA) and ceftipime (Europe), have similar activity against Gram-negative bacteria as third-generation cephalosporins, and have enhanced activity against Gram-positive bacteria. Fifth-generation cephalosporins, such as Ceftaroline, have enhanced activity against newer variants of Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA)⁸⁹.

Third generation cephalosporins have been used in clinical settings since the 1980s with the most frequently prescribed being cefotaxime, ceftriaxone, and ceftazidime⁹⁰. Shortly after their use became common in the 1980s, the first variants of ESBL Enterobacteriaceae (ESBL-E) were reported⁹¹. Organisms producing CTX-M-type ESBLs were first reported sporadically in the late 1980s in Japan, Europe, and Argentina⁹².

1.3 Antimicrobial resistance

Antimicrobials are essential for modern medicine; however, we are currently in an AMR crisis. Once susceptible organisms are now becoming resistant, posing a serious threat to human health due to infectious diseases becoming untreatable⁹³. The consequences of AMR are not only critical in LMICs but also have serious repercussions for developed countries through rapid globalization. Indeed, it is estimated that the total mortality rate due to AMR in the next 30 years will be >10 million deaths per annum, globally⁹⁴. This scenario is likely being driven by poor sanitation, a high demand for antimicrobials, large burdens of infectious diseases, and the irrational use of antimicrobial treatment.

The association between antimicrobial consumption and AMR at the population level has been well-established⁹⁵. Having an assumption that antimicrobials used for diarrhoea is common (chapter 4), I investigate the presence of AMR through the prevalence of AROs and ARGs among healthy children in chapter 5. In this section I provide an overview of AMR.

1.3.1 Definition

AMR is a phenomenon where bacteria can survive and replicate in the presence of an active antimicrobial compound, and this process can be natural (intrinsic, innate, or inherent) or acquired^{96,97}. Innate resistance, which is normal in some genera, species, or groups of bacteria, arises because either there is a lack of a particular antimicrobial target, or the drug cannot reach their targets because of outer membranes^{98,99}. For example, *Enterococci* are naturally resistant to all cephalosporins because they lack penicillin binding proteins that effectively bind, and are inhibited by, beta lactam antibiotics⁹⁸.

Another type of resistance that has more impact on public health is acquired resistance. Acquired resistance happens when a few isolates acquire or inherit some mutation, such as, for example, a mutation of a chromosome or gene that causes rifampicin or ciprofloxacin resistance^{100,101}. Additionally, acquired resistance may be associated with isolates that acquire resistance-conferring DNA. This is an example of “horizontal gene transfer” or “horizontal spread” through the exchange of DNA, such as a plasmid, which is a typical mechanism¹⁰². This type of DNA can be transferred by three mechanisms: transformation, transduction, or conjugation.

Acquired bacterial AMR can result from either the mutation of genes in the pathways targeted by the drug, the acquisition of resistance genes through horizontal gene transfer, or a combination of these mechanisms. ARGs have been shown to accumulate on mobile elements such as plasmids, transposons, and integrons, leading to a situation where multidrug resistance (MDR) phenotypes can be transferred to a susceptible recipient via a single genetic event¹⁰².

1.3.2 Mechanisms of AMR

Figure 1.1 depicts mechanisms of antimicrobials and AMR⁹⁶. Bacteria can become resistant to the antimicrobials due to several mechanisms including (A) impermeability, in which the bacteria can prevent the drug from getting into the cell (reduced uptake) by either decreasing influx or increasing efflux, (B) alteration or replacement of the target (such as target amplification by overproducing a target enzyme) or mutation, and (C) enzyme inactivation, in which the bacteria can either destroy antimicrobials completely or modify them chemically^{103,104}. ARGs encoding the resistance mechanisms can be located in the chromosomal DNA or on transferable elements integrated in the chromosome (integrons, transposons) or separated plasmids, which can be transferable through horizontal spread¹⁰⁵. As a consequence, some factors that affect the AMR include the natural level of resistance of the species, the resistance gene or mutation involved, the expression of the gene in a particular strain, and the presence of other genes or mutations¹⁰⁶.

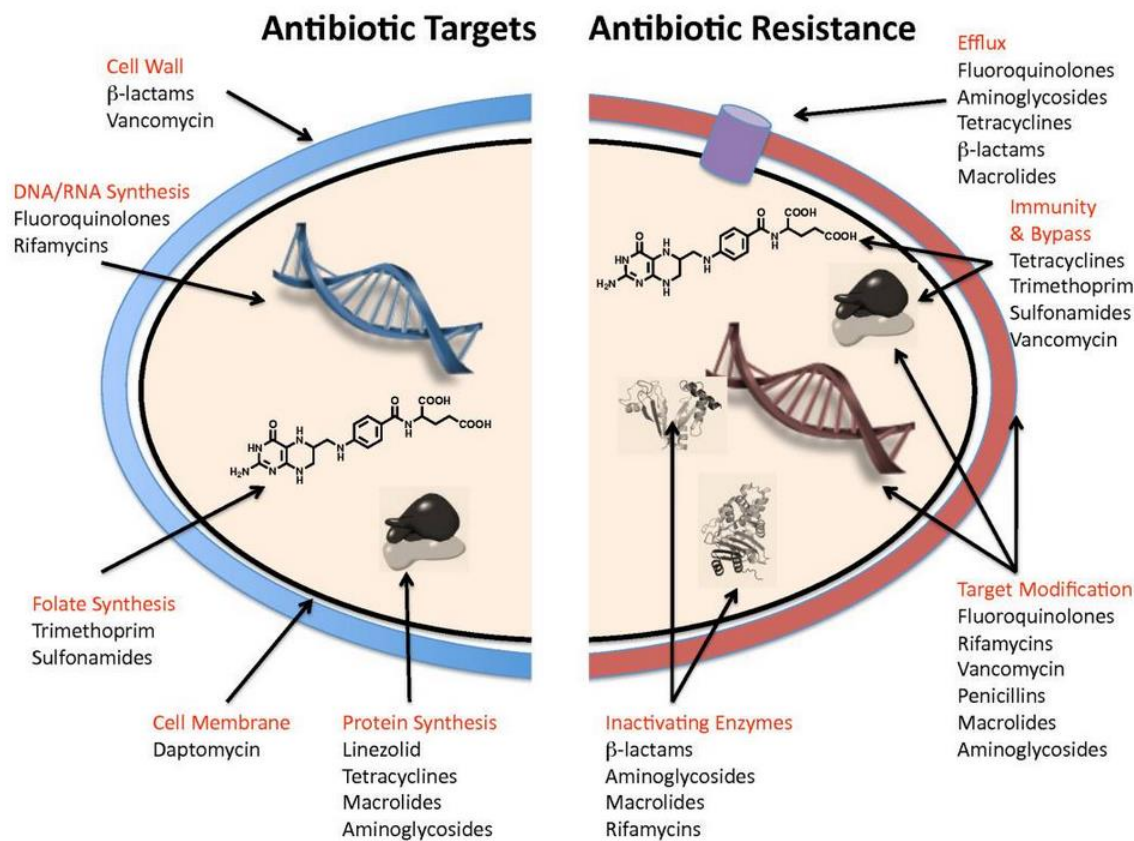


Figure 1.1 AMR mechanisms. Five main antibiotic targets are displayed on the left and four general mechanism of antibiotic resistance are explained on the right. These processes can occur at different parts of the bacterial cell: cell membrane, protein, enzyme, ribosome, and DNA/RNA. (Source: Wright in BMC Biology⁹⁶)

1.3.2.1 Mechanisms of AMR to fluoroquinolones

Fluoroquinolones resistance mechanisms can be summarised into three categories, which include (i) alterations in the target enzymes, comprised of DNA gyrase and topoisomerase IV as stated in section 1.2.2, (ii) alterations in drug permeability, and (iii) plasmid-mediated quinolone resistance determinants. In the first mechanism, alterations in specific domains,

called QRDR (Quinolone Resistance Determining Region), reduce the affinity for fluoroquinolone binding. The common QRDR mutation positions include the codon 83 and codon 87 within the *gyrA* gene and codons 80 and 86 within the *parC* gene, the latter of which is less common. In the secondary mechanism, resistance to fluoroquinolones is associated with a reduction in porins in the membrane, which reduces the accumulation of the drug. Consequently, fluoroquinolones cannot cross the cytoplasmic and outer membranes. The bacterial cell can also active an endogenous efflux system which pumps the fluoroquinolones out of the cytoplasm, and requires three components. These components include efflux pumps in the cytoplasmic membrane, an outer membrane protein, and membrane fusion proteins that connect inner and outer membrane proteins. In the last mechanism, which was more recently discovered, antimicrobial resistance genes are carried on plasmids and these genes can block the activity of fluoroquinolones. This mechanism was first described in 1998 in the USA in *K. pneumonia* from a patient with urinary tract infection in the USA. Since then, the plasmid mediated quinolone resistance (PMQR) determinants in Enterobacteriaceae and other species have been reported¹⁰⁷.

1.3.2.2 Mechanisms of AMR to third generation cephalosporins

As noted in section 1.2.3, β -lactam antimicrobials have a β -lactam ring, and work by interfering cell wall synthesis by impeding enzymes that are responsible for the formulation of the peptidoglycan layer¹⁰⁸. However, the production of several enzymes, including Extended spectrum β -lactamase (ESBLs), can block antimicrobial activity by cleaving the hydrolytically susceptible chemical bonds in the β -lactam ring¹⁰⁹. ESBLs mediate resistance to all penicillins, third generation cephalosporins, and aztreonam¹¹⁰.

1.3.3 The epidemiology of AMR

AMR is rapidly increasing as a public health challenge¹¹¹. AMR is currently estimated to account for >700,000 annual deaths worldwide⁹⁷. In the USA, “the large majority of 99,000 deaths each year associated with hospital acquired infections are caused by AMR bacteria; the annual cost of managing these infections may be as much as \$34 billion per year”⁸. In Europe, AMR organisms account for an estimated 25,000 deaths per year at a cost of 2.5 million extra hospital days¹¹². The cost of these infections is approximately 1.5 billion euros per year, which is associated with, in part, 600 million days of lost productivity^{112,113}. Developing countries are also hit hard by resistance. In Thailand >140,000 AMR infections each year cause 30,000 deaths¹¹³. In Pakistan 71% of infections in neonates are caused by AMR bacteria, and in Peru and Bolivia the majority of hospital infections are caused by AMR bacteria (51%)¹¹³. In its 2013 Global Risks Report, the World Economic Forum put AMR on par with unrestricted proliferation of weapons of mass destruction and global economic meltdown¹¹³.

Additionally, AMR rates of current antimicrobial drugs are increasing much faster than the production of new drugs, whilst antimicrobials are overly used for the prevention and treatment of illness. Many first-line antimicrobials have lost their effectiveness in treating infections caused by important bacterial pathogens; their activity has been eroded over time due to the reduced susceptibility of pathogens^{114,115}. For example, enteric fever, which is caused by the bacterium *Salmonella enterica* serovar Typhi, is a common cause of bacterial febrile disease in LMICs¹¹⁶, and used to be reliably treated with ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. However, these antimicrobials are no longer active against enteric fever and the disease is being treated with third-generation

cephalosporins and fluoroquinolones^{117,118}. A further example is shigellosis, caused by bacteria belonging to the genus *Shigella*, which was recommended to be treated with nalidixic acid and then ciprofloxacin by the WHO^{46,119}. First, nalidixic acid resistance emerged, and now ciprofloxacin-resistant *S. sonnei* is increasing throughout Asia^{120–122}. It has additionally been observed that these ciprofloxacin-resistant *S. sonnei* are spreading out of Asia into other areas of the world due to globalization^{123–125}. This observation is concerning as it demonstrates that AMR is not restricted to hospital settings, as it was commonly observed a few decades ago, but is now rampant in the community.

AMR is a complex scientific, political, economic, and social issue, and the greatest impact of AMR occurs in LMICs in Asia and Africa^{126–129}. However, there is a general lack of consistent AMR surveillance systems in these areas¹³⁰. The AMR issue is amplified in LMICs because the principal driver of AMR is antimicrobial usage⁹⁵. Unrestricted access to low-cost antimicrobials combined with a high burden of infectious disease, limited capacity for diagnostics, and large urbanizing populations demanding better medical support means that AMR bacteria are prospering in LMICs across Asia^{131,132}.

1.3.4 AMR and the resistome

The resistome, which is the totality of ARGs in the microbiome, may be directly impacted by the diversity of bacterial populations found within the gastrointestinal microbiota¹³³. For example, in densely populated urban areas, the exposure of pathogenic bacteria to the gastrointestinal resistome may result in lateral exchange of ARGs¹³⁴. Antimicrobial exposure, whether from environmental exposure or treatment, causes selective pressure and thus reduces microbial diversity, and exacerbates ARG exchange. The development of the

human resistome is likely to occur in early childhood¹³³⁻¹³⁵, particularly in urban settings with intense environmental exposure. Improving our understanding of how AMR develops in the gastrointestinal microbiota in LMIC settings is vital to enable preservation of future antimicrobial options and to develop intervention strategies to optimise individual, population, and environmental managements.

1.3.5 Current challenges in the AMR to global health

AMR is a sustained global health problem across countries including both high-income countries and LMICs. The burden of AMR is associated with both medical and financial issues as AMR infections pose a large threat to individuals in LMICs. Current challenges in AMR are not only related to antimicrobial consumption but also improving sanitation, access to clean water, public governance, and public health expense¹³⁶.

1.4 The setting of high usage and high resistance to antimicrobials in Viet Nam

Antimicrobials are essential in such LMICs as Viet Nam where infectious diseases are among the leading causes of morbidity and mortality^{137,138}. However, antimicrobials are used inappropriately in Viet Nam. Despite antimicrobials being officially prescription only drugs in Viet Nam, the majority (78%) of antimicrobials are bought from pharmacies without a prescription¹³⁹. A recent study in the urban north of Viet Nam found that 24% of transactions from pharmacies were for antimicrobials, of which 88% occurred without a prescription¹⁴⁰.

Children in LMICs are the most vulnerable group that are highly exposed to antimicrobials⁹⁵. Children are more susceptible to diarrhoeal disease due to naivety of the

immune system and a higher-level of exposure to environmental pathogens compared to adults. In a cross sectional study from Viet Nam, approximately 75% of children in the community were treated with antimicrobials within one month prior to interview¹³⁹ and >85% of children attending a hospital for diarrhoeal disease were prescribed an antimicrobial¹⁴¹.

AROs and ARGs have a substantial impact on the exchange of acquired AMR determinants. Antimicrobial exposure induces a selective pressure and may therefore facilitate the exchange of ARGs. A recent report of AMR in Viet Nam found that bacteria isolated from children with diarrhoea are highly resistant¹⁴². Additionally, there is evidence that a pathogen may acquire ARGs from the commensal bacteria that constitute the microbiota in the human gastrointestinal tract⁷⁰. Therefore, commensal *E. coli* are likely an important reservoir for AMR determinants¹⁴³.

1.5 Breastfeeding

I aim to tackle AMR in such LMICs as Viet Nam, and I hypothesize that an affordable and pragmatic solution in the community, such as breastfeeding, may have an impact on infectious disease incidence. In turn, breastfeeding could impact antimicrobial usage and have knock-on consequences for AMR. Data suggests that mortality and morbidity from diarrhoea can be reduced by exclusive breastfeeding^{76,144–146}. Moreover, gastrointestinal immunity against enteric pathogens can be enhanced by breast milk^{76,147}. Making use of data from a birth cohort that was conducted prior to the diarrhoeal cohort from 2014, I investigated risk factors associated with suboptimal breastfeeding, which is described in chapter 6.

1.5.1 The practice of breastfeeding

Breastfeeding is the method of feeding an infant with human breast milk to provide them with the nutrients they need for healthy growth and development¹³⁰. Current practices of breastfeeding vary according to initiation, intensity, and duration. The most common definitions of breastfeeding used in clinical and research settings are suggested by the WHO¹⁴⁸. Breastfeeding patterns are classified into different categories based on their duration and exclusivity; these categories are shown in table 1.3.

1.5.1.1 Breastfeeding initiation and early breastfeeding

The first few days of breastfeeding after delivery are a very important time for mothers and their infants. Both mother and infant are learning how to breastfeed and are getting familiar with one another. The WHO recommends that breastfeeding should be initiated as soon as possible after birth, provided that mother and infant are well enough to do so¹⁴⁹. Following this recommendation, the infant should be placed, and remain, in direct skin-to-skin contact with the mother and latched on during the first half-hour after delivery¹⁵⁰. This process enhances milk production and improves the bonding between mother and infant. Liquids other than breast milk should not be given to the infant unless a medical indication requires so¹⁵¹. The infant may take some time to latch on and begin suckling or may even need assistance. Milk supply is determined by the frequency of feeding and effective removal of milk from the breast¹⁵². Lactation performance during the first few weeks is critical to the establishment of an adequate milk supply¹⁵³.

1.5.1.2 Exclusive breastfeeding and duration of breastfeeding

The WHO recommends that infants should be exclusively breastfed for the first six months of life, which means that infants receive only breast milk and fluid medication, such as drops and syrups, during the first six months of life¹⁴⁹. In addition, children are recommended to be breastfed for at least 24 months and beyond, together with complementary food¹⁵⁴.

Table 1.3. Definition of some common breastfeeding practices recommended by the WHO.

Feeding category	Infant receives	May include	Does not include
Early breastfeeding initiation	Human milk within the first hour post-partum	Human milk	Anything else
Exclusive breastfeeding	Human milk	ORS, drops, syrups (vitamins, minerals, medicines)	Anything else
Predominant breastfeeding	Human milk as a predominant source of nourishment	Certain liquids (water and water-based drinks, fruit juice), ritual fluids and ORS, drops or syrups (vitamins, minerals, medicines)	Anything else (in particular, non-human milk, food-based fluids)
Complementary feeding	Human milk and solid or semi-solid food	Anything else: any food or liquid including non-human milk	NA
Breastfeeding	Human milk and solid or semi-solid food	Anything else: any food or liquid including non-human milk	NA
Bottle-feeding	Any liquid or semi-solid food from a bottle with nipple/teat	Anything else: any food or liquid including non-human milk	NA

In chapter 6, I explore the practice of early breastfeeding during hospital stays and breastfeeding at the routine follow up visit in the child's first year. The protective benefits of breastfeeding come from the components of breast milk and the practice of breastfeeding^{155,156}. Continued breastfeeding practice in the later months of infancy depends on the early practice of breastfeeding within a few days after birth¹⁵⁷. One possible reason is the physiology of secreting different components of human milk in the early stages after birth¹⁵³. Various components and mechanisms of breast milk secretion in the postpartum period could impact the success of milk secretion in later months. Specifically, as long as women start breastfeeding their child after birth, they experience two stages of lactation, which are lactogenesis I (colostrum is secreted) and lactogenesis II, following transition from colostrum (transitional milk) to the secretion of mature milk. Initially, colostrum is secreted during the first few days after birth. The milk production (lactogenesis) is induced during the first two to four days after delivery by the high levels of prolactin¹⁵⁸. Next, at the transition stage milk production and secretion are stimulated in the neuroendocrine pathway involving the release of prolactin and oxytocin—both hormones have key roles in human milk synthesis and secretion—by stimulation of sensory nerves of the breast by the suckling infant. Therefore, suckling should be established in the first three days to ensure sufficient milk production¹⁵³. Mature milk is secreted after 10 to 15 days after birth.

1.5.2 Benefits of breastfeeding and gastrointestinal infections

Breastfeeding offers many positive health benefits because of its unique components which confer nutritional and immunological effects to infants¹⁵². Exclusive breastfeeding during

the first six months of life provides both short- and long-term advantages that protect children against several childhood illnesses as well as offers positive benefits in later life^{147,159}. Table 1.4 outlines a summary of protective effects of breastfeeding to infant health.

Components of breast milk are various. Nutritional factors in breast milk include macronutrients (such as lipids, protein and carbohydrates), minerals and trace elements (such as sodium, potassium, calcium, magnesium, phosphate, chloride and citrate), vitamins (such as water soluble vitamins: vitamin B1, B3 [nicotinic acid], B5 [pantothenic acid], B6, biotin, folic acid, B12 and C, and fat-soluble vitamins: vitamin A, vitamin D [in low concentration], vitamin E, and vitamin K)^{152,160}. In addition, human milk has an abundance of bioactive factors (such as bile-salt simulated lipase and epidermal growth factor including leptin, adiponectin, ghrelin, resistin and obestatin, which help to digest and absorb nutrients), which are elements from food that have an impact on biological processes, bodily functions and health¹⁵². Furthermore, **colostrum, the first milk, is rich in immunoglobulins specifically, secretory immunoglobulin A (sIgA)**. The bioactive factors in breast milk provide infants with protection from infections by various microorganisms, hormones, growth factors that affect growth and development, agents that modulate immune function, and anti-inflammatory components¹⁶¹. Moreover, human milk contains growth promoters of protective microorganisms and inhibitors of harmful bacteria. For example, acetic acid produced by *B. bifidum* discourages the growth of some enteropathogenic bacteria¹⁶².

Table 1. 4. Evidence for a few advantages of breastfeeding to the infant that have positive evidence.

Disease outcome	Effects	Levels of evidence ¹⁶³	Settings of evidence	A few typical or high-quality relevant studies
Gastrointestinal infection	Exclusive breastfeeding in the first six months of life have major protection against diarrhoea morbidity and admissions to hospital	Strong evidence	Mostly from LMICs	Large body of studies ^{76,164,165}
Respiratory infections (pneumonia)	Breastfeeding have effect on reduction in severe respiratory infections	Strong evidence	Mostly from LMICs	Large body of studies ¹⁵⁹
Obesity	Breastfeeding provides protection effects	Suggestive evidence	LMICs and HICs	High-quality systematic review ^{147,159,163}
Cognitive development	Increasing IQ points	Consistent evidence	Mostly in HICs	Large observational studies and a RCT study ¹⁶⁶
Acute otitis media	Longer duration of breastfeeding reduces acute otitis media during the first two years	Consistent evidence	LMICs and HICs	Large body of studies ¹⁶⁷
Type II diabetes	Breastfeeding have protective effect	Restricted evidence (11 study)	LMICs (2 out of 11 studies) HICs	Evidence from 11 studies ¹⁵⁹

Some suggested mechanisms that enable breastfeeding to prevent gastrointestinal infections include the composition of antimicrobial or immunological factors in breast milk, nutritional status of breastfed infants, and the absence of exposure to contamination from non-human milk¹⁴⁷. In addition, breast milk contains antimicrobial and anti-inflammatory factors, hormones, digestive enzymes, and growth modulators that protect against infection¹⁴⁷. It has been suggested that glycans in their free and conjugated species, such as those found in human milk oligosaccharides, form part of the natural immunological mechanism that account for the way in which human milk protects breastfed infants against diarrhoeal disease⁷⁶. Additionally, by avoiding formula feeding an infant's exposure to bacterial contamination is reduced, which helps ensure adequate nutrition and contributes to non-specific immunity^{76,146}. Breastfeeding helps protect against diarrhoea incidence, prevalence, hospitalization, diarrhoea mortality, and all-cause-mortality¹⁴⁶.

1.6 Research questions and objectives

Improving our knowledge on how antimicrobial exposure impacts AMR is essential, and diarrhoeal disease in children represents an ideal model for understanding the interplay of antimicrobial usage and AMR in the community. This work aims to contribute to the effort of understanding AMR in a LMIC where there is extensive exposure to both antimicrobials and infectious pathogens.

1.6.1 Aims and hypothesis

I hypothesise that diarrhoeal disease is a common infection in the community in the urban area of HCMC, Viet Nam, and that a large proportion of healthy children in this setting are

colonised by drug resistance bacteria, which may be facilitated by the common use of antimicrobials to treat diarrhoeal diseases. Therefore, the general aims of this thesis are to measure the incidence of diarrhoea in a cohort of children in HCMC, to assess antimicrobial usage in the local population, and to evaluate the prevalence of ARGs and AROs. A secondary aim is to explore risk factors of suboptimal breastfeeding, which is a pragmatic response to the problem of community diarrhoea.

1.6.2 Objectives

My thesis had four main objectives, which correspond with the four result chapters (3, 4, 5, and 6). Table 1.5 outlines the framework of my thesis. Figure 1.2 defines the relationship between these four objectives.

- **Objective 1:**

I hypothesized that diarrhoea is a common disease in children in the community in Viet Nam and is predominantly associated with *Salmonella* and *Shigella*. To test this hypothesis I conducted a community cohort study in 748 children to investigate the clinical symptoms, disease aetiology (pathogen-specific diarrhoeal episodes), risk factors, and population-based incidence of diarrhoeal disease among children in the community in Ho Chi Minh City, Viet Nam (Chapter 3).

- **Objective 2:**

I hypothesized that childhood diarrhoeal disease is commonly managed in the community with antimicrobials in Ho Chi Minh City. To test this hypothesis I measured antimicrobial usage in the community for treatment of childhood diarrhoea in HCMC by performing a

mixed approach study including healthcare utilization, geospatial mapping, simulated client modelling, and incidence data from the cohort study (Chapter 4).

- **Objective 3:**

I hypothesized that the carriage of AROs and ARGs is common in children in HCMC and associated with antimicrobial usage and nutrition. To test this hypothesis I quantified the prevalence AROs and ARGs carriage in healthy children in the community in HCMC and identify risk factors associated with carrying genes associated with resistance to fluoroquinolones and third generation cephalosporins (Chapter 5).

- **Objective 4:**

I hypothesized that optimum breastfeeding practices are limited in the population of mothers in Ho Chi Minh City that is associated with an increase in diarrhoea. To test this hypothesis I measured the prevalence of optimum breastfeeding practices in this population in Viet Nam and then assessed the impact of breastfeeding on diarrhoeal disease. Increasing breastfeeding may be a simplistic approach for decreasing AMR carriage in children (Chapter 6).

Figure 1. 2. DPhil thesis structure

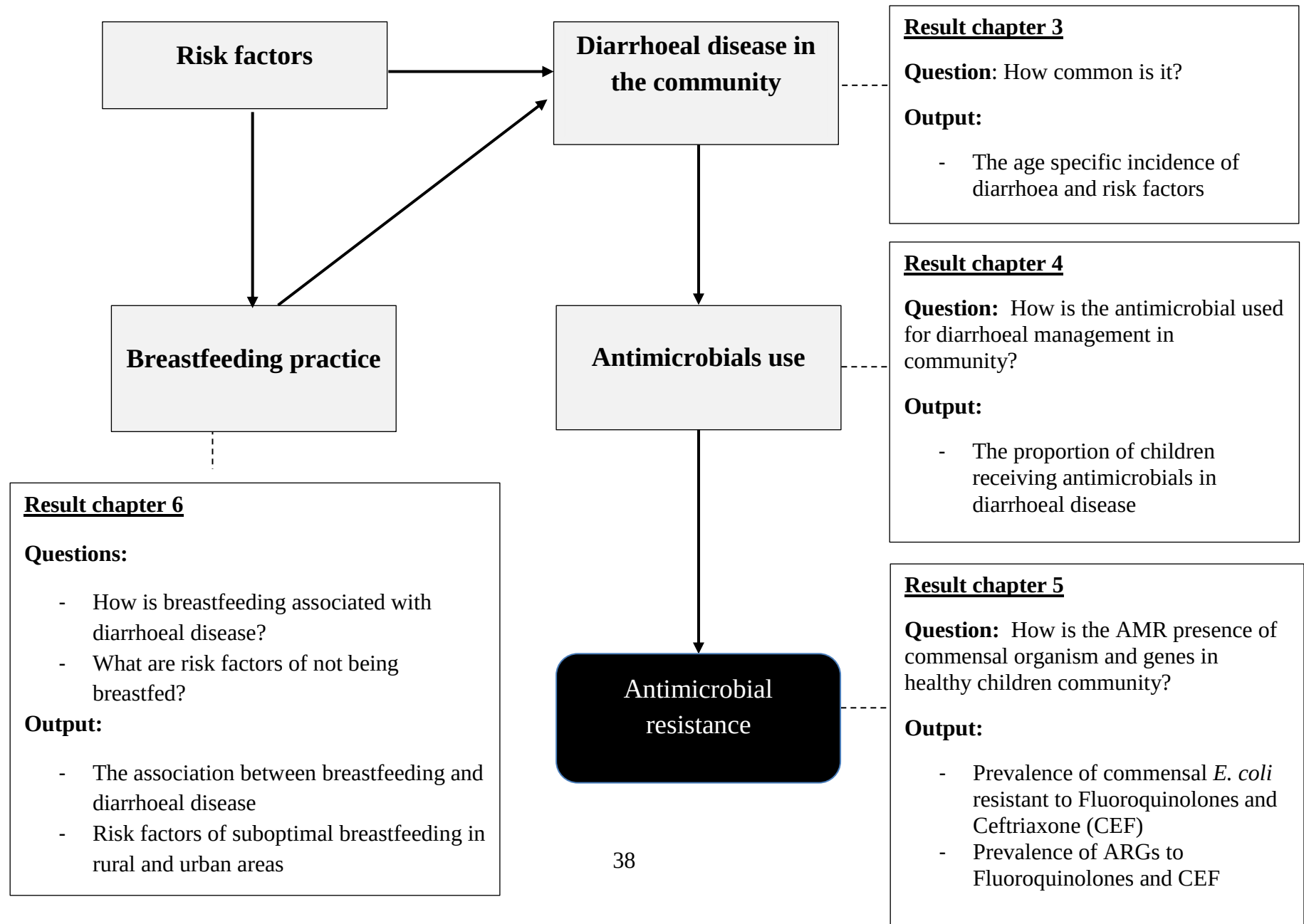


Table 1. 5. Logical framework for DPhil thesis.

Objectives	Outputs	Activities	Results
To determine the burden of diarrhoeal disease in an urban children community of HCMC, Viet Nam	Incidence and age-specific incidence of diarrhoeal disease among children age 1 to 5 in District 8, HCMC, Viet Nam including: Mild diarrhoeal disease Moderate to severe diarrhoeal disease	<i>Project 2:</i> Diarrhoeal cohort A longitudinal prospective population based study of 748 children in District 8, HCMC, Viet Nam conducted from 2014 to 2016.	Chapter 3
To investigate the antimicrobials usage in the community regarding diarrhoeal disease treatment	Pharmacy density in District 8, HCMC Proportion of antimicrobials dispensed by pharmacy retailer in community Proportion of children receiving antimicrobials in diarrhoeal disease management in community	<i>Project 3:</i> Multiple approaches Mapping health care services in District 8, HCMC, Viet Nam in 2016 Secret shopper in pharmacies in Ward 5, District 8, HCMC, Viet Nam, in 2016 Community survey of a representative group of parents and caregivers in District 8, HCMC, in 2016	Chapter 4

Objectives	Outputs	Activities	Results
To identify the problem of resistant <i>E. coli</i> and associated genes among healthy children in District 8	Prevalence of fluoroquinolones and third generation cephalosporin resistant <i>E. coli</i> Prevalence of associated fluoroquinolones and third generation cephalosporin resistant genes	<i>Project 2: Diarrhoeal cohort</i> Healthy rectal swabs taken from healthy children are used for laboratory method to identify the prevalence of fluoroquinolones and third generation cephalosporin resistant <i>E. coli</i> and associated genes.	Chapter 5
To determine the associations between breastfeeding, as a pragmatic solution to AMR in community, and diarrhoeal disease. To identify risk factors of not exclusive breastfeeding in hospital in urban area compared to semi-rural area.	Prevalence of exclusive breastfeeding in hospital in urban and semi-rural areas Risks factors of not being exclusive breastfeeding before hospital discharge Association between breastfeeding and the first time to diarrhoeal disease Association between breastfeeding and number of diarrhoeal disease in the first year of life	<i>Project 1: Birth cohort.</i> A prospective birth cohort conducted since 2009 in HCMC, an urban areas and Dong Thap province, a semi-rural area in Mekong Delta of Viet Nam	Chapter 6

2. Methods

In this chapter I describe in detail all of the materials and methods used throughout my thesis. This chapter describes how studies have been designed and conducted, statistical and geospatial analysis strategies as well as microbiology procedures, materials, methods, and techniques targeting four aims in my thesis. Data used for the results chapters are based on three epidemiological projects including a prospective birth cohort conducted from 2009 to 2013 (Chapter 6), a continuing longitudinal study afterwards conducted between 2014 and 2016 (Chapter 3), a community cross sectional study investigating self-reported behaviour of parents and care givers in taking care of children with diarrhoeal disease and a simulated survey to explore pharmacy practice in dispensing antimicrobials for paediatric in 2016 (4). Materials and methods for the result chapter 5 are also described in the microbiology section under this chapter. The relationship of the study projects used for this thesis is shown in figure 2.1.

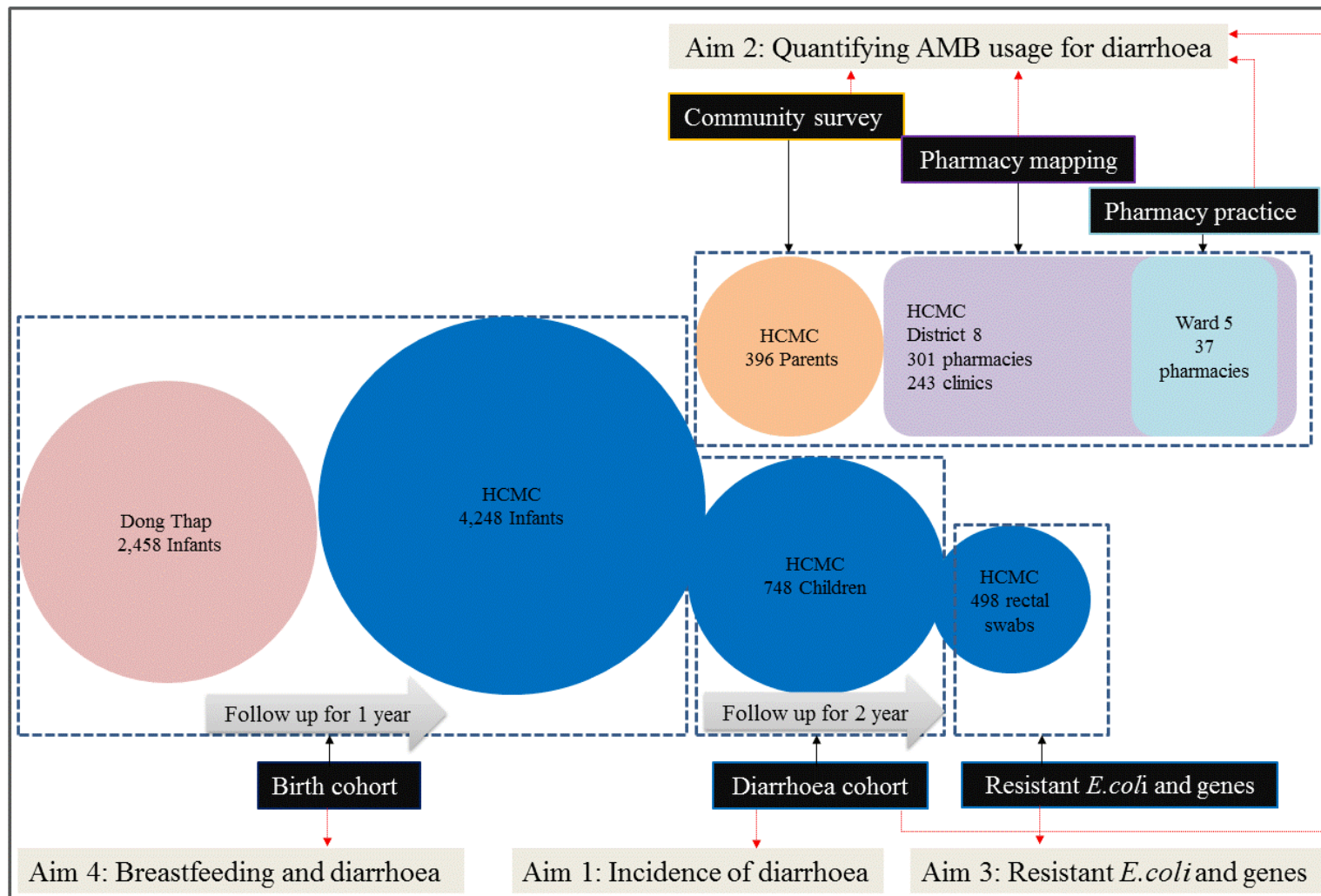


Figure 2.1 Relationship of studies that data have been used in my thesis including study name, participants and size of the sample population (AMB: antimicrobials)

2.1 Study settings of epidemiological studies

2.1.1 Project 1: Birth cohort

2.1.1.1 Study summary

With the original aim of investigating childhood infectious diseases with dengue fever focus in the South Viet Nam, a birth cohort was established in 2009. Specifically, this study aimed to investigate immunity and pathogenesis in young children exposed to dengue fever and to study other infectious causes such as respiratory and diarrhoeal disease in the first year of life. The study areas included HCMC and Dong Thap province, where research connection had been made with the OUCRU. Mothers and infants were recruited since during pregnancy or on the delivery date. A total of 6,706 children in both areas were enrolled from 2009 to 2013 and were followed for 12 months for infectious diseases. I used data from this birth cohort to investigate the proportions of breastfeeding before infants were discharged and the association of breastfeeding with diarrhoeal disease. I also explored the risk factors of not having optimal breastfeeding practices. (Statistical analysis is described in section 2.2.4 and results are shown in chapter 6)

2.1.1.2 Ethical approval

Ethical approval for this study was provided by the OxTREC and the Institutional Review Boards (IRBs) at the local hospitals where the study was conducted and included the Hospital for Tropical Diseases (HTD), Hung Vuong Hospital (HVVH), District 8 Hospital and Children's Hospital No.1 in HCMC; and Dong Thap Hospital - a provincial hospital in Dong Thap province the Mekong Delta region. Written informed consent was obtained from parents and caregivers of infants.

2.1.1.3 Study location

Mothers enrolled in this study resided in District 8 in HCMC (urban area) and Cao Lanh Township and Cao Lanh District, Dong Thap province (semi-rural area). HCMC is the largest city in southern Viet Nam with a population of 7,820,000 in 2013 (this becomes more than 8 million in 2017) and a mean population density of 3,731 person/km² ¹⁶⁸.

District 8 in HCMC is an inner city area with >400,000 people and > 20,000 person/km² ¹⁶⁹. District 8 was selected because it is a typical inner-city district and is in close proximity to the Hospital for Tropical Diseases (HTD)..

The population in Dong Thap province, located 165km Southwest of HCMC, was estimated to be 1,678,000 people in 2013, with a population density of 496 person/km² ¹⁶⁸.

Cao Lanh Township, the location of Dong Thap hospital, is the provincial capital, and in 2013 had a population of 163,030 people with a population density of 1,523 person/km²; 44.7% of the population live in a rural area ¹⁷¹. Cao Lanh District is larger geographical area with a population of 202,117 people and a population density of 412 person/km²; 93.6% of the population live in a rural area ¹⁷¹.

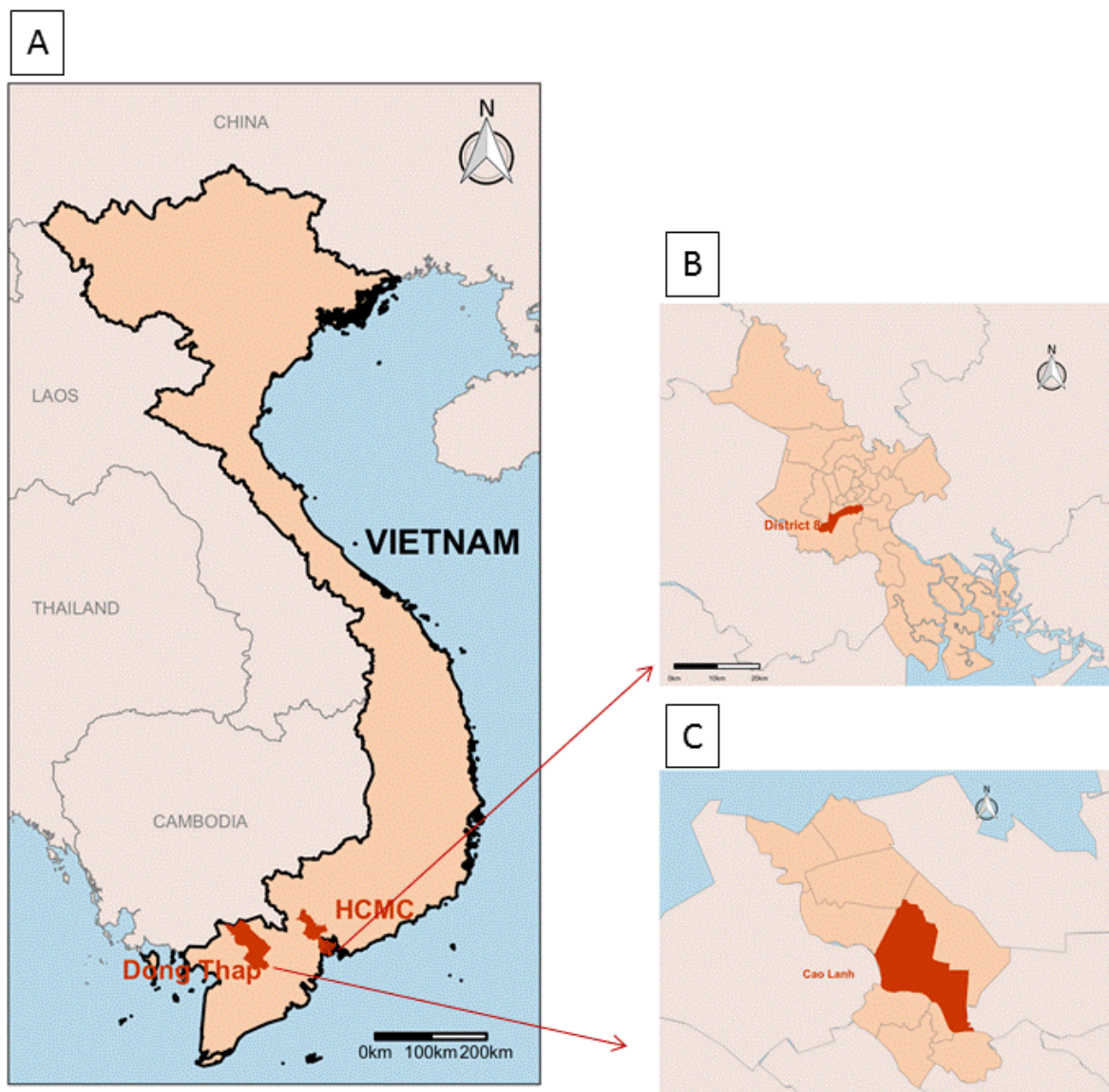


Figure 2.2 Location of study areas. 6,706 infants have been enrolled from HCMC (4,248) and Dong Thap province (2,458).

A) Map of Viet Nam including HCMC and Dong Thap province B) Enhance map to show District 8 in HCMC C) Enhance map to show Cao Lanh Township and Cao Lanh District in Dong Thap province

2.1.1.4 Study design and participants

This was a prospective birth cohort study in which infants were enrolled from HVH and Dong Thap hospital. Briefly, mothers were invited to participate at the time of delivery in hospital or during their last trimester visit these hospitals. Mothers were aged >15 years and had to have been residing in either HCMC or Dong Thap province for at least 12 months prior to enrolment. A total of 6,706 infants (4,248 from HCMC and 2,458 from Dong Thap province) were enrolled. Children attended routine follow up visits in these hospital at 1, 2, 4, 6, 9, and 12 months of age for a health check-up and interviews for demographic and history of diseases questions.

2.1.1.5 Study procedures and data management

After providing informed consent, mothers were enrolled; infants from enrolled mothers who delivered at study hospitals were also enrolled before hospital discharge at birth. Upon enrolment, the study nurses collected information regarding socio-economics, obstetrics history, the characteristics of parents and infants, delivery information and breastfeeding. All of these data were collected prior to discharge from hospital (within three days of vaginal birth and within seven days of other delivery methods).

The study nurses additionally conducted routine follow up visits of enrolled infants in these hospitals at 2, 4, 6, 9, and 12 months of age (infants in HCMC had additional visits at 1 and 3 months of age when they received a routine check-up and scheduled immunizations). At each follow up visit, mothers or caregivers were interviewed with questions regarding the health status of the infants, changes in demographic information, current feeding practice,

and diarrhoeal disease episodes. At 4, 9 and 12 months of age, blood samples (1 ml) were collected for serological testing against various viral pathogens. Additional data on diarrhoeal episodes was obtained from follow up records through passive surveillance at the assigned study hospitals in both study areas.

Data were collected through face-to-face interviews conducted by trained study nurses using a standardized electronic questionnaire. All study nurses were senior hospital staff with recent training (prior to conducting recruitment and interviews) for Good Clinical Practice, standard operating procedures, and communication skills. Data collected at enrolment and at routine follow-up visits were stored in an encrypted web-based database. Additional clinical data at illness presentation was recorded in a standard case report form (CRF), which was double entered into the web database. When the data collection was finalised, all data was checked and corrected for errors. Variables were derived and coded according to pre-defined definitions to produce datasets suitable for analysis. Figure 2.3 shows a diagram of enrolment and follow –up activities in the birth cohort conducted since 2009.

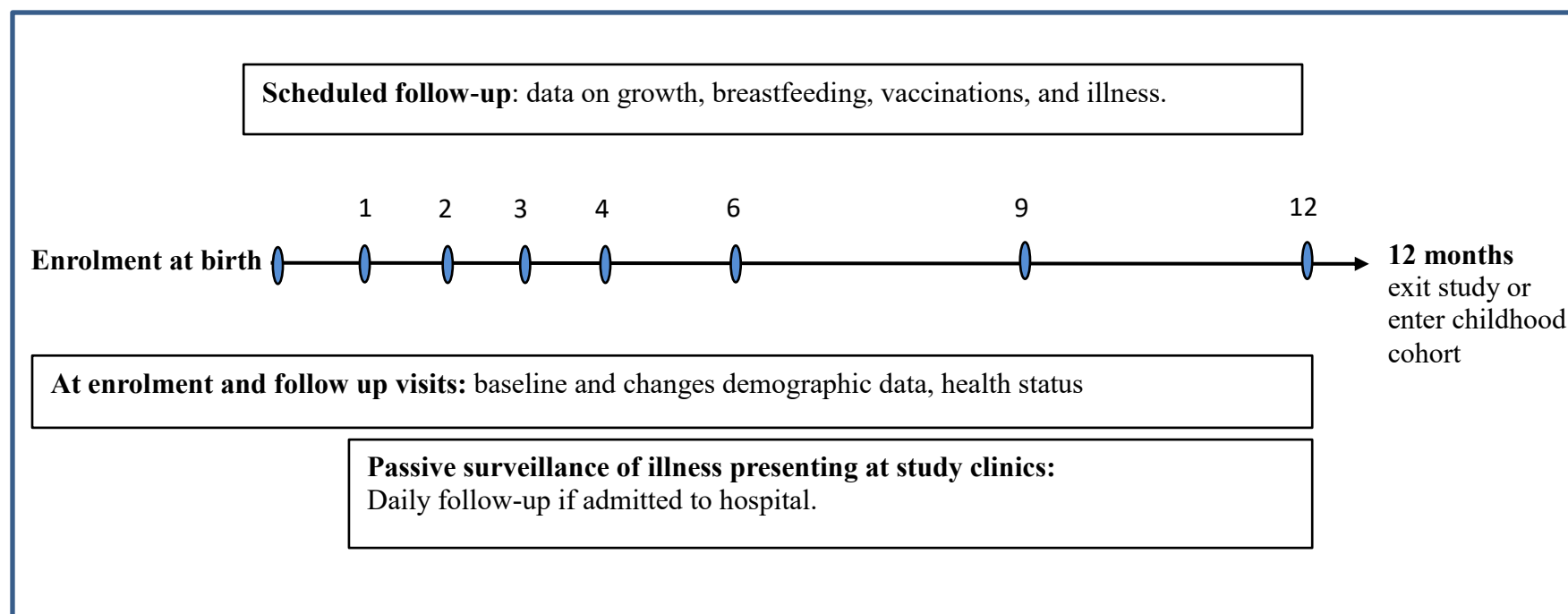


Figure 2.3 Birth cohort procedures. Children were enrolled at birth with routine follow up visits (shown in blue circles) during the first year of life. During enrolment and routine follow up visits, interviews were conducted to collect demographics data at baseline and changes over time as well as health status of the children. Follow up were continued for the first 12 months. Any sickness during the first year of life was collected through passive surveillance at study clinics.

2.1.2 Project 2: Diarrhoea cohort

2.1.2.1 Study summary

With the aim of investigating the burden of diarrhoeal disease in the community, this study was a continuation of the previously described birth cohort (section 2.1.1 Birth cohort). These children were resident in HCMC and when participated in the diarrhoea cohort, they were followed for another two more to assess diarrhoeal disease. This was a continuous longitudinal population-based prospective study, shortly named as “diarrhoeal cohort”, in which diarrhoeal disease in an urban area (HCMC) was the focus. The children were one to three years old at the time of recruitment into this diarrhoea cohort in 2014. Children were asked to attend routine follow up visits every six months at HVH and were examined for growth, vaccinations, and illness. The main outcome of the study was diarrhoeal disease episodes, which were investigated through both passive and active surveillance. The passive part covered mostly hospital admissions, in which the participants visited the study hospital; the latter was comprised of telephone message (SMS) to participants biweekly. Questionnaires regarding risk factors for diarrhoeal disease were conducted at each diarrhoeal episode recorded. Stool samples were collected (if available) at each diarrhoeal episode during the seven days captured through active surveillance were processed at HTD for microbiology at the laboratory. The relationship between diarrhoea cohort and the prior birth cohort is shown in Figure 2.1.

2.1.2.2 Ethical approval

Ethical approvals for this study were provided by the OxTREC of the United Kingdom (OxTREC approval 1058-13) and local ethical approvals were provided by the Ethics Committee of the HTD and HVH in Viet Nam. Written informed consent was obtained

from parents and caregivers of children at the enrolment.

2.1.2.3 Study design and participants

This was a prospective, community-based longitudinal study of the diarrhoeal disease and was based on a previous birth cohort. 748 children aged 1- 3 years were enrolled into this cohort from June to December 2014. Diarrhoea cohort participants included children, who participated in the previous birth cohort, and were one to three years of age at the time of recruitment of 2014 and were followed for two years (until 2016) for diarrhoeal disease episodes. The study nurses either invited these children and their parents to join the diarrhoea cohort when they visited HVH for the last routine visit from the birth cohort or invited those who met the recruitment criteria via phone. Every six months after enrolment participants were requested to attend HVH for a routine follow-up visit (January 2015 to December 2016). Healthy routine visits were conducted every six months and diarrhoeal episodes were examined actively via SMS. Children whose parents did not give the written informed consent, and have moved out of the study area of District 8, HCMC for more than six months, were excluded.

2.1.2.4 Sample size calculation

The sample size was calculated based on the annual incidence of diarrhoea in Vietnamese children and the proportion of *Shigella* infections in Viet Nam because the preliminary objective of the diarrhoea cohort was to investigate the incidence of the *Shigella* infections among children in Viet Nam. With the convenient and practical sample of 700 participants from the previous birth cohort, and the incidence of 1.5 diarrhoeal events per child each year, it was estimated that 2,100 diarrhoeal episodes over two year of follow up. The

estimations were considered conservative due to the facts that diarrhoea determinants in Viet Nam have been improve over years and *Shigella* infections do not always present with dysentery in clinical setting. The sample size method and sample population were presented in the Appendix of this thesis under the diarrhoea study protocol.

2.1.2.5 Study procedures

Diarrhoea was defined as three or more loose stool in a 24 hours period as defined by the WHO ¹⁹. Additionally, a diarrhoeal event was defined as an increase in frequency, reduction in consistency or notable change in quality of the stools, compared to what the family considers to be normal for the child ¹⁷². Two separated diarrhoeal episodes were distinguished by an interval of seven days from the date of onset. Figure 2.4 presents the study procedures and sample collection method.

2.1.2.5.1 CRF and specimen collection

Data were collected using the same methods and the same trained nursed in the previous birth cohort (section 2.1.1). Study nurses using a standardized electronic questionnaire at enrolment and at each 6-month follow up visit to ask for information regarding the participants' characteristics on socio-economy, demography, water and food usage, sanitation and food consumed during the prior three days of the visit. History of health during the six months prior to the visit and children's height and weight were also recorded.

Children with diarrhoea presented at hospital or visited by nurses at home were asked a short questionnaire about their diarrhoeal episode, which included clinical symptoms, diarrhoea in the household and the disease management. Fresh stool samples were collected

at any time diarrhoeal diseases presented at hospital actively or captured by SMS. These CRFs were presented in the Appendices of this thesis.

Throughout the study, all specimens were collected on enrolment and routine visits every six months including rectal swab and blood samples. Venous blood samples from the antecubital fossa area of the arm were collected in an EDTA tube and stored at room temperature. A total of 2ml were collected from children aged 12–35 months, and 3ml for children ≥ 36 months of age. Table 2.1 summaries the specimens collected through the diarrhoea cohort.

Table 2.1 Sample types and time points for collection throughout the cohort study

Sample	Enrolment	Routine visit	Diarrhoea:	Diarrhoea:
			Home visit	HTD visit
Blood*	X	X		
Rectal swab	X	X		
Fresh stool			X	X

*a total of 2ml from children <36 months and 3ml for children $\geq$ 36 months

2.1.2.5.2 Active and passive diarrhoeal surveillance

Diarrhoeal diseases were accessed using both passive and active surveillance. Given a diarrhoeal episode, the participants either came to a designated hospital of HTD passively on their own choice or informed our study nurses. Passive surveillance of diarrhoeal disease was to cover the frequency of children at admission to the hospital due to diarrhoeal diseases during the interval time prior to the scheduled visits every six months. At each routine healthy visit, parents or child guardians were asked if their child had been admitted to the hospital due to diarrhoeal in the prior six months. No stool sample was collected at these routine visits. In addition, study participants presented any time during the study time at the HTD with diarrhoeal disease were asked a short questionnaire on their clinical symptoms and family members' status of health related to their current diarrhoeal episode and a fresh stool sample was collected in the hospital by our study nurse. Information of diarrhoea treatment and antimicrobials used for each episode were also recorded.

Active surveillance was performed to identify diarrhoeal diseases of participants. Additionally, text messages (SMS) were sent biweekly to ask if the children had diarrhoeal diseases in the previous seven days of SMS date (Figure 2.4). Participants were also encouraged to contact our study nurses or come to HTD when they had diarrhoeal diseases. At the time of sending SMS, if the child's diarrhoeal diseases had recovered, a short version of questionnaire was asked through phone call (*phone record*). In case the child's diarrhoeal disease was identified by SMS, we sent a nurse to their home to collect stool sample and asked some questions about their diarrhoeal event (*home visit*). If the child's disease developed more serious or the nurse evaluated them as moderate to severe, the child was sent to HTD for doctors' examination (*hospital visit*). The child also had hospital

visit spontaneously without notice by SMS (*hospital visit*). At each diarrhoeal event, a stool sample was collected for a microscopy and cultured for bacterial pathogens. Further, a new technology for detecting multiple pathogens (Luminex xTAG) was used to detect 15 viral, bacterial and parasitic agents of diarrhoea sample. Figure 2.5 shows a description on how diarrhoeal diseases were detected.

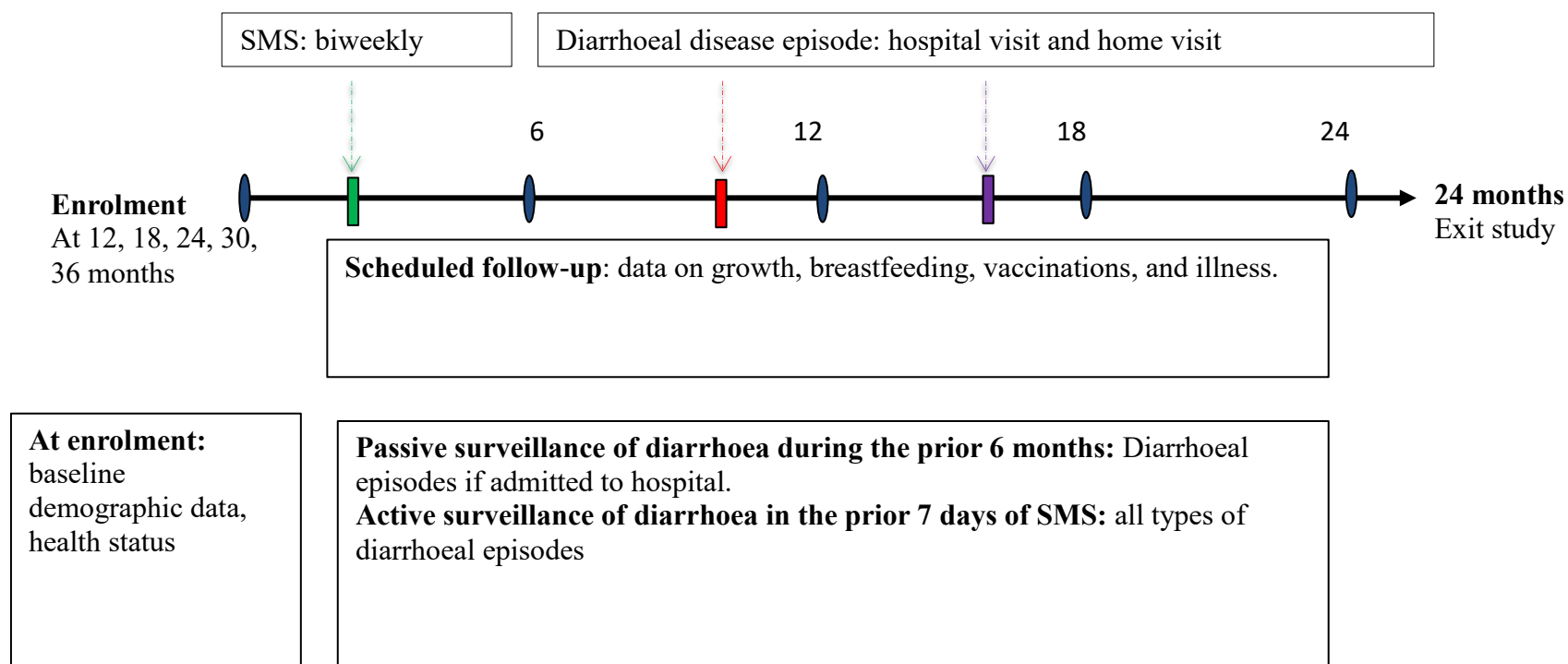


Figure 2.4 Flowchart of diarrhoea cohort. Children were enrolled at age 12 to 36 months with routine follow up visits every six months (shown in blue circles). During enrolment and routine follow up visit, a rectal swab was collected in addition to a CRF detailing growth and health information. Stool samples were collected during diarrhoeal episodes at hospital and home visits (shown in red and purple bar.) SMS were sent every two weeks to ask about diarrhoea episodes within 7 days prior to the day SMS sent (shown in green bar.)

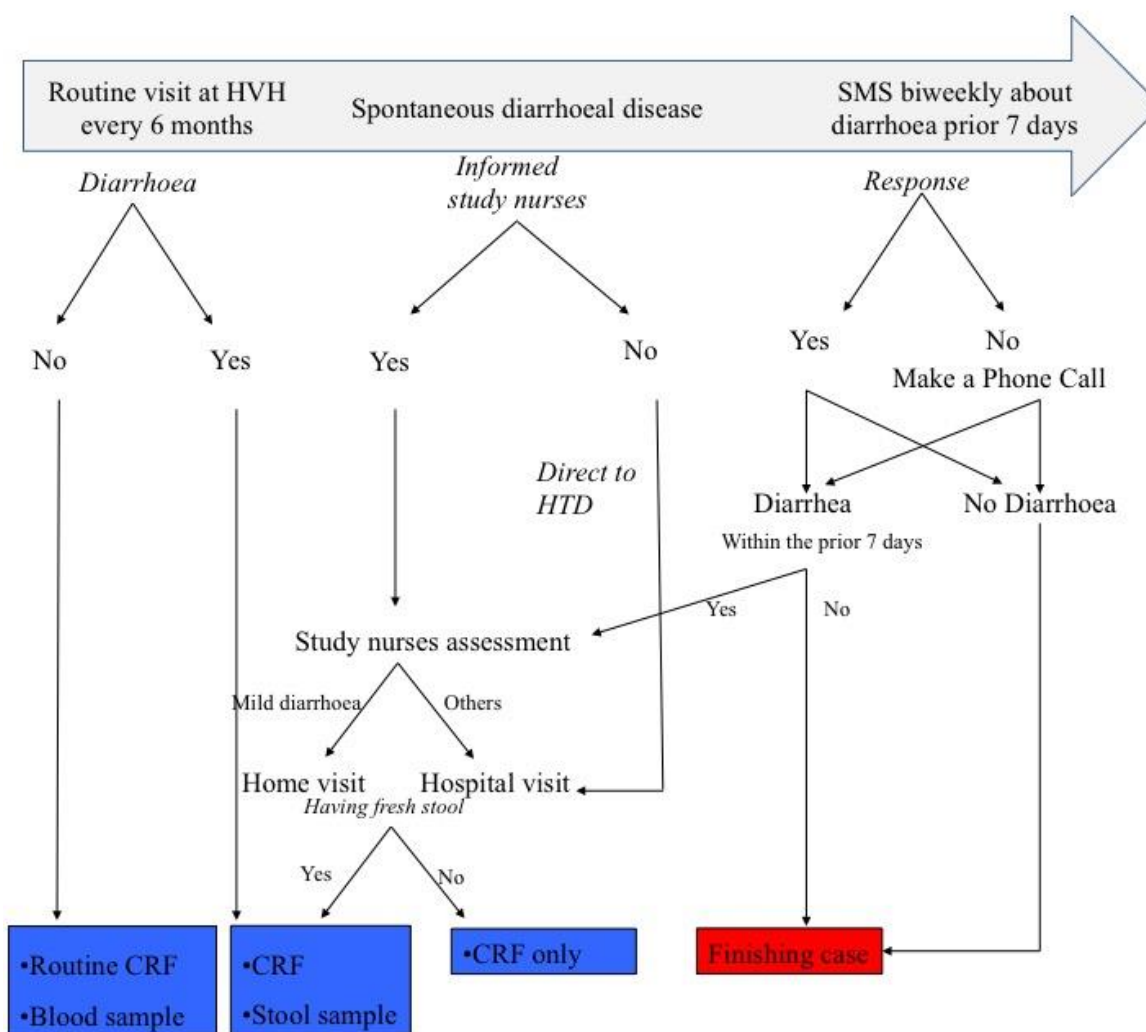


Figure 2.5 Flowchart of diarrhoeal record system in the diarrhoea cohort. Children were followed up every six months at HVH with routine CRF recorded and a blood sample collected. Diarrhoea information from children was obtained either spontaneously (passive at the study hospital) or through SMS (active surveillance). A short CRF were recorded and a stool sample was collected if available.

2.1.3 Project 3: Cross sectional studies in community

2.1.3.1 Study summary

Aiming to investigate the antimicrobials management in the community, I used a multi approach method including a cross-sectional community survey to describe the behaviour of using antimicrobials for paediatric diarrhoea among parents and guardians which in short will be called “community survey” (1), a pharmacy practices study to describe the practice of antimicrobials dispense, which is called the “pharmacy practice study” or “mystery shopper study” (2). In addition, I described the density of pharmacies in the urban area to explore the access of the community to antimicrobials by geographically mapping the health services provided at the locale (Mapping) (3).

2.1.3.2 Ethical approval

Ethics approvals for the mapping of the pharmacies, pharmacy practices, and community surveys were provided through a minimal risk application by the OxTREC (OxTREC approval 5110-16) and the local IRB of the UMP HCMC (No. 220/DHYD-HD). Written informed consent was obtained from all participants over 18 years, or from parents and guardians if participant was less than 18 years.

2.1.3.3 Study location

With an estimated population of >8 million people, HCMC is the largest city in southern Viet Nam ¹⁶⁹. The city is divided into five rural districts and 19 urban districts spread over an area of 19 km²; the urban districts had a mean population density of 13,394 people/km² in 2016 ¹⁶⁹. We selected District 8 in which to quantify access to vendors selling antimicrobials within HCMC. District 8 is located in the central part of the city and is

divided into 16 wards (Figure 2.6) , and had a population density of 22,522 people/km² as of 2016 ¹⁶⁹.

2.1.3.4 Study design

A cross-sectional study was conducted including three components, i) geographical mapping and of all pharmacies and healthcare facilities in District 8 of HCMC, ii) a pharmacy practice study to assess medication suggested by retail pharmacies for paediatric diarrhoea, and iii) a self-reported caregiver community survey on the management and antimicrobial treatment of paediatric diarrhoea.

2.1.3.4.1 Geographical mapping of pharmacies in District 8

Addresses of all registered health clinics and pharmacies in District 8 were obtained from the HCMC Department of Health. Global Positioning System (GPS) coordinates of all pharmacies in District 8 were measured using hand-held electronic devices, such as mobile phones and tablets, and recorded using the geo-location tool EpiCollect5 ¹⁷³.

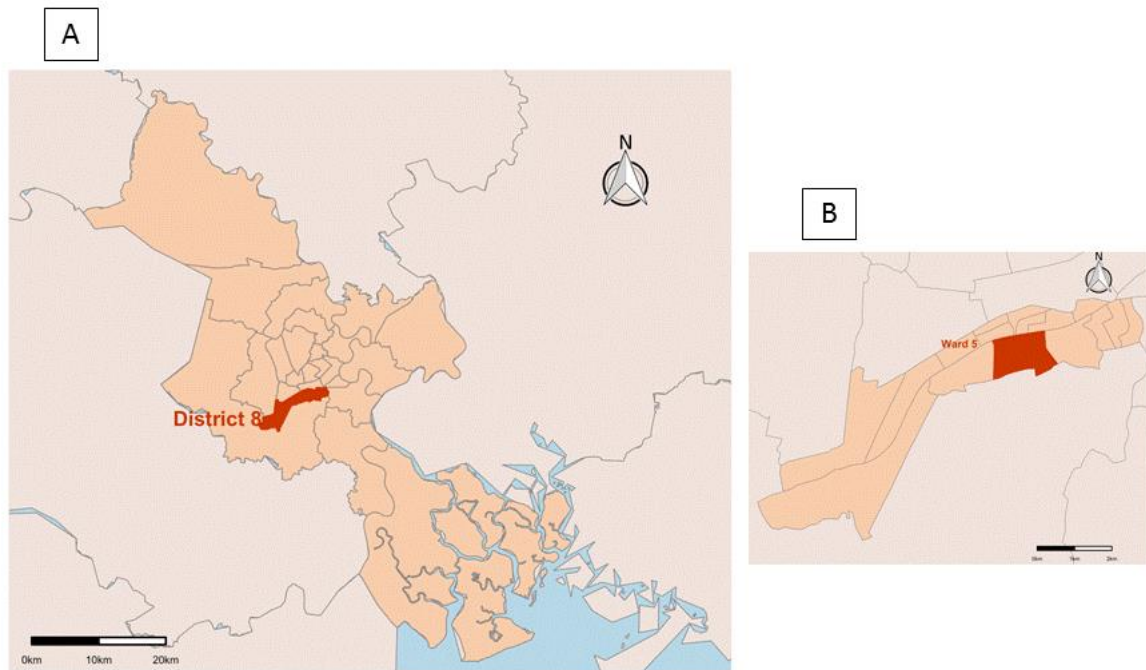


Figure 2.6 The geographical location for this study.

A) Map of HCMC showing the districts 8 (the study area); B) Enhanced map of district 8 of HCMC highlighting ward 5 as the location for the “mystery shopper study” simulated client’s method in red.

2.1.3.4.2 Pharmacy practice study

A pharmacy behaviour study was conducted using an unbiased observational technique for assessing dispensed medication called simulated client method or mystery shopper methodology^{174–177}. Two “mystery shoppers” with no healthcare training role-played as caregivers and approached pharmacies in a sub-region (Ward 5) of the study area to seek medical advice and treatment for two childhood diarrhoea scenarios. *Scenario 1* (watery diarrhoea): A mother attending the pharmacies in the study area to buy medication for a child that is 2 years of age. The child has had 4–5 loose stool episodes within the previous 24 hours. The child has no fever, and no blood or mucus in stools. *Scenario 2* (mucous diarrhoea with fever): A mother attending the pharmacies in the study area to buy medication for a child that is 2 years of age. The child has had 4–5 loose stool episodes within the previous 24 hours. The child had a mild fever and mucus in the stool. Pharmacy-prescribed drugs were labelled anonymously and then were classified by a clinician and confirmed by a qualified pharmacist. Scripts were presented in Table 2.2. In addition, a mystery shopper visited pharmacies in the study area to buy ciprofloxacin, aiming to investigate if antimicrobials are sold without doctor’s prescription. This was a specific scenario in which the shopper requested this antimicrobial and did not provide any information about what or whom the treatment was required for.

Table 2.2 Scripts for the mystery shopping in the pharmacies survey

Scenario	Who	How
Scenario 1: Buying medication for 2-year old child having watery diarrhoeal disease	Mother (M)	M: Greeting and asking for buying medication for my son.
	Drug vendor (V)	V: May ask for age and symptoms of the son. M: Response with these following information: - 2 years old or 27–28 months - 4–5 loose stool since the last 24 hour V: May ask for other symptoms such as blood or mucus in stool. M: Response with “No blood or mucus seen”. In case V doesn’t ask for this information, M doesn’t answer it. V: May ask for other symptoms such as vomit and fever. M: Response with these following information: - No vomiting - No fever V: May ask for how many days of medication the mother would want. M: Response with “Any is fine.” Closing the conversation by saying good-bye and leaving.
Scenario 2: Buying medication for 2-year old child having mucous diarrhoeal disease	Mother (M)	M: Greeting and asking for buying medication for my son
	Drug vendor (V)	V: May ask for age and symptoms of the son M: Response with these following information: - 2 years old or 27–28 months - 4–5 loose stool since the last 24 hour V: May ask for other symptoms such as blood or mucus in stool. M: Response with “Some mucus seen” In case V doesn’t ask for this information, M added this information later in the conversation. V: May ask for other symptoms such as vomit, fever.

M: Response with these following information:

- No vomiting
- Mild fever

V: May ask for how mild the fever is.

M: Response with “ Warmer than usual”

V: May ask for how many days of medication the mother would want.

M: Response with “Any is fine.”

Closing the conversation by saying good-bye and leaving.

2.1.3.4.3 Self-reported behaviour survey in the community

A cross-sectional study was performed in District 8 to capture the self-reported behaviour of parents and caregivers in the community in managing paediatric diarrhoea. The study population consisted of parents and caregivers of children (aged <5 years) residing in District 8, HCMC. I combined proportional sampling techniques including stratified random and simple random sampling. Firstly, a stratified random sampling technique was used to capture a representative sample of the identified population stratified by 16 wards and by age of the children. Secondly, a simple random sampling technique was applied to the currently registered children aged <5 years in the area to identify available individuals for the interviews. Local health authorities accompanied and assisted in community visits. A second home visit was made if caregiver was absent during the first visit. Those who had relocated from District 8 were replaced using a child of similar age chosen by random sampling. Mothers or caregivers were asked a short questionnaire regarding their choice of care if their child had diarrhoea in simulated scenarios similar to those described in the pharmacy behaviour study; this is provided in supplementary information. We surveyed 396 parents or caregivers.

2.2 Variables definition and statistical, microbiology and geospatial analysis

2.2.1. Diarrhoeal incidence (Method for Chapter 3)

The primary aim of this analysis was to describe the incidence of paediatric diarrhoea in the community. Diarrhoeal disease was actively monitored through fortnightly SMS messages sent to the phone of the children's parent/care provider. During diarrhoeal episodes nurses visited homes to confirm the case, collect samples and provide supportive care. Cases were

treated according to local standard of care and were required to attend the HTD in central HCMC for medical care.

2.2.1.1 Specimen laboratory investigations

All stool samples underwent microscopy for parasites. A fresh smear of faecal specimen was prepared in phosphate buffered saline; 10µl of this solution was examined at 400 times magnification to identify *Giardia lamblia* and *Entamoeba histolytica* and *Cryptosporidium* cysts was detected by Ziehl–Neelsen (ZN) stain.

All faecal samples were cultured on MacConkey Agar (MCA), Xylose-Lysine-Deoxycholate Agar (XLD), Selenite broth, and *Campylobacter* media. The purpose of these plates was to identify and isolate *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., and *E. coli*. The MCA, XLD, and Selenite broth were incubated at 37°C overnight and the *Campylobacter* plates were incubated micro-aerophically at 42°C. The identification of potential pathogens was performed using conventional biochemical (API E20 tests) and serological testing. All isolated pathogens were stored for further testing and the isolated organisms were recorded into the study database. To potentially increase the diagnosis rate of *Shigella*, *Salmonella*, and *Campylobacter* a multiplex real-time polymerase chain reaction (PCR) was additionally performed on extracted nucleic acid to detect these pathogens.

To identify pathogenic *E. coli* variants (ETEC, EPEC, EHEC, and EIEC) sweeps of Gram-negative colonies indicative of *E. coli* were collected from MCA plates and stored in 20% glycerol at -80°C. Samples were subjected to PCR to identify pathogenic *E. coli* variants after DNA extraction.

2.2.1.2 Antimicrobial resistance profiling

Antimicrobial susceptibility testing of the pathogens isolated was performed by the disk diffusion method using guidelines established by the Clinical and Laboratory Standards Institute (CLSI 2014) and, when required, by minimum inhibitory concentrations (MICs) by Etest. Antimicrobial susceptibilities tests were performed against Nalidixic acid, Ciprofloxacin, Ceftriaxone, Ceftazidime, Augmentin, Ampicillin, Trimethoprim-sulfamethoxazole, Azithromycin, Chloramphenicol, Imepenem and Amikacin.

The production of ESBL was investigated using the double-disc synergy test by comparing zone sizes between ceftazidime discs against ceftazidime-clavulanic acid discs and cefotaxime discs against cefotaxime-clavulanic acid discs. Isolates with an increase in diameter of inhibitory zone of equal to or more than 5 mm by the synergy of clavulanate were considered ESBL positive.

2.2.1.3 PCR and Luminex procedures

The Luminex xTAG gastrointestinal pathogen panel (GPP) is a qualitative multiplex test which is able to identify 15 separated enteric pathogens every six hours¹⁷⁸. Approximately 200µl of each stool sample was subjected to nucleic acid extraction with 10µl xTAG MS2 (internal control) by MagNA Pure-96 machine (Roche). Each 15µl-master mixture contained 7.5µl of 5X xTAG One Step Buffer, 2.5µl of xTAG GPP Primer Mix, 0.5µl of xTAG BSA (10mg/ml), 2µl of xTAG One Step Enzyme Mix, and water up to 15µl. Approximately 10µl of the extracted nucleic acid sample was subjected to multiplex PCR amplification. The PCR reaction was performed at 53°C for 20 minutes and the thermal cycler conditions used were enzyme activated at 95°C for 15 min; 95°C for 30s; 38 cycles

of 95°C for 30s, 58°C for 30s, and 72°C for 30s; a final extension at 72°C for 2 min; and holding at 4°C.

The xTAG GPP Bead mix and Reporter Buffer containing 0.15M NaCl were thawed and the 0.22 SAPE was diluted 75-fold with the xTAG Reporter Buffer and kept away from light until use. Each reaction contained 20µl xTAG Bead Mix, 5µl PCR product and 75µl of reporter solution. The hybridisation was performed at 600C for 3 min, 450C for 45 min, and holding at 450C. When the hybridisation was complete, the plate was placed on the Luminex plate heater block and read by Luminex® 100/200TM software.

2.2.1.4 Quality assurance

All laboratory experiments in my thesis were conducted in the laboratory at OUCRU Viet Nam. The OUCRU lab management group oversees the laboratories running the same GCLP guidelines (March 2015), and follows the requirements of the Vietnamese MoH. OUCRU laboratory has Biosafety level 2 laboratories certified by the Department of Health HCMC. In addition, the OUCRU Vietnam laboratory is located in the same area as the clinical HTD laboratory, which is accredited by the Bureau of Accreditation of Vietnam on ISO15189: 2012 - VILAS MED 006.

2.2.1.5 Statistical analysis

Diarrhoea incidence rates were calculated based on the number of diarrhoea episodes during the time of following up. Age-specific incidence was calculated based on the event and the follow-up time at certain age group of less than two, from two to three and above three. In addition, pathogen-specific incidences were calculated by using events of diarrhoea episodes with at least 1 stool sample positive with a certain pathogen by

Luminex. Associations between diarrhoeal disease and child age, calendar months, and season were analysed using univariable and multivariable assessment with Poisson regression model.

Diarrhoea incidence was calculated using this following formula:

$$\text{Incidence rate} = \frac{\text{total diarrheal episodes (A)}}{\text{number of children have been followed through a period of time (B)}}$$

From the diarrhoea cohort, diarrhoeal diseases were captured in three manners including hospital visits, home visits, and phone records. Table 2.3 shows a summary of diarrhoeal disease episodes features capture from the diarrhoea cohort.

Incidence of diarrhoea was estimated based on the data from the diarrhoea cohort. A diarrhoea episode was estimated to last an average of 4–8 days, and a new episode was measured by 2–3 consecutive diarrhoea-free days. As only start date of a diarrhoea record (reported by participant) was available, I employed a pragmatic approach to define a diarrhoea episode with two criteria: (1) the start date of an episode was either the first date of diarrhoea records or the date of diarrhoea record with at least 2 consecutive diarrhoea-free days from the previous diarrhoea record, (2) duration of a diarrhoea episode was at least 7 days. This definition is described in the 4 scenarios in Figure 2.7.

Table 2.3 Features of diarrhoea episodes captured from the diarrhoea cohort

Diarrhoea captured from	Method	Stool sample	Diarrhoea severity	Potential bias
Hospital visit	Passive	Sample available	Reflect moderate to severe diarrhoea	Underestimate if participate came to different hospital
Home visit	Passive (trigger by patient), active (trigger by SMS)	Sample available if diarrhoea is ongoing	Reflect mild to moderate diarrhoea	Underestimate if patient forgot
Phone	Active (trigger by SMS) (Only capture diarrhoea within 7 days before SMS + day when SMS sent (7.5 days period))	Sample not available	Reflect all type of diarrhoea	Underestimate mild diarrhoea (recall bias, forgot), overestimate severe diarrhoea (recall bias)

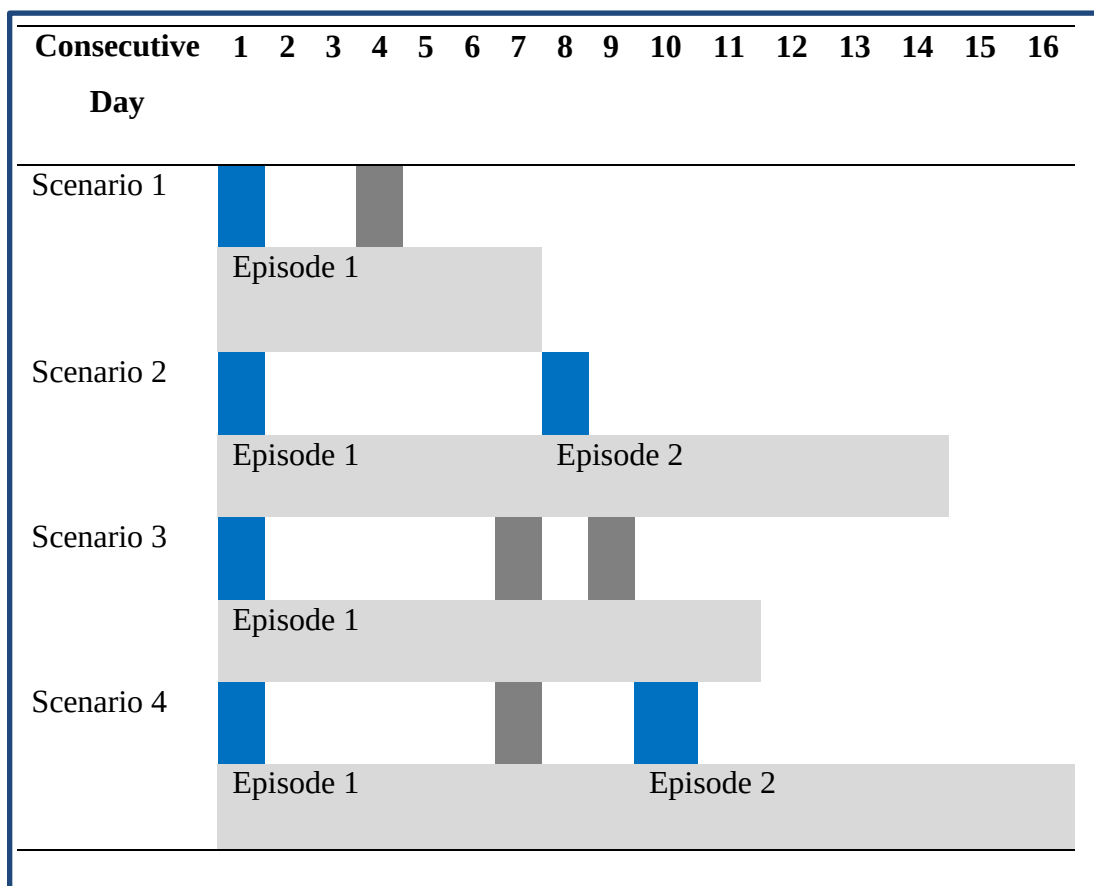


Figure 2.7 Definition of diarrhoea episode in 4 scenarios.

A new episode starts with at least 2 consecutive diarrhoea-free days from the previous diarrhoea record. Duration of a diarrhoea episode is at least 7 days. Counted diarrhoea episodes are in blue.

Considering the potential bias of different sources of diarrhoea in this study (Table 2.3), I used two approaches to estimate incidence of diarrhoea. In the first approach, all diarrhoea episodes were included (*event*) and the follow-up period was total follow-up time from study enrolment to study exit (*follow-up time*) (*Approach 1- all available information used*). In the second approach, I only used information captured by SMS (*Approach 2*). This approach used all diarrhoea episodes that were captured in the periods covered by SMS (*event*), with the follow-up time include all periods covered by SMS (*follow-up time*). As SMS was sent biweekly to collect diarrhoea information within the prior 7 days and assuming that this SMS was read at the midday on average, each SMS covered a 7.5 follow-up days. Overlapping SMS periods were combined a single SMS period and the total follow-up time covered by these SMS periods can be used to calculate incidence rate of diarrhoea captured within these periods.

As the active surveillance was based on SMS communication with SMS sent biweekly but only covered diarrhoea information within the prior 7 days, incidence of diarrhoea based on approach 1 may underestimate the true number. Given assumption that the occurrences of diarrhoea are similar between weeks covered and uncovered by SMS, approach 2 may provide a more realistic estimate of diarrhoea incidence. I used Poisson regression to calculate overall diarrhoea incidence as well as incidence by sources of information (home visit or hospital visit), by age (as continuous variable or categorical variable with 3 groups: 12–23, 24–35 and ≥ 36 months) and by pathogens (as detected by Luminex assay). To compare performance of Luminex versus cultures in detecting pathogen from stool samples, I described detection rate of each pathogen by each method. I also calculated the

sensitivity of Luminex with culture as a reference and vice versa, as well as positive agreements between these diagnostic methods.

2.2.2 Geospatial analysis and quantifying antimicrobial use for diarrhoeal treatment in the community and hospital (Method for Chapter 4)

2.2.2.1 Geospatial analysis

Viet Nam administrative boundaries were downloaded from Global Administrative Divisions Map (GADM). Viet Nam population data rasters for 2015 (i.e. population per 0.01 Km²) were downloaded from WorldPop (100 m resolution), and satellite imagery of Viet Nam was downloaded from ESRI basemaps. All geospatial mapping was conducted using ArcGIS v10.2 (Redlands CA, USA). Euclidean distance to the nearest pharmacy and kernel density plots for pharmacy density (i.e. pharmacies per 0.01 Km²) was estimated for District 8 using the “*Euclidean distance*” and “*Kernel*” tools in ArcGIS, respectively. Raster map depicting pharmacies per 10,000 people was computed by dividing the pharmacy density raster by the 2015 population raster using “*Raster Calculator*” in ArcGIS.

2.2.2.2 Quantifying antimicrobial use for diarrhoeal treatment in the community and hospital

Considering that antimicrobial usage for diarrhoeal disease was dependent on the number of diarrhoeal episodes seeking medical treatment and how likely antimicrobials were to be prescribed for diarrhoeal treatment, I aim to quantify the actual use of antimicrobials in the community in chapter 4. Figure 2.8 outlines the parameters, the source of data and the calculations used to quantify antimicrobial usage for diarrhoea in children aged 1- 5 years.

The annual number of diarrhoeal episodes presenting at pharmacies was the product of (A) the total number of children aged 1–5 years in the study area, (B) the incidence rate of diarrhoea episodes per child-year, and (E) the proportion of parents or caregivers selecting to firstly take their child to a pharmacy. The annual number of diarrhoeal episodes presenting at hospitals was multiplied by (A) the total number of children aged 1–5 years living in the study area and (C) the incidence rate of diarrhoea episode presenting at hospital per child-year. Parameter (A) was derived from recent census data from the HCMC Demographic department (30/06/17). Incidence rates (B) and (C) were estimated from a prospective diarrhoeal cohort conducted between 2014 and 2016 that actively followed 748 children in residing in the study area 17. The proportion of parents or caregivers seeking pharmacies as their first choice (E) was derived from the self-reported behaviour survey of parents and caregivers. To quantify the likelihood of prescribing antimicrobials for diarrhoeal treatment at a hospital or a pharmacy, we used antimicrobial treatment data from (D) the prospective cohort study 17 (from hospital files) and (F) the pharmacy behaviour study, respectively.

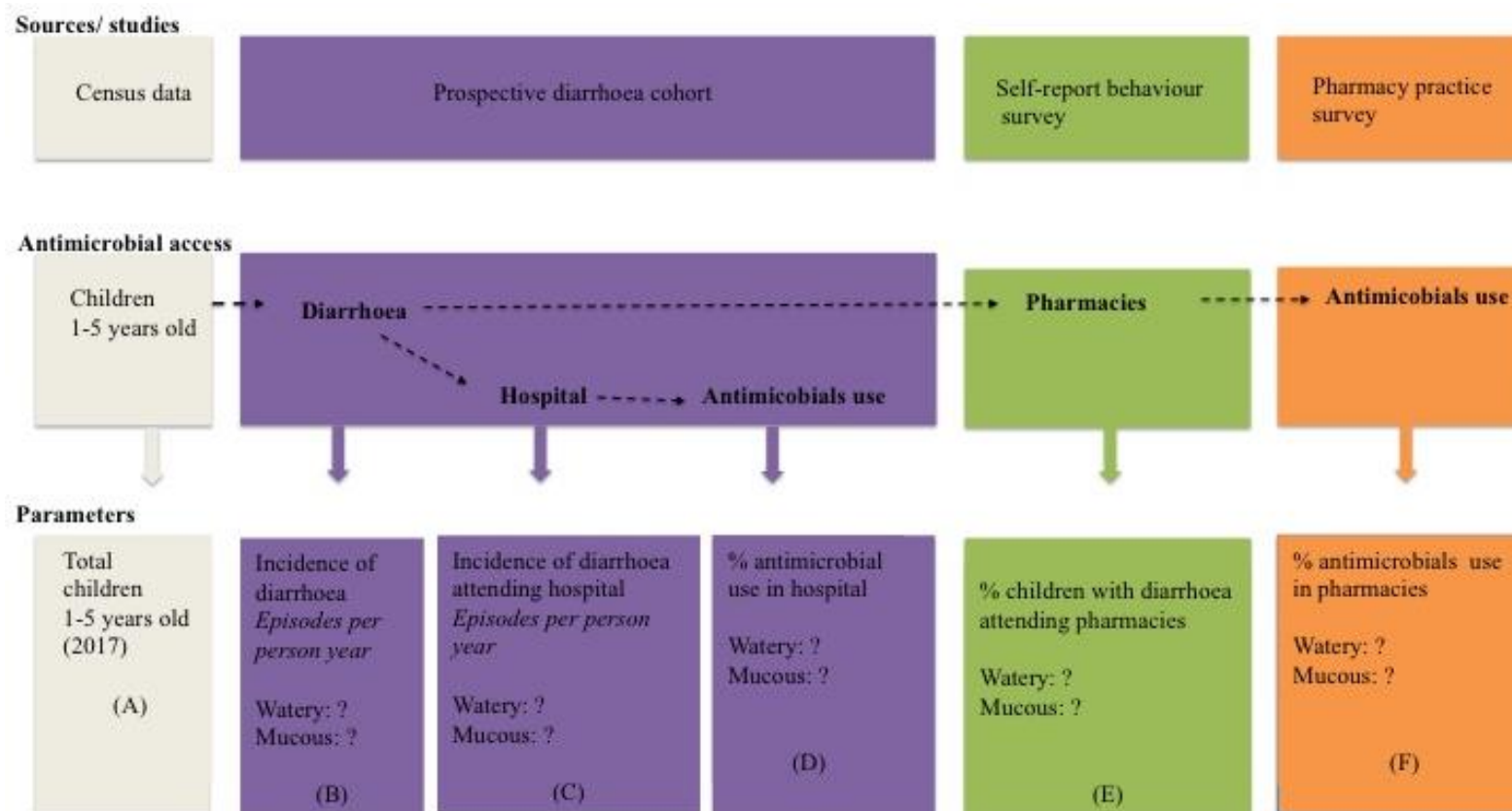


Figure 2.8 Methods and sources for estimating antimicrobial usage for diarrhoea in children aged 1 to 5 years in pharmacies and hospital. Diagram outlining the sources of data, parameters, and calculations used to quantify antimicrobial usage for diarrhoea in children aged 1- 5 years in this population. Note each of the parameters have been given an initial (A-F), which inform the calculations in Result chapter 4 (Table 4.5).

2.2.3 Resistant commensal *E. coli* and resistant genes (Method for Chapter 5)

Aiming to describe the prevalence of AMR bacteria and the diversity of associated AMR genes in the commensal microbiota of healthy children living in an urban community, to identify epidemiological risk factors associated with the carriage of phenotypic (AROs) and genotypic (ARGs) resistance in these urban children, I measured phenotypic and genotypic resistance against fluoroquinolones and third generation cephalosporins among a cohort of 498 healthy children resident in HCMC. These participants were from the diarrhoea cohort.

2.2.3.1 Study population

All rectal swabs were collected from healthy children who were participants of the diarrhoea cohort that previously described in section 2.1.2 (Diarrhoea cohort). In brief, infants were recruited into the birth cohort at HVH in HCMC, the second largest obstetric hospital in South Viet Nam, and followed for 12 months for febrile disease through passive surveillance, which has been described in section 2.1.1 (Birth cohort). A subset of these children were followed beyond 12 months and recruited into a continuous cohort conducting active surveillance for diarrhoeal disease. The children attended scheduled routine follow up visits at HVH every six months. Information on demographics, household water sources, sanitation, and health issues in the intervening six months between visits, and food consumption habits in the three days prior to the visit was collected. A rectal and nasopharyngeal swab was collected at the time of the visit. There were 748 children enrolled from June 2014 to December 2014 and followed to December 2016 in the diarrhoea cohort.

2.2.3.2 Identification of antimicrobial resistant *E. coli*

From March to August 2016, we collected 498 rectal swabs from healthy children (no diarrhoea or other symptoms of illness) at routine follow up visits for the purposes of this investigation. At routine follow up visits, we recorded information regarding their general well being and regarding their antimicrobial practices in the prior six months. All rectal swabs were inoculated onto MCA to pick all suspected *E. coli* colonies (e.g. lactose fermenting and non-lactose fermenting, circular, elevated, entire margins, smooth texture, pink or colourless, dry, and shiny). These meta *E.coli* population were then inoculated into brain heart infusion (BHI) media supplemented with 20% glycerol and stored at -80°C until being subjected to inoculation onto MCA agars supplemented with either ciprofloxacin (4mg/l) or ceftriaxone (6mg/l) to select for resistant organisms against these antimicrobials. *E. coli* strain ATCC 25922 (MIC against ciprofloxacin and ceftriaxone 0.008 mg/l and 0.094 mg/l, respectively) and a clinical *E. coli* strain 03_0334 (MIC against ciprofloxacin and ceftriaxone with >256mg/l) were used as negative and positive controls for testing the culture media. All suspected *E.coli* colonies of each plate were then confirmed by MALDI-TOF.

2.2.3.3 Detection of antimicrobial resistant genes

The meta *E. coli* population in frozen BHI were subjected to nucleic acid extraction and multiplex PCR amplification. Approximately 200µl of each meta-*E. coli* in frozen BHI was removed and aliquoted into a 96-well plate and centrifuged for 10 minutes at 4,000rpm. The supernatant was removed and discarded. The remains in the wells were added 200µl sigma water and the bacterial solution was used to gentle agitation to ensure mixing.

Nucleic acid was extracted by heating the solution to 100°C on a heating block for 10 minutes, then cooling to ambient temperature. The solution was subjected to centrifugation for 10 minutes at 4,000 rpm in a microfuge. The supernatant was removed and used as template DNA in all PCR amplifications. We conducted a series of multiple PCR amplifications aiming to detect PMQR genes (*aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, and *qnrS*), ESBL genes (*bla_{CTX-M}*, *bla_{TEM}*, *bla_{SHV}*, and *bla_{OXA}*), and AmpC lactamase genes (MOX, DHA, EBC, FOX, ACC, CIT). The primer sequences and the predicted amplification sizes are shown in Table 2.4. The conditions were as previously described^{179–182} a positive PCR result was determined by the detection of a PCR amplicon of an appropriate size (using a UV transilluminator and gel documentation system) for each target in the presence of appropriate positive and negative controls.

2.2.3.4 Statistical analyses

Categorical and continuous variables were described using frequencies (%) and medians (interquartile range), respectively. Bar plots were used to describe the prevalence of ciprofloxacin and ceftriaxone resistant *E. coli* and the various ESBL and PMQR genes. I used univariable and multivariable logistic regression to assess the relationship between participants' characteristics and the presence of AMR *E. coli* and AMR associated genes. These relationships were described in odds ratios (OR) and their corresponding 95% confidence intervals (CI). The significance level for statistical tests was 0.05. All analyses were performed in R v3.4.0 (R Foundation, Vienna, Austria) and companion packages.

Table 2.4 PCR primers, targets, and predicted amplification size

Target gene	Primer name	Primer sequence (5' → 3')	Predicted size of amplicon (bp)
<i>qnrA</i>	QnrA-F	TCAGCAAGAGGATTTCTCA	627
	QnrA-R	GGCAGCACTATTACTCCCA	
<i>qnrB</i>	QnrB-F	GGMATHGAAATTCGCCACTG	264
	QnrB-R	TTTGCGYGYCGCCAGTCGAA	
<i>qnrS</i>	QnrS-F	ATGGAAACCTACAATCATAC	491
	QnrS-R	AAAAACACCTCGACTTAAGT	
<i>aac(6')-Ib</i>	aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	482
	aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT	
<i>qepA</i>	qepA_F	GCAGGTCCAGCAGCGGGTAG	199
	qepA_R	CTTCCTGCCCCGAGTATCGTG	
CTX-M	CTX-M-1F	AAAAATCACTGCGCCAGTTC	415
	CTX-M-1R	AGCTTATTCATCGCCACGTT	
	CTX-M-2F	CGACGCTACCCCTGCTATT	552
	CTX-M-2R	CCAGCGTCAGATTTTTCAGG	
	CTX-M-9F	CAAAGAGAGTGCAACGGATG	205
	CTX-M-9R	ATTGGAAGCGTTCATCACC	
	CTX-M-8F	TCGCGTTAAGCGGATGATGC	666
	CTX-M-25F	GCACGATGACATTCGGG	
	CTX-M-8/25R	AACCCACGATGTGGGTAGC	327
MOX	MOXF	GCTGCTCAAGGAGCACAGGAT	520
	MOXR	CACATTGACATAGGTGTGGTGC	
CIT	CITF	TGGCCAGAACTGACAGGCAAA	462
	CITR	TTTCTCCTGAACGTGGCTGGC	
DHA	DHAF	AACTTTCACAGGTGTGCTGGGT	405

	DHAR	CCGTACGCATACTGGCTTTGC	
ACC	ACCF	AACAGCCTCAGCAGCCGGTTA	346
	ACCR	TTCGCCGCAATCATCCCTAGC	
EBC	EBCF	TCGGTAAAGCCGATGTTGCGG	302
	EBCR	CTTCCACTGCGGCTGCCAGTT	
FOX	FOXF	AACATGGGGTATCAGGGAGATG	190
	FOXR	CAAAGCGCGTAACCGGATTGG	
TEM	TEMF	CATTTCCGTGTCGCCCTTATTC	800
	TEMR	CGTTCATCCATAGTTGCCTGAC	
SHV	SHVF	AGCCGCTTGAGCAAATTAAAC	713
	SHVR	ATCCCGCAGATAAATCACCAC	
OXA	OXAF	GGCACCAGATTCAACTTTCAAG	564
	OXAR	GACCCCAAGTTTCCTGTAAGTG	

2.2.4 Breastfeeding and diarrhoeal disease (Method for Chapter 6)

I aimed to assess the prevalence of various breastfeeding practices, including exclusive breastfeeding before hospital discharge at birth, and any breastfeeding activity among infants who have participated in the birth cohort that was described under section 2.1.1. I aimed to explore patterns of infant feeding during the first year of life and identify risk factors associated with inadequate breastfeeding practices at birth in urban and semi-rural areas. A secondary aim was to investigate whether breastfeeding practice at birth was associated with time until a primary diarrhoeal episode and the number of diarrhoeal episodes in the first year of life.

2.2.4.1 Breastfeeding data collection

At enrolment, breastfeeding practices were assessed using a multiple-choice question regarding whether infants were exclusively breastfed, breastfed partially, or formula-milk fed after delivery. At each routine follow up visit, breastfeeding practices were again assessed by asking a using a multiple-choice question on whether infants were currently breastfed, exclusively breastfed, or fed by different combinations of breast milk, formula-milk and solid food.

2.2.4.2 Diarrhoea information collection

In the birth cohort, information on diarrhoeal diseases during the first year of life was collected through two main sources. The first source was that diarrhoeal diseases covered the period prior to the visit through questions at each routine visits (follow-up data). In addition, diarrhoeal episodes were passively detected when the infants came to visit any clinics or hospitals with diarrhoeal diseases (passive data). In case the child was admitted to

the hospital, there was a detailed clinical evaluation recorded, otherwise a brief clinical report was reported.

2.2.4.3 Main variable definitions

In the birth cohort that described in section 2.1.1, breastfeeding practices were collected at each routine interview as (i) “exclusive breastfeeding” if only receiving breast milk, or (ii) “partial breastfeeding” if receiving breast milk in combination with other types of food, or (iii) “no breastfeeding” if not receiving breast milk. Table 1.3 under section 1.5.1.2 in the Introduction chapter of this thesis has provided a definition of some common breastfeeding practices recommended by the WHO. For the purpose of the analysis in result chapter 6, the variables of breastfeeding practices included (1) “exclusive breastfeeding at birth”, (2) “any breastfeeding at birth”, (3) “any breastfeeding”, (4) “exclusive breastfeeding for 4 months” and (5) “exclusive breastfeeding for 6 months”. (1) Exclusive breastfeeding at birth was defined as exclusive breastfeeding initiated before hospital discharge after delivery, (2) “any breastfeeding at birth” was defined as either exclusive or partial breastfeeding before hospital discharge after delivery, (3) “any breastfeeding” was defined as either exclusive or partial breastfeeding at any time-point from birth to the last follow-up visit. (4) “Exclusive breastfeeding for 4 months” and (5) “exclusive breastfeeding for 6 months” were defined by the duration of exclusive breastfeeding among infants from birth to the 4 months and 6 months follow-up visits, respectively.

The selected outcome for the main analysis was exclusive breastfeeding at birth. The explanation variables were pre-defined as those that could affect breastfeeding practice at birth. These variables included socioeconomic status (household income), general

characteristics of the mothers (first live child, age, education [high education meaning >9 years at school], occupation, ethnic group, marital status, living arrangements, complications during pregnancy, and infection with either HIV or Hepatitis B), general characteristics of the fathers (education, occupation and ethnic group), general characteristics of the infants (age at birth, sex, delivery method (Caesarean section or vaginal birth), low birth weight (birth weight <2,500g), premature birth (gestational age at birth < 37 weeks) and neonatal complications). These variables were selected based on subject-matter knowledge and data availability. The Social Economic Status (SES) score was derived using the Demographic and Health Surveys Program using principle component analysis which was categorized into quintile levels ¹⁸³.

For the secondary analysis, I investigated whether breastfeeding practices at birth had any potential association with diarrhoeal disease. The main outcomes were the frequency of diarrhoeal episodes during the first 12 months of life and time to the first reported diarrhoeal episode. Each diarrhoeal episode was defined as at least three loose stool or one mucus or bloody stool within 24 hours , and occurring within seven days between the onset dates of symptoms as described in a previous study ¹⁸⁴. The pre-defined explanation variables were breastfeeding practices (exclusive breastfeeding and any breastfeeding at birth). I adjusted the association by maternal level factors (maternal age and education), and infant level factors (sex, low birth weight status, and mode of delivery).

2.2.4.4 Statistical analysis

Data generated in HCMC and Dong Thap were analysed separately due to differences in context and follow-up schedules. As missing data in variables of interest was low (the

maximum number of missing data per variable was 11 and the number of cases with at least one missing value (%) was 19/6706 (<1%)), I conducted a complete-case analysis.

Descriptive analyses were presented by frequencies and proportions for categorical variables and medians and interquartile ranges for continuous variables. Comparisons between groups of participants were performed using the Kruskal-Wallis test for continuous variables, and Chi-squared or Fisher's exact test for categorical variables. Univariable and multivariable logistic regression were performed to determine factors associated with not initiating exclusive breastfeeding at birth.

I used Cox proportional hazard regressions models, with adjustment for maternal age and education, infant sex, low birth weight status, and mode of delivery to investigate the association between breastfeeding practices (exclusive breastfeeding and any breastfeeding at birth) and time to the first episode of diarrhoeal disease. I used negative binomial regression models with adjustment for similar factors to explore the association between breastfeeding practices and the number of diarrhoeal episodes. These analyses were stratified by type of data collection on diarrhoeal diseases (follow-up and passive data), and urban/semi-rural areas. Statistical significance was defined as a p value <0.05; all analyses were performed on R statistical software¹⁸⁵.

3. The incidence and aetiology of diarrhoea in children: a two-year active surveillance cohort study in an urban community in Viet Nam

Diarrhoeal diseases remain amongst the primary causes of mortality among children under five years in LMICs^{2–4,6}. In Viet Nam, the burden of hospitalised paediatric diarrhoeal disease is well-described^{29,119,141,186–189}; however, the true burden and aetiology of diarrhoeal disease in the community are not well documented. In addition, previous estimations of diarrhoea incidence from population-based studies have been conducted without an optimal surveillance method^{184,190–192}. Between 2014 and 2016, we prospectively followed a cohort of 748 children aged between one and three years living in an urban area in southern Viet Nam. Active surveillance with SMS messaging and phone calls were conducted bi-weekly to caregivers to acquire further diarrhoeal disease episodes in their children in the preceding seven days. Children with severe diarrhoea were advised to visit a study hospital. Faecal samples were collected during the diarrhoeal episodes at home or at hospital. These samples were then examined by microscopy for parasites, by culturing for bacterial pathogens, and by the Luminex xTAG GPP assay to identify 15 pathogens. I used data from this cohort (Method section 2.1.2 Diarrhoea cohort) to investigate the true burden of diarrhoeal diseases in the community and characteristics of mild to moderate diarrhoea among children aged one to five years in urban HCMC.

3.1 Background

While studies on diarrhoeal diseases are generally historically focused on clinical infections with specific pathogens and disease manifestations, the true burden of diarrhoeal disease in the community remains largely underestimated. A recent notable case-controlled study, the

GEMS study, investigated population-based diseases in seven areas in Africa and Asia since 2013 and focused on the aetiology of moderate to severe diarrhoea¹⁵. A further prospective longitudinal study, the MAL-ED study, investigated diarrhoeal diseases in eight high-disease incidence sites in Africa, Asia, and North America, and followed children up to the age of 24 months¹⁹³.

Viet Nam is among the most densely populated urbanizing countries in Southeast Asia^{1,194}. Improvements in hygiene and sanitation as well as increased quality in health policies and practices have contributed to the decrease of the disease burden; however, diarrhoea still plays a crucial role regarding the primary causes of deaths among children under five, in which the mortality rate was approximately 24 per 1,000 live births in 2013¹⁹⁵. Further, the trend of diarrhoeal diseases in urban areas, such as HCMC, the largest city in the south of Viet Nam, remains elusive despite the implementation of the Law on prevention and Control of Infectious Diseases in 2008⁷⁸. Diarrhoeal disease remains a common reason for hospital visits with more than 20,000 children annually admitted to hospital for diarrhoea¹⁹⁶. The purpose of this study was to determine the overall incidence rate and the age-specific incidence rate of diarrhoeal disease among children under five in the community in the urban area of HCMC. Additionally, I investigated the pathogen-specific aetiology associated with diarrhoeal disease in this area.

3.2 Results

3.2.1 Baseline features of the cohort and diarrhoeal diseases surveillance

Between June 2014 and December 2016 there were 748 healthy children aged one to three years enrolled in a cohort study, which had a follow-up rate of 94% (Figure 3.1). Among

the 45 (45/748; 6%) drop outs, one (1/45, 2%) had died of severe pneumonia and cerebral palsy and one (1/45, 2%) had died of accident (drowning) while the majority (28/45; 62%) dropped out due to a fear of having blood drawn during scheduled visits at HVH. Other reasons included moving out of the study area (9/45; 20%) and a loss of contact information (6/45; 13%). The majority of participants (704/748; 94%) finished all four routine follow-up visits. A total of 35,626 SMS messages were sent to participants during two years of following up, most of them (33,191/35,626; 93%) being within the follow-up time. The median of SMS messages per participant was 50 (IQR 46-52) per two years, with the median time lag between these SMS messages being 14 days (IQR 14–15). These SMS messages produced 35,439 SMS periods (33,106 [93%] were in the follow-up time), which covered a median of 50% (IQR 46%-51%) of the follow-up time per participant. The Lexis diagram in Figure 3.2 shows an overview of the total follow-up time, the follow-up time covered by SMS periods, and the routine follow-up visit of each participant. Of note, the time lags between SMS periods were longer in February 2015 and February 2016 due to the national New Year holidays (Tet). There were 2,333 SMS periods when the follow-up durations were stopped. As participants were not formally followed up during these late periods, these periods and any diarrhoea information which occurred during these periods were excluded from the following analyses.

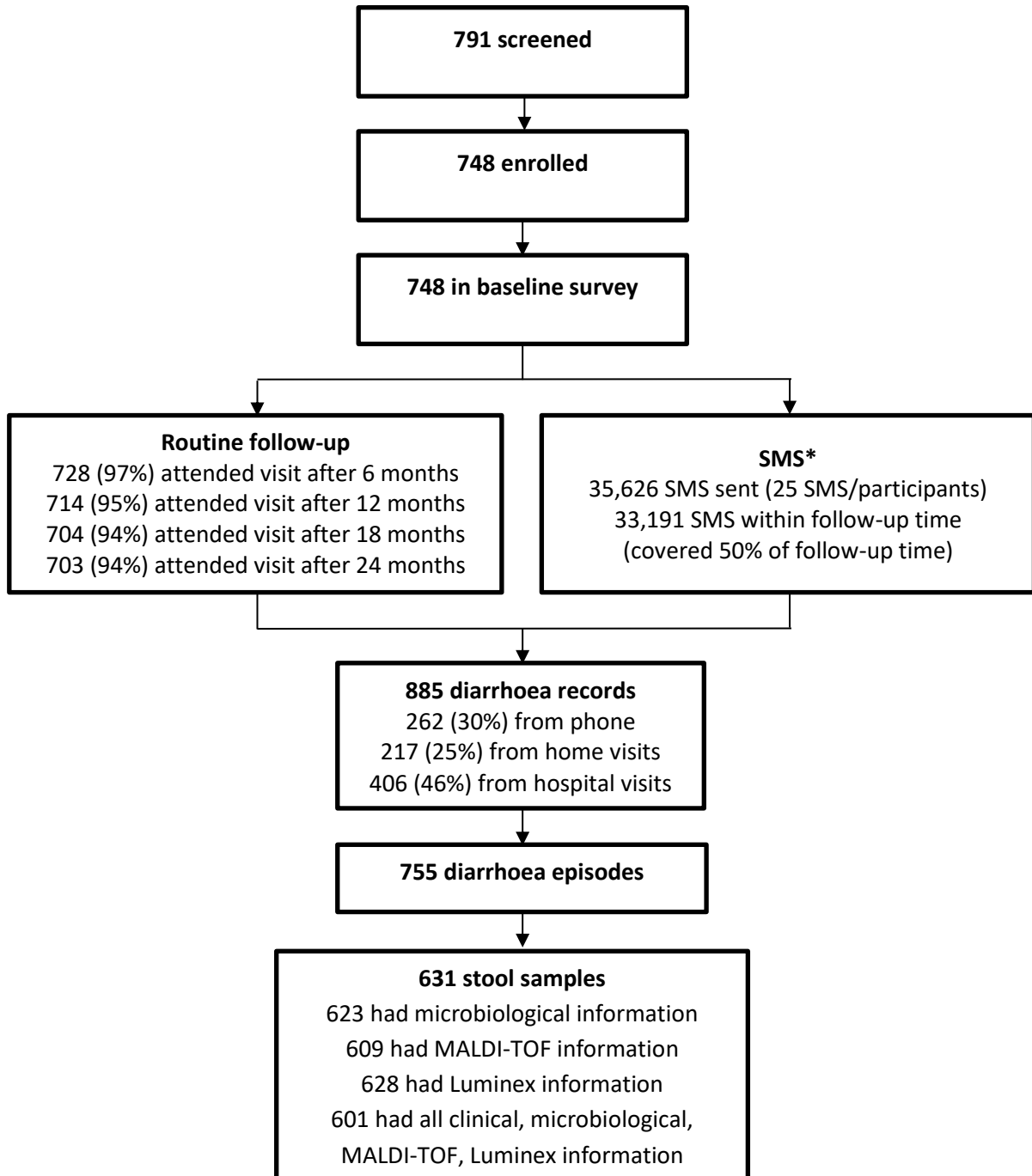


Figure 3.1 Diarrhoea cohort profile. Text messages (SMS) were sent to the parents and caregivers biweekly to ask about their children’s diarrhoea in the prior 7 days. 755 diarrhoea episodes were calculated from 885 diarrhoea records as defined in Figure 2.7 under section 2.2.1.4 in the Method (Chapter 2).

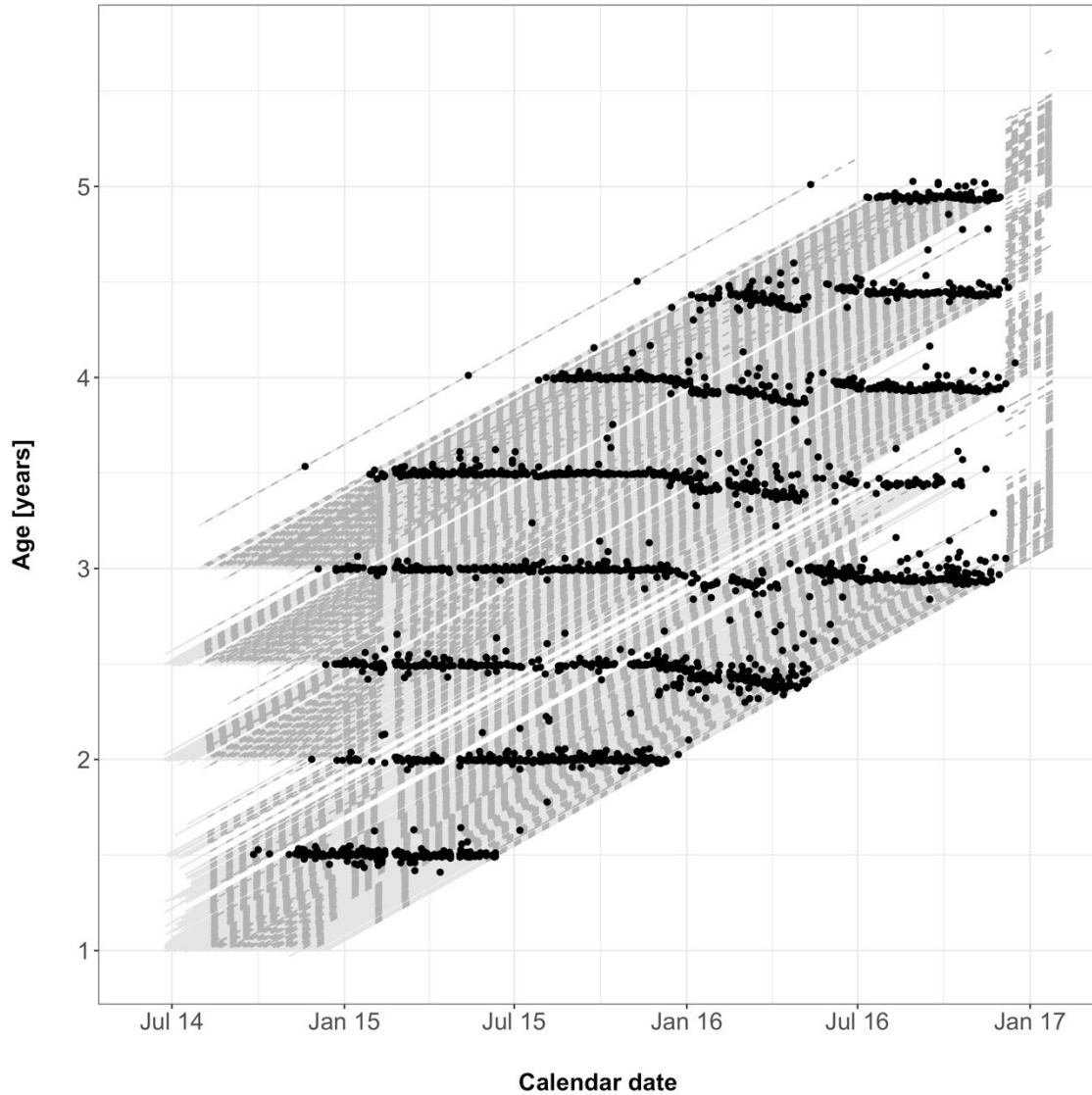


Figure 3.2 Lexis diagram of all participants during follow-up visits. Grey lines are follow-up time. Dark grey segments are periods covered by SMSs. Black dots are routine follow-up visits.

The median age of 748 children at enrolment was 24 months (IQR: 12 to 30 months) (Table 3.1). Almost half (350/748; 47%) of the participants were female. Forty-three percent were born by C-section (325/748; 43%), and at the time of delivery 14% (104/748) were preterm and 4% (29/748) had a low birth weight. Almost every child in the cohort (694/748; 93%) had been breastfed at least once by the time of enrolment. Rotavirus vaccination had been given in two doses to almost half of the participants at enrolment (304/748, 41%). A quarter of the children (179/748; 24%) were attending kindergarten/school at the time of enrolment. Two-thirds of the participants (491/748; 66%) had piped water to their residence. A small proportion of households were exposed to flooding (164/748; 22%), and 60% (450/748) were commonly exposed to animals around the home (Table 3.1). Approximately half of the households (416/748; 56%) regularly bought toilet paper. Notably, almost every household had soap routinely in their toilet room (739/748; 99%) but only 53% (400/748) of parents reported that their children always washed hands after defecating and 86% (641/748) reported washing hands after changing diapers.

Table 3.1 Baseline characteristics of participants at enrolment (n = 748).

Characteristics	Frequency (%)
Child age (months)*	24 (12, 30)
Child age group	
12–23 months	382/748 (51%)
24–35 months	253/748 (34%)
>= 36 months	113/748 (15%)
Female	350/748 (47%)
Caesarean section	325/748 (43%)
Premature at birth	31/748 (4%)
Low birth weight	29/748 (4%)
Ever breastfeeding	694/748 (93%)
School/kindergarten attendance	179/748 (24%)
Age of mother (years) (n= 746)	28 (25, 32)
Low maternal education (<=9y)	394/748 (53%)
Crowding (> = 2 people/room)	519/748 (69%)
Main water source	
Piped to residence	491/748 (66%)
Bottled water	248/748 (33%)
Well	9/748 (1%)
Defecate	
Chamber pot	400/640 (62%)
Toilet	171/640 (27%)
Floor	55/640 (9%)
Outside	3/640 (<1%)
Other	11/640 (2%)
Hand washing after defecating	
Always	400/748 (53%)
Sometimes	346/748 (46%)
Never	2/748 (<1%)
Toilet paper	416/748 (56%)
Toilet soap	739/748 (99%)
Diaper wear	126/748 (17%)
Handwashing after changing diaper	
Always	641 (86%)
Sometimes	107 (14%)
Exposure to flooding around the house	
None	584/748(78%)
Seldom	91/748(12%)
Moderate	67/748(9%)
Severe	6/748(1%)
Exposure to animal around the house	450/748 (60%)

* age in median (range)

At enrolment, 210/748 (28%) had a medical condition at clinical examination. Common illnesses were respiratory infections (21/210, 10%), asthma (10/210, 5%), and diarrhoea (5/210, 2%). Eight children were considered to be malnourished (8/210, 4%, weight-for-age Z-score <-1.5) and 29/210 (14%) were considered as obese (weight-for-height Z-score >1.5). From all participants, there was one child ($<1\%$) that was severely underweight and stunted and two children ($<1\%$) that were severely wasted. Notably, 6% (44 children) of children were classified as being obese based on weight-for-height Z-score (Table 3.2). The majority of the children received vitamin A during a government supplement program (574/748; 77%). There were 22 children (3%) whose parents reported having dysentery, which means having mucous or bloody stool, prior to the enrolment visit.

Table 3.2 Health related characteristics of participants at enrolment (n=748).

Characteristics	Frequency (%)
Weight-for-age Z-score	0.2 (-0.5, 1.1)
≥ -2	736/748 (98%)
[-3, -2) (Underweight)	11/748 (1%)
< -3 (Severely underweight)	1/748 (<1%)
Height-for-age Z-score	0.0 (-0.8, 0.6)
≥ -2	729/748 (97%)
[-3, -2) (Stunted)	18/748 (2%)
< -3 (Severely stunted)	1/748 (<1%)
Weight-for-height Z-score	0.4 (0.3, 1.3)
> 3 (Obese)	44/748 (6%)
(2, 3] (Overweight)	65/748 (9%)
(1, 2] (Risk of overweight)	126/748 (17%)
[-2, 1]	502/748 (67%)
[-3, -2) (Wasted)	9/748 (1%)
< -3 (Severely wasted)	2/748 (<1%)
Rotavirus vaccination	
1 dose	314/748 (42%)
2 doses	304/748 (41%)
Sickness at enrolment	210/748 (28%)
Abnormal conditions defined at enrolment	
Respiratory infection	21/748 (3%)
Diarrhoeal disease	5/748 (1%)
Hand foot mouth disease	1/748 (<1%)
Asthma	10/748 (1%)
Malnutrition	8/748 (1%)
Obese (determined by study nurse and doctors at the visit)	29/748 (4%)
Previous dysentery	22/748 (3%)
Vitamin A supplementation within 6 months of enrolment	574/748 (77%)
Probiotic use within 6 months of enrolment	259/748 (35%)
Antimicrobial use within 6 months of enrolment	311/748 (42%)
Hospitalization within 6 months of enrolment	105/748 (14%)
Food consumed within 3 days of enrolment	
Poultry	274/748 (37%)
Beef	338/748 (45%)
Shrimp	485/748 (65%)
Pork	634/748 (85%)
Fish	580/748 (78%)
Shellfish	118/748 (16%)
Pate	37/748 (5%)
Unpasteurised milk	11/748 (1%)
Egg	353/748 (47%)

3.2.2 Clinical features and management of diarrhoeal disease

The total number of diarrhoeal episodes recorded in the cohort is shown in Table 3.3.

During the two years of individual follow-ups, a total of 885 diarrhoea records were recorded from 356/748 (48%) participants. The occurrence of these diarrhoeal episodes is described in Figure 3.3. A fresh stool sample was collected from almost all of the diarrhoeal episodes during hospital visits (403/406, 99%) but only in 44% (213/479) of the diarrhoeal episodes from the community visits; this resulted in at least 616 records from 885 diarrhoea records (70%). The age at diarrhoea illness was slightly less in hospital episodes in comparison to the home/phone records ($p=0.007$) (Table 3.3). The majority of diarrhoeal episodes captured in the community were watery; >50% of diarrhoeal episodes from the hospital visits were mucous or bloody diarrhoea ($p<0.001$). Diarrhoeal diseases from the hospital visits were also more likely to be accompanied by vomiting (219/406; 54% versus 161/479; 34%, $p<0.001$). Notably, antimicrobials were prescribed by doctors in 54% (221/406) of hospitals visits.

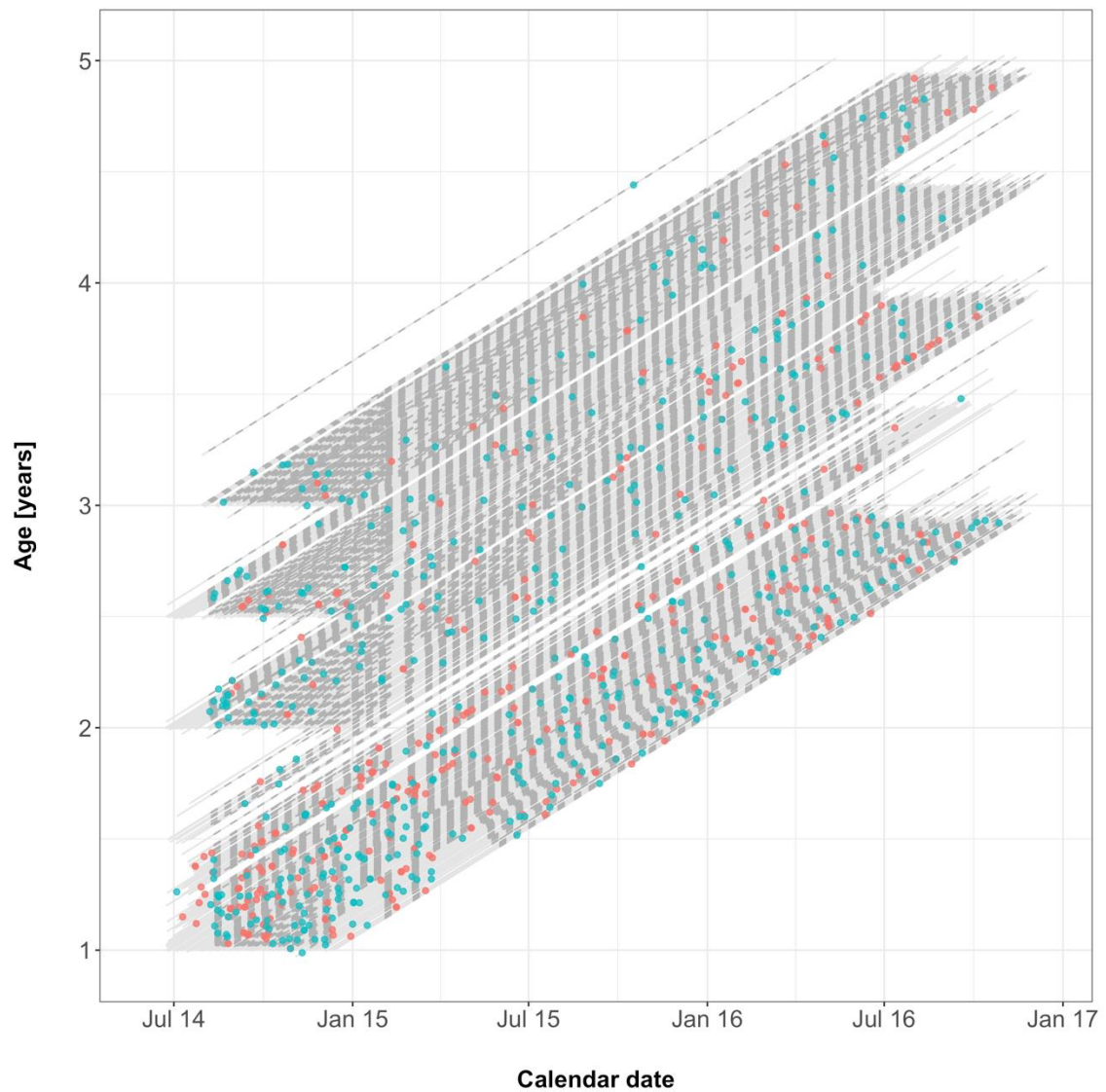


Figure 3.3 Lexis diagram of all participants in active diarrhoea surveillance. Grey lines are follow-up time. Dark grey segments are periods covered by SMSs. Dots are diarrhoea records (red: from hospital visit, blue: from phone/home visit).

Table 3.3 Clinical characteristics of the diarrhoeal records from home visits and hospital visits.

Characteristics	All (n=885)	Home (n=479)	Hospital (n=406)	Comparison
	Summary	Summary	Summary	p
Age at illness (months)	27 (19, 35)	28 (20, 36)	26 (19, 34)	0.007
Type of diarrhoea				
Watery	784/879 (89%)	454/473 (96%)	330/406 (81%)	<0.001
Mucous	294/885 (33%)	87/479 (18%)	207/406 (51%)	<0.001
Bloody	31/879 (4%)	6/473 (1%)	25/406 (6%)	<0.001
Abdominal pain	352/885 (40%)	179/479 (37%)	173/406 (43%)	0.114
Vomiting	380/885 (43%)	161/479 (34%)	219/406 (54%)	<0.001
Fever	154/885 (17%)	79/479 (16%)	75/406 (18%)	0.477
Dehydration	2/623 (<1%)	1/217 (<1%)	1/406 (<1%)	-
Probiotic	579/885 (65%)	212/479 (44%)	367/406 (90%)	<0.001
Zinc	385/885 (44%)	206/479 (43%)	179/406 (44%)	0.786
Rehydration	407/885 (46%)	191/479 (40%)	216/406 (53%)	<0.001
AMB	-	-	221/406 (54%)	

Using the definition of diarrhoea episode described previously, a total of 755 diarrhoea episodes were recorded, and 34% (254/755) of them were mucous/bloody diarrhoea. As described in Table 3.4, mucous diarrhoea occurred at younger age and was more likely to be accompanied by vomiting, abdominal pain, and fever compared to watery diarrhoea. Presenting with a more severe manifestation, mucous diarrhoea was more likely to be treated with probiotics, zinc, rehydration, and antibiotics. Of note, antibiotics were used in 60 episodes with watery diarrhoea only. Not all of these 60 diarrhoea episodes had severe manifestations (Table 3.5); in fact, symptoms like fever, vomiting, and abdominal pain did not occur in 11/60 of these episodes.

Table 3.4 Clinical characteristics and management of the diarrhoeal episodes stratified by type of diarrhoea.

Characteristics	All diarrhoea episodes (n=755)	Mucous diarrhoea (n=254)	Watery diarrhoea only (n=501)	p
Age at illness (months)	28/755 (20, 35)	26/254 (18, 34)	29/501 (21, 36)	0.038
Abdominal pain	319/755 (42%)	135/254 (53%)	184/501 (37%)	<0.001
Vomiting	334/755 (44%)	132/254 (52%)	202/501 (40%)	0.002
Fever	148/755 (20%)	68/254 (27%)	80/501 (16%)	<0.001
Dehydration	2/497 (<1%)	2/214 (1%)	0/283 (0%)	0.185
Probiotic	483/755 (64%)	209/254 (82%)	274/501 (55%)	<0.001
Zinc	356/755 (47%)	142/254 (56%)	214/501 (43%)	<0.001
Rehydration	370/755 (49%)	156/254 (61%)	214/501 (43%)	<0.001
AMB	179/301(59%)	119/167 (71%)	60/134 (45%)	<0.001

Table 3.5 Clinical characteristics of 60 diarrhoeal episodes with watery diarrhoea only but received antimicrobials.

All watery diarrhoea receiving antimicrobials(n = 60)	
Characteristics	Summary
Age at illness (months)	30/60 (22, 36)
Abdominal pain	28/60 (47%)
Vomiting	35/60 (58%)
Fever	19/60 (32%)
Dehydration	0/59 (0%)
Probiotic	54/60 (90%)
Zinc	26/60 (43%)
Rehydration	35/60 (58%)

3.2.3 The aetiology of diarrhoea using microbiology and Luminex

In total, 631 stool samples were collected from the diarrhoeal episodes (14 were collected after the follow-up period) (Table 3.6). The majority of stool samples from the diarrhoeal cohort were subjected to diagnostic testing by using Luminex (628/631; 99%) and conventional culture (623/631; 99%) to detect pathogens. Suspected bacterial pathogens were identified using a bacterial Bruker MALDI-TOF bacterial identification system. 69% (433/628) of faecal samples tested were positive by Luminex (Table 3.7).

Of all 628 samples, 42% (265) were positive for at least one viral pathogen, 47% (297) were positive for at least one bacterial pathogen, and 8% (53) were positive for at least one parasitic pathogen. The most common viral aetiological agent detected in samples from diarrhoea episodes was norovirus (163; 26%), followed by adenovirus (75; 12%), and rotavirus (69; 11%). The most frequently identified bacteria included *Salmonella* (140, 22%), followed by ETEC (82, 13%), *Clostridium difficile* (*C. difficile*) (80, 13%), *Campylobacter* (63, 10%), and *Shigella* (30, 5%). *Cryptosporidium* (44, 7%) was the most frequently detected parasite.

Table 3.6 Characteristics of 631 stool samples.

Characteristics of stool samples	All (n = 631)
Had clinical information	626/631 (99%)
Had Luminex information	628/631 (99%)
Had conventional culture information	623/631 (99%)
Had MALDI-TOF identification	609/631 (97%)
Had culture OR MALDI-TOF information	623/631 (99%)
Had Luminex AND (culture/MALDI-TOF information)	620/631 (98%)
Had clinical AND Luminex AND (culture/MALDI-TOF information)	615/631 (97%)
Had all information	601/631 (95%)
Sample from hospital visit	414/630 (66%)
Type of sample 625 samples (those had clinical information)	
Pot	57/625 (9%)
Swab	568/625 (91%)

Table 3.7 Summary of diagnostic results using the Luminex xTAG diarrhoeal pathogen panel from 628 stool samples.

Organism	Luminex positive
Adenovirus	75/628 (12%)
Norovirus	163/628 (26%)
Rotavirus	69/628 (11%)
Any virus	265/628 (42%)
<i>E. coli</i> O157	9/628 (1%)
ETEC	82/628 (13%)
STEC	5/628 (1%)
<i>Salmonella</i>	140/628 (22%)
<i>Shigella</i>	30/628 (5%)
<i>Campylobacter</i>	63/628 (10%)
<i>C. difficile</i>	80/628 (13%)
<i>V. cholerae</i>	0/628 (0%)
<i>Y. enterocolitica</i>	0/628 (0%)
Any bacteria	297/628 (47%)
<i>Cryptosporidium</i>	44/628 (7%)
<i>Entamoeba</i>	2/628 (<1%)
<i>Giardia lamblia</i>	11/628 (2%)
Any parasite	53/628 (8%)
Any positive^b	433/628 (69%)

Table 3.8 shows results from diagnostic testing among 601 samples with all information from both microbiology and Luminex. Only 41/601 (7%) stool samples were positive with at least one pathogen using conventional culture and MALDI-TOF identification, whereas 399/601 (66%) were positive with at least one pathogen by Luminex, of which 43% (258/601) were positive with at least one bacterial pathogen. While conventional culture and MALDI-TOF detected *E. coli* and selected bacteria, Luminex detected a wider spectrum of pathogens including viruses. The cross-match between Luminex and microbiological diagnosis by pathogens in all 601 samples and stratified by source of samples is described in Table 3.8. In general, the agreement in detecting selected pathogens (*Salmonella*, *Shigella*, *Campylobacter*, and *Entamoeba*) between two diagnostic approaches was low, especially for *Entamoeba*, *Campylobacter*, and *Salmonella*. Agreement between these diagnostic methods was slightly increased among samples collected through the home visits. Luminex was more sensitive than microbiological diagnosis in detecting these pathogens as the sensitivity of Luminex (microbiological test as reference) outperformed the comparative sensitivity of microbiological testing (Luminex as reference) (Table 3.9). Furthermore, among 18 stool samples that were negative by microbiological test, 10 (56%) positive confirmed by Luminex, in which most pathogens were virus such as Adenovirus (4/18; 22%), norovirus (5/18; 28%), and rotavirus (3/18; 17%) (Table 3. 10). Given the higher sensitivity of Luminex, results from this diagnostic approach were used as the basis for all subsequent pathogen-related analyses.

Table 3.8 Summary of diagnostic results among 601 samples with all information.

Organism	Culture positive	Luminex positive
Adenovirus	-	71/601 (12%)
Norovirus	0 (%)	150/601 (25%)
Rotavirus	0 (%)	66/601 (11%)
Any virus	0 (0%)	250/601 (42%)
<i>E. coli</i> O157	NA	9/601 (2%)
<i>E. coli</i>	580/601 (97%) ^a	
ETEC		78/601 (13%)
STEC		5/601 (1%)
<i>Salmonella</i>	21/601 (3%)	136/601 (23%)
<i>Shigella</i>	8/601 (1%)	29/601 (5%)
<i>Campylobacter</i>	12/601 (2%)	63/601 (10%)
<i>C. difficile</i>	-	79/601 (13%)
<i>V. cholerae</i>	-	0/601 (0%)
<i>Y. enterocolitica</i>	-	0/601 (0%)
Any bacteria ^b	40/601 (7%)	258/601 (43%)
<i>Cryptosporidium</i>	-	42/601 (7%)
<i>Entamoeba</i>	2//601 (<1%)	1/601 (<1%)
<i>Giardia lamblia</i>	-	9/601 (1%)
Any parasite	2//601 (<1%)	48/601 (8%)
Any positive ^b	41/601 (7%)	399/601 (66%)

^a *E. coli* in general^b Not including *E. coli*/*E. coli* O157/ETEC/STEC

NA: not applicable

Table 3.9 Luminex vs. microbiological results among 601 samples.

Luminex		Culture and confirmed byMALDI-TOF				
Organism		Positive	Negative	Sensitivity 1	Sensitivity 2	PA
All						
<i>Salmonella</i>	Positive	14	122	0.67	0.10	0.18
	Negative	7	458			
<i>Shigella</i>	Positive	7	22	0.88	0.24	0.38
	Negative	1	571			
<i>Campylobacter</i>	Positive	5	58	0.42	0.08	0.13
	Negative	7	531			
<i>Entamoeba</i>	Positive	0	1	0.00	0.00	0.00
	Negative	2	592			
Home visit						
<i>Salmonella</i>	Positive	6	48	0.75	0.11	0.19
	Negative	2	154			
<i>Shigella</i>	Positive	2	4	0.67	0.33	0.44
	Negative	1	203			
<i>Campylobacter</i>	Positive	1	23	0.50	0.04	0.08
	Negative	1	185			
<i>Entamoeba</i>	Positive	0	0	-	-	-
	Negative	0	210			
Hospital visit						
<i>Salmonella</i>	Positive	8	74	0.62	0.10	0.17
	Negative	5	304			
<i>Shigella</i>	Positive	5	18	1.00	0.22	0.36
	Negative	0	368			
<i>Campylobacter</i>	Positive	4	35	0.40	0.10	0.16
	Negative	6	346			
<i>Entamoeba</i>	Positive	0	1	0.00	0.00	0.00
	Negative	2	382			

Sensitivity 1: Luminex result as test, Sensitivity 2: Luminex result as reference

PA: positive agreement = conditional probability that one test is positive given the other test is also positive.

Table 3.10 Luminex results of 18 samples negative by microbiology.

Pathogen	Luminex positive and microbiology negative
Adenovirus	4/18(22%)
Norovirus	5/18 (28%)
Rotavirus	3/18 (17%)
<i>E. coli</i>	1/18 (6%)
ETEC	4/18 (22%)
STEC	0/18 (0%)
<i>Salmonella</i>	4/18 (22%)
<i>Shigella</i>	1/18 (6%)
<i>Campylobacter</i>	0/18 (0%)
<i>C. difficile</i>	0/18 (0%)
<i>V. cholerae</i>	0/18 (0%)
<i>Y. enterocolitica</i>	0/18 (0%)
<i>Cryptosporidium</i>	3/18 (17%)
<i>Entamoeba</i>	0/18 (0%)
<i>Giardia lamblia</i>	0/18 (0%)
Any positive	10/18 (56%)

3.2.4 Baseline and clinical characteristics of diarrhoea by pathogens

The baseline characteristics of 252 participants who had stool samples that were positive with a pathogen after Luminex screening were described and compared to all 748 participants in Table 3.11. Participants with detected pathogens were younger than the general study population (13, 14 and 16 months among detected pathogens compared 24 months in all participants). Caesarean section was common among all participants (325/748; 43%) and more prevalent among participants with *Cryptosporidium* (18/37; 49%) and *C. difficile* (27/57; 47%), while premature births were more prevalent among participants with *Cryptosporidium* (5/37; 14%), *Shigella* (3/25; 12%), and *C. difficile* (6/57; 11%). Similarly, low birth weights were more common among participants with *Shigella* (3/25; 12%), *Cryptosporidium* (4/37; 11%), and *C. difficile* (6/57; 11%). Rotavirus vaccinations were more prevalent among individuals with *Cryptosporidium* (19/57; 51% finishing two doses of rotavirus) but less prevalent among individuals with an Adenovirus (17/53; 32%) or ETEC infection (19/54; 35%). Table 3.12 shows that diarrhoea episodes positive with *Shigella* had the highest proportions of mucous/bloody diarrhoea (17/29; 59%), abdominal pain (16/29; 55%), and fever (13/29; 45%). Diarrhoea with *Campylobacter* also had high prevalence of mucous/bloody diarrhoea and abdominal pain (no fever). Vomiting was more prevalent among diarrhoea with Rotavirus and *C. difficile*.

Table 3.11 Baseline characteristics of participants by pathogen (n = 252).

Characteristics	Adenovirus	Norovirus	Rotavirus	ETEC	Sal	Shigella	Camp	C. diff	Crypto	All
N (participants)	53	94	55	54	89	25	53	57	37	748
Age (month)*	14 (12, 24)	13 (12, 24)	13 (12, 24)	13 (12, 24)	13 (12, 24)	16 (12, 30)	14 (12, 24)	12 (12, 16)	14 (12, 24)	24 (12, 30)
Female	26 (49%)	32 (34%)	23 (42%)	21 (39%)	33 (37%)	8 (32%)	22 (42%)	16 (28%)	7 (19%)	350 (47%)
Caesarean birth	22 (42%)	35 (37%)	24 (44%)	21 (39%)	38 (43%)	8 (32%)	15 (28%)	27 (47%)	18 (49%)	325 (43%)
Premature	4 (8%)	4 (4%)	5 (9%)	4 (7%)	6 (7%)	3 (12%)	2 (4%)	6 (11%)	5 (14%)	31 (4%)
Low birth weight	2 (4%)	4 (4%)	3 (5%)	3 (6%)	5 (6%)	3 (12%)	1 (2%)	6 (11%)	4 (11%)	29 (4%)
Breastfeeding	45 (85%)	85 (90%)	48 (87%)	45 (83%)	78 (88%)	22 (88%)	51 (96%)	53 (93%)	30 (81%)	694 (93%)
Anthropometry										
Weight-for-age	0 (-1, 1)	0 (0, 1)	0 (-1, 1)	0 (0, 1)	0 (0, 1)	0 (-1, 1)	0 (0, 1)	0 (0, 1)	0 (-1, 1)	0 (0, 1)
Height-for-age	0 (-1, 1)	0 (-1, 1)	0 (-1, 1)	0 (-1, 1)	0 (-1, 1)	0 (0, 1)	0 (-1, 1)	0 (0, 1)	0 (-1, 1)	0 (-1, 1)
Weight-for-height	0 (0, 1)	0 (0, 1)	0 (0, 1)	0 (0, 1)	0 (0, 1)	0 (0, 1)	0 (0, 1)	0 (0, 1)	0 (-1, 1)	0 (0, 1)
Rotavirus vaccination										
None	36 (68%)	53 (56%)	32 (48%)	33 (65%)	53 (60%)	15 (60%)	30 (57%)	30 (43%)	17 (46%)	434 (58%)
1 dose	17 (32%)	41 (44%)	23 (42%)	19 (35%)	36 (40%)	10 (40%)	23 (43%)	27 (47%)	20 (54%)	314 (42%)
2 doses	17 (32%)	39 (41%)	22 (40%)	19 (35%)	35 (39%)	9 (36%)	23 (43%)	25 (44%)	19 (51%)	304 (41%)
Age (mother, year)	28 (24, 31)	28 (23, 32)	29 (25, 31)	28 (25, 33)	28 (24, 32)	26 (23, 30)	28 (25, 32)	28 (24, 31)	28 (25, 32)	28 (25, 32)
Low maternal education	28 (53%)	57 (61%)	34 (62%)	33 (61%)	49 (55%)	8 (32%)	28 (53%)	27 (47%)	20 (54%)	394 (53%)
Household crowding	38 (72%)	65 (69%)	35 (64%)	40 (74%)	62 (70%)	17 (68%)	37 (70%)	36 (63%)	23 (62%)	519 (69%)
Main water sources										
Piped to home	32 (60%)	64 (68%)	39 (71%)	41 (76%)	58 (65%)	15 (60%)	38 (72%)	41 (72%)	24 (65%)	491 (66%)
Bottled water	21 (40%)	30 (32%)	16 (29%)	13 (24%)	30 (34%)	10 (40%)	13 (25%)	16 (28%)	13 (35%)	248 (33%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1%)	0 (0%)	2 (4%)	0 (0%)	0 (0%)	9 (1%)
Toilet										
Toilet	10 (25%)	14 (20%)	8 (21%)	12 (30%)	17 (25%)	6 (30%)	9 (22%)	9 (21%)	8 (31%)	171 (27%)
Chamber pot	25 (62%)	45 (63%)	24 (62%)	23 (57%)	37 (54%)	9 (45%)	26 (32%)	28 (67%)	13 (50%)	400 (62%)
Floor	5 (12%)	7 (10%)	3 (8%)	4 (10%)	10 (15%)	4 (20%)	4 (10%)	2 (5%)	4 (15%)	55 (9%)
Other	0 (0%)	5 (7%)	4 (10%)	1 (2%)	4 (6%)	1 (5%)	2 (5%)	3 (7%)	1 (4%)	14 (2%)

Sal: *Salmonella*; Camp: *Campylobacter*; C.diff: *C. difficile*; Crypto: *Cryptosporidium*

Missing data: adenovirus (mother's age 1, toilet 13); norovirus (toilet 23); rotavirus (toilet 16); ETEC (toilet 14); *Salmonella* (toilet 21); *Shigella* (toilet 5); *Campylobacter* (toilet 12); *C. difficile* (toilet 13); *Cryptosporidium* (toilet 9); All (mother's age 2, toilet 108)

* Median (IQR);

Table 3.12 Clinical characteristics of samples by pathogen (n = 601).

Characteristics	Adenovirus	Norovirus	Rotavirus	ETEC	Salmonella	Shigella	Campylobacter	C. difficile	Cryptosporidium
N (samples)	71	150	66	78	136	29	63	79	42
Source									
Home visit	27 (38%)	55 (37%)	12 (18%)	26 (33%)	54 (40%)	6 (21%)	24 (38%)	25 (32%)	18 (43%)
Hospital visit	44 (62%)	95 (63%)	54 (82%)	52 (67%)	82 (60%)	23 (79%)	39 (62%)	54 (68%)	24 (57%)
Age (month)*	30	24	28	27	29	33	28	22	35
	(22, 35)	(17, 33)	(21, 34)	(22, 36)	(20, 36)	(27, 36)	(22, 37)	(15, 31)	(29, 42)
Type of diarrhoea									
Watery	66 (93%)	136 (91%)	61 (92%)	68 (87%)	120 (88%)	26 (90%)	51 (81%)	66 (84%)	37 (88%)
Mucous	28 (39%)	48 (32%)	25 (38%)	33 (42%)	51 (38%)	17 (59%)	37 (59%)	38 (48%)	16 (38%)
Bloody	1 (1%)	4 (3%)	1 (2%)	1 (1%)	4 (3%)	3 (10%)	7 (11%)	5 (6%)	1 (2%)
Abdominal pain	28 (39%)	53 (35%)	29 (44%)	34 (44%)	62 (46%)	16 (55%)	36 (57%)	29 (37%)	21 (50%)
Vomiting	37 (52%)	83 (55%)	42 (64%)	39 (50%)	62 (46%)	14 (48%)	28 (44%)	45 (57%)	19 (45%)
Temperature**	37	37	37	37	37	37	37	37	37
	(37, 37)	(37, 37)	(37, 38)	(37, 37)	(37, 37)	(37, 38)	(37, 37)	(37, 37)	(37, 37)
Fever	7 (10%)	19 (13%)	16 (24%)	9 (12%)	18 (13%)	13 (45%)	9 (14%)	9 (11%)	9 (21%)
Dehydration	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)
Therapies									
Probiotic	65 (92%)	136 (91%)	60 (91%)	72 (92%)	124 (91%)	27 (93%)	62 (98%)	72 (91%)	38 (90%)
Zinc	44 (62%)	87 (58%)	35 (53%)	50 (64%)	87 (64%)	20 (69%)	45 (71%)	47 (59%)	30 (71%)
Rehydration	47 (66%)	92 (61%)	44 (67%)	51 (65%)	87 (64%)	14 (48%)	44 (70%)	48 (61%)	23 (55%)
Antimicrobials	24/44	45/95	25/54	30/52	45/82	13/23	26/39 (67%)	24/54	12/24 (50%)
usage	(55%)	(47%)	(46%)	(58%)	(55%)	(57%)		(44%)	

* Median (IQR); ** Mean (range)

3.2.5 Pathogen coinfections

Figure 3.4 presents results from pathogen detection using Luminex screening and the combinations between pathogens. Coinfections were detected among 120 patients with 184 stool samples, resulting in 550 pairs of pathogens. Of all 628 samples having results from Luminex screening, 29% (184/628) had more than one pathogen, including 101 samples positive with two pathogens, 45 samples with 3 pathogens, 23 samples with 4 pathogens, 11 samples with 5 pathogens, 3 samples with 6 pathogens, and 1 sample with 7 pathogens. The prevalence of pairs of pathogens is shown in Figure 3.5. Coinfection was most common between norovirus and *Salmonella* (9%; 49/550), followed by *Salmonella*/ETEC (6%; 35/550), *Salmonella*/adenovirus (6%; 34/550), *Salmonella*/*Cryptosporidium* (5%; 28/550), and norovirus/*C. difficile* (5%; 28/550).

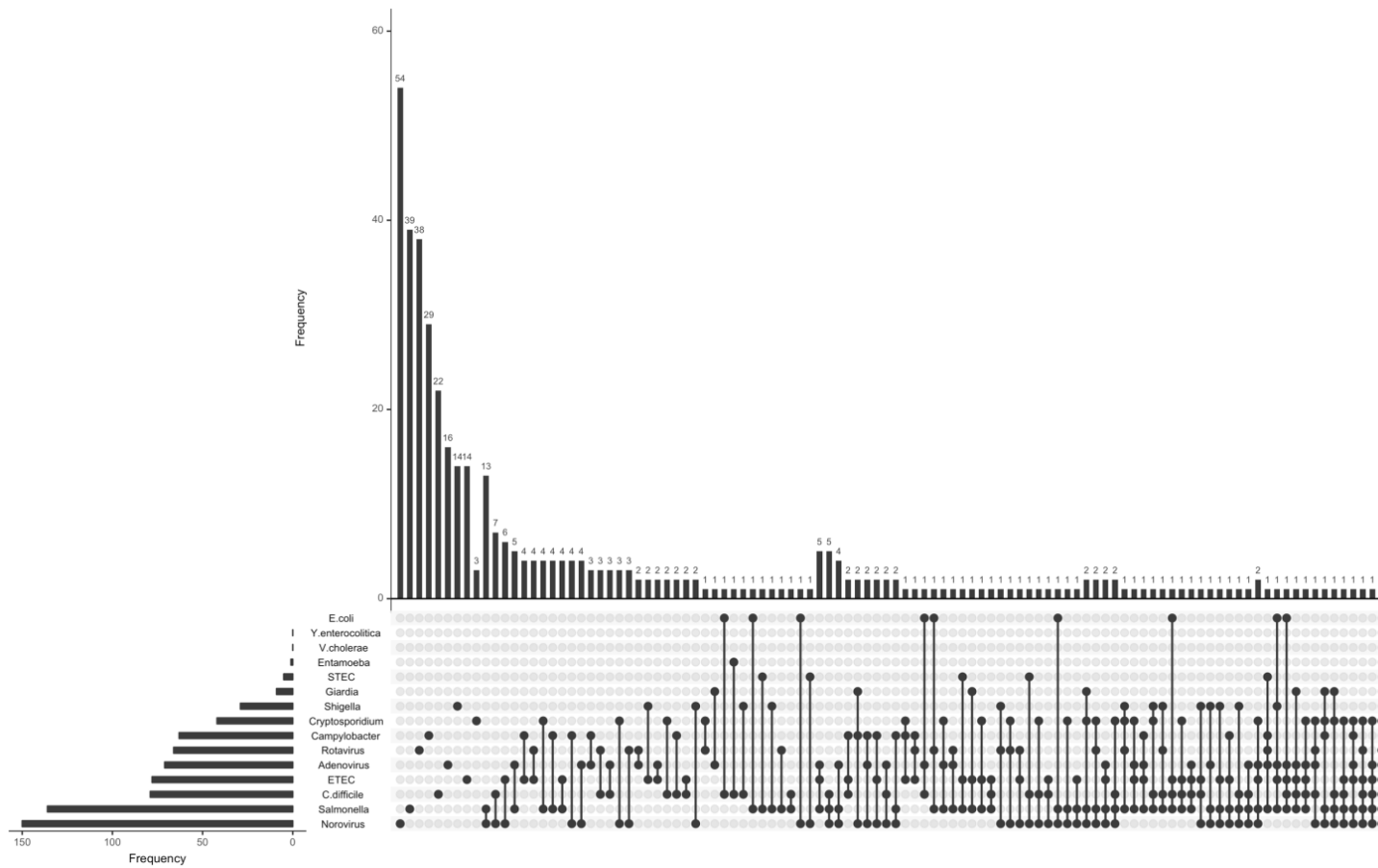


Figure 3.4 Pathogens identified by Luminex among 601 stool samples with all information. Pathogens are ordered by their frequencies (represented as horizontal bar chart at the bottom left). All combinations of pathogens are displayed in the bottom-right panel and their frequencies are displayed in the top-right bar chart.

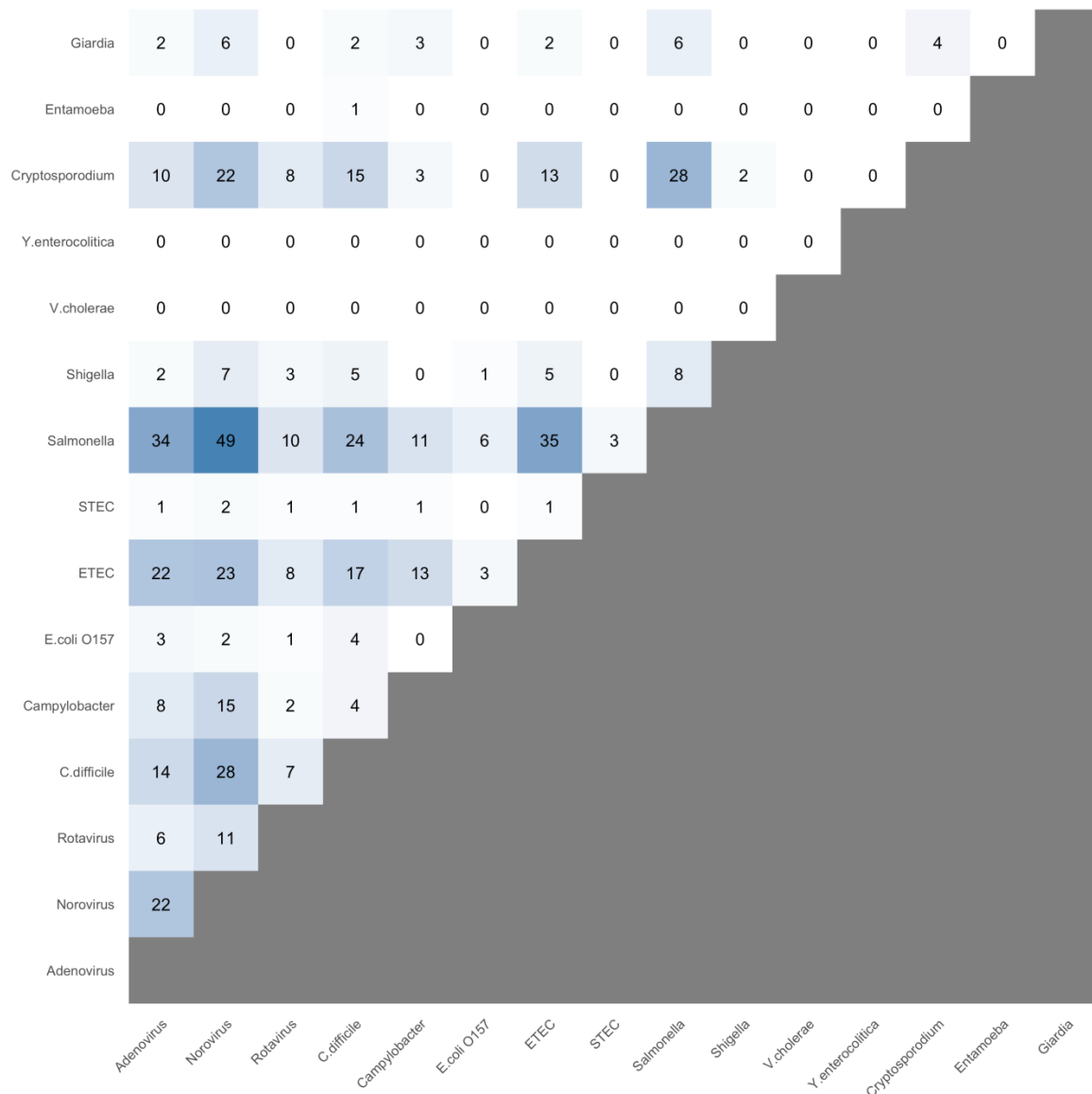


Figure 3.5 Matrix of coinfections. Distribution by pathogen of 550 pairs of pathogens from 184 stool samples with coinfection, collected from 120 patients. Each number is frequency of each pair of pathogens. These numbers are also represented as colour: higher numbers have darker colours. The lower triangle is dismissed and represented in grey to avoid replication of results in the upper triangle.

3.2.6 Reinfection

Reinfection was defined as the detection of the same pathogens among stools of two or more diarrhoea episodes during the two years of follow-up, in which each episode lasted 7.5 days as described in the methods section. A single participant in the diarrhoeal cohort may have multiple diarrhoeal episodes during two years of follow-up from 2014 to 2016. Among 46 patients who had reinfection, the most frequent pathogens were norovirus (47%; 22/46 patients) and *Salmonella* (46%; 21/46 patients) (Table 3.13). There were no cases having reinfection with *E. coli* O157, STEC, and *Y. enterocolitica*. The median number of reinfections per patient was 1 with a range from 1 to 11 times. The median interval between reinfection was 79 days (range: 8–638, IQR: 17.5–210.75).

Table 3.13 Summaries by organisms for the number of reinfections per patients and the interval between reinfections.

Organism	Times of reinfection per patients	Interval between reinfections
	Median(range)	Median (Range) (IQR) (days)
Adenovirus	1 (1–8)	55 (8–536) (37–180)
Norovirus	1 (1–6)	112 (9–566) (25–279)
Rotavirus	1 (1–2)	122 (9–160) (78–147)
<i>C. difficile</i>	1 (1–4)	88 (15–412) (64–156)
<i>Campylobacter</i>	1 (1–1)	432 (379–484) (405–458)
ETEC	1(1–6)	144 (11–535) (74–236)
<i>Salmonella</i>	1 (1–11)	144 (9–638) (57–329)
<i>Shigella</i>	0	0
<i>Cryptosporidium</i>	1 (1–1)	9
<i>Giardia lamblia</i>	1 (1–1)	34

3.2.7 General diarrhoeal incidence, age-specific diarrhoeal incidence, and pathogen-specific diarrhoeal incidence

In this analysis I used two approaches for calculating the incidence of diarrhoea in this community cohort. In the first approach, the follow-up period was the total follow-up time from study enrolment to study exit, meaning that all diarrhoea episodes were included. In the two years of follow-up, which was equivalent to 1,417 child-years of observation, there were a total of 755 diarrhoeal episodes captured during this period; 434 from home visits and 321 from hospital visits. Therefore, the overall diarrhoeal incidence was 53.29/100 child-years of observation (95%CI: 49.58, 57.19), which decreased as the children got older (Table 3.14). The incidence of diarrhoea collected by phone/home visit was higher than by hospital visit. The diarrhoea incidences (53.29/100 child-years of observation) likely underestimated the true incidences as active surveillance using SMSs only covered half of the follow-up time.

To improve the incidence estimation, I used diarrhoeal records that were covered by the biweekly SMS system. This approach included 488 diarrhoeal episodes. The time of observation was the total follow-up period covered by SMS, meaning that only diarrhoeal episodes that occurred within these interval periods were counted. Diarrhoeal episodes detected within SMS periods were less likely to be captured by hospital visits and were more likely at older age. There was no significant difference in the clinical characteristics of diarrhoea episodes detected and undetected within SMS periods (Table 3.15). On average, most participants received 50 SMS messages (IQR: 46–52) during the time of following up. The total follow-up time covered by SMS was 679 child-years of observation. Therefore, the overall incidence of diarrhoea in this cohort was 71.87/100

child-years of observation (95%CI: 65.68, 78.44). In a comparable manner to the results of incidence from approach 1, the incidence rate of diarrhoea episodes decreased with age and was higher with phone/home visits (Table 3.14). In comparison to approach 1, incidence estimated by the second approach was 1.3 times higher (Table 3.14 and Figure 3.6). The highest incidence of diarrhoeal diseases was among children of 12–23 months with 100.18/100 child-years of observation (95%CI: 88.66, 112.66) (1st approach), and 132.64/100 child-years of observation (95%CI: 112.28, 155.34) (2nd approach). The age-specific incidence gradually decreased with age (Table 3.14).

Table 3.14 Diarrhoea incidence: overall and stratified by age groups.

	All		12–23 months		24–35 months		≥36 months	
Approach 1								
Follow-up (100 CY)	14.17		2.68		5.08		6.41	
Group	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)
Total	755	53.29 (49.58, 57.19)	268	100.18 (88.66, 112.66)	307	60.39 (53.89, 67.40)	180	28.09 (24.18, 32.39)
Home visit	434	30.64 (27.84, 33.61)	134	51.09 (42.08, 59.06)	184	36.20 (31.21, 41.68)	116	18.10 (15.00, 21.60)
Hospital visit	321	22.66 (20.27, 25.23)	134	51.09 (42.08, 59.06)	123	24.20 (20.17, 28.73)	64	9.99 (7.74, 12.64)
Approach 2								
Follow-up (100 CY)	6.79		1.10		2.48		3.21	
Group	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)
Total	488	71.87 (65.68, 78.44)	146	132.64 (112.28, 155.34)	210	84.59 (73.66, 96.55)	132	41.16 (34.54, 48.59)
Home visit	318	46.83 (41.87, 52.17)	88	79.95 (64.39, 97.84)	133	53.57 (44.98, 63.20)	97	30.25 (24.62, 36.67)
Hospital visit	170	25.04 (21.46, 28.99)	58	52.69 (40.27, 67.44)	77	31.02 (24.59, 38.47)	35	10.91 (7.69, 14.94)

Approach 1: Follow-up time is the total follow-up time from study enrolment to study exit (all diarrhoea episodes were counted).

Approach 2: Follow-up time is the total follow-up time covered by SMSs (only diarrhoea episodes occurred within this period were counted).

Incidence rates and 95% confidence intervals were estimated using univariable Poisson regression models. CY: child-years of observation.

Table 3.15 Characteristics of diarrhoeal episodes detected and undetected by SMS periods.

Characteristics	Diarrhoea episode detected by SMS periods		p
	Yes (n=488)	No (n=267)	
Visited hospital	170/488 (35%)	151/267 (57%)	<0.001
Age at illness (months)	30/488 (22, 37)	25/267 (17, 33)	<0.001
Abdominal pain	209/488 (43%)	110/267 (41%)	0.700
Vomiting	203/488 (42%)	131/267 (49%)	0.055
Fever	94/488 (19%)	54/267 (20%)	0.774
Dehydration	2/278 (1%)	0/219 (0%)	0.185

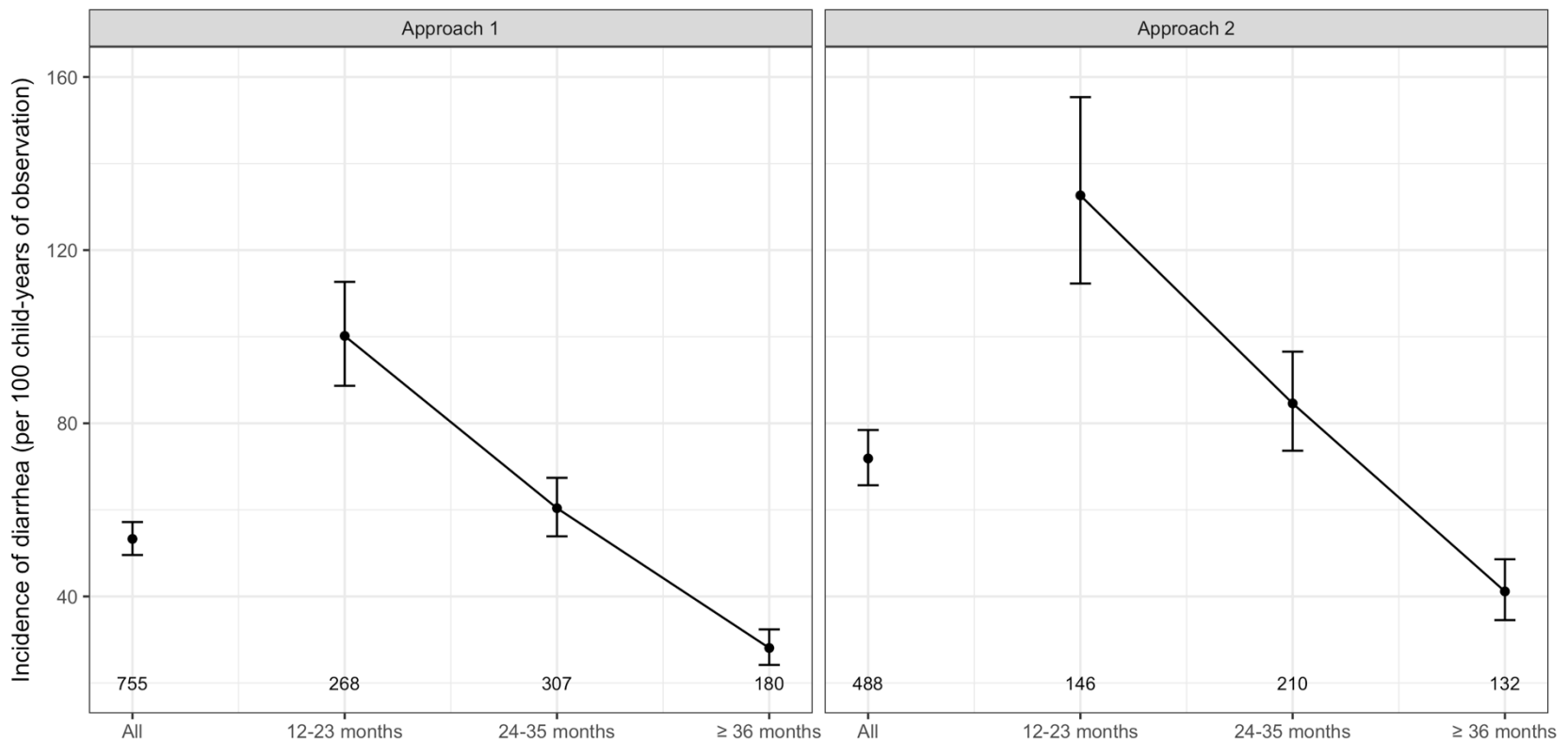


Figure 3.6 Incidence of diarrhoea for all participants and for each age group by estimation approach.

Approach 1 was based on the total follow-up time, approach 2 was based on the follow-up time covered by SMS periods only.

Numbers at the bottom describe number of diarrhoea episodes.

Figure 3.7 illustrates a significant decreasing trend of diarrhoea incidence with age (likelihood ratio test based on Poisson regression models that modelled age as natural cubic spline with 4 degrees of freedom with and without adjustment for calendar time and age at enrolment: $p < 0.001$). Examining effects of age and calendar time on diarrhoea incidence (with adjustment for age group at enrolment) suggests a significant effect of calendar time (likelihood ratio test based on multivariable Poisson regression model that modelled calendar time linearly: $p = 0.021$). Figure 3.8 shows significantly higher diarrhoea incidences from August to October (during the rainy season) compared to March (during the dry season).

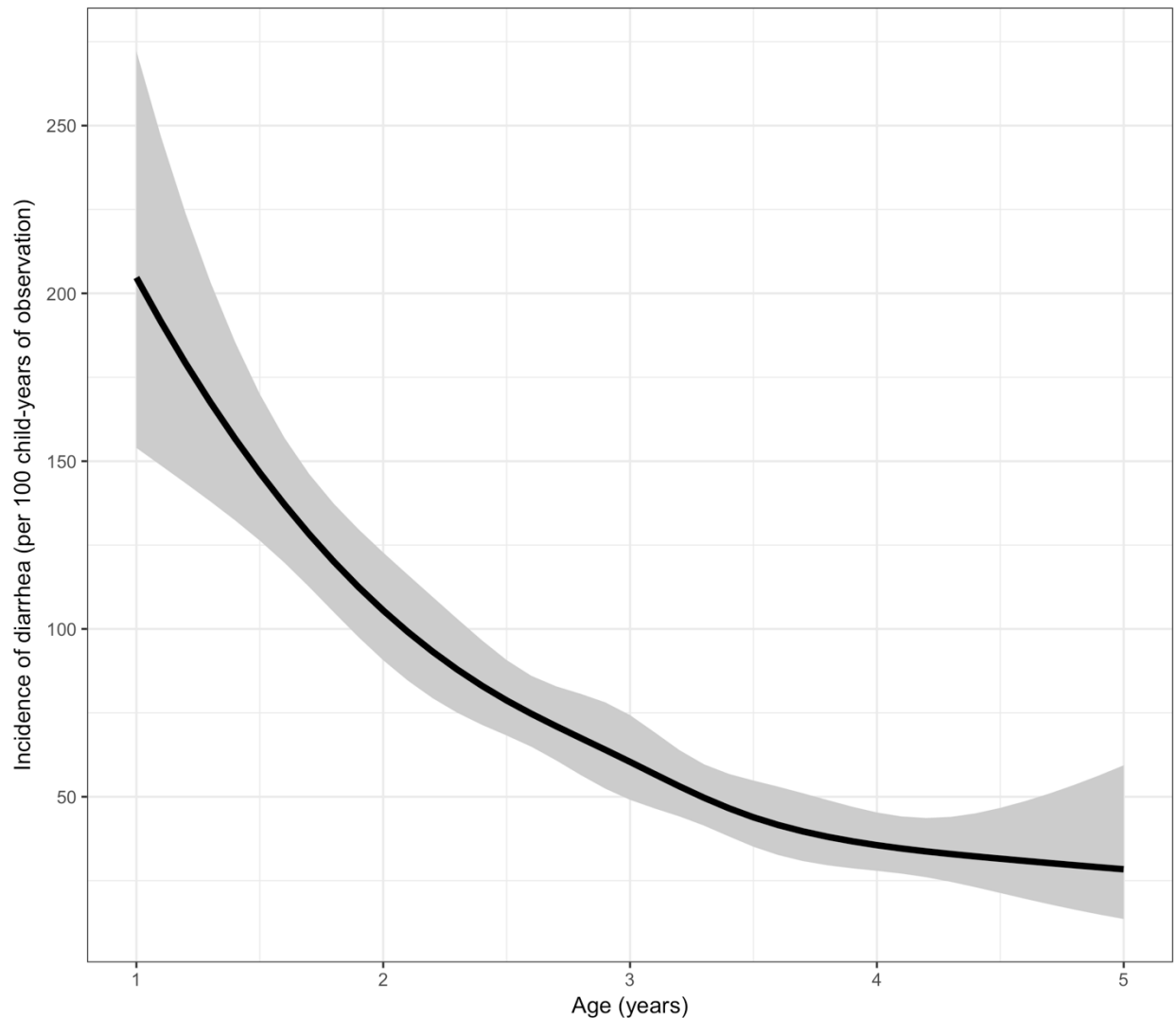


Figure 3.7 Incidence of diarrhoea by age, based on approach 2 (using the follow-up time covered by SMS periods only).

Solid line is point estimate, while grey area reflects 95% confidence interval region.

Estimation based on univariable Poisson regression model with occurrence of diarrhoea at each SMS period as outcome, age at start of SMS period as covariate, length of SMS period as offset.

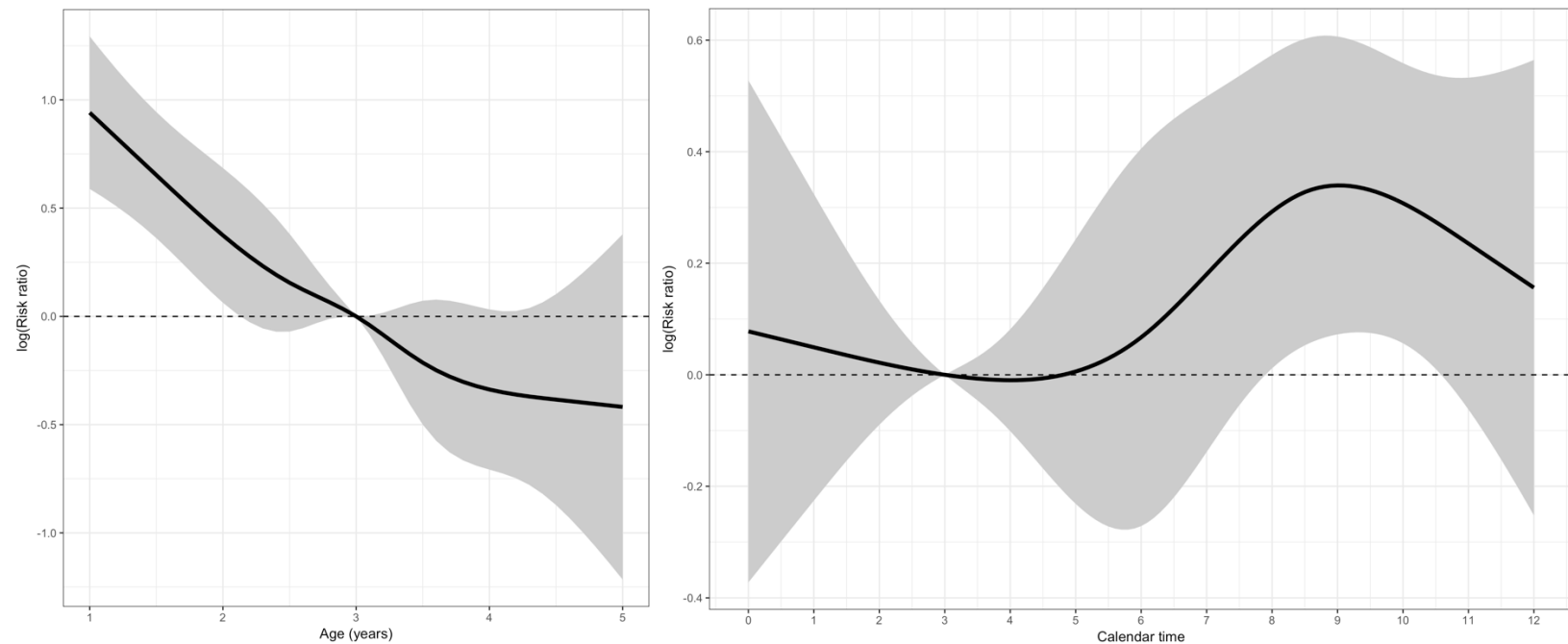


Figure 3.8 Log (Risk ratio) of diarrhoea by age and by calendar time.

Solid lines are point estimates, while grey areas reflect 95% confidence interval regions. Left panel: reference is age of 3, right panel: reference is at the end of March. These figures were based on a multivariable Poisson regression model with occurrence of diarrhoea at each SMS period as outcome, age at start of SMS period, age group at enrolment, and calendar time as covariate, and length of SMS period as offset.

Table 3.16 and Figure 3.9 show the pathogen-specific incidence of diarrhoea. In both approaches, Norovirus, *Salmonella* and *C. difficile* were the pathogens with the highest incidence rate. There was also a general decreasing trend in pathogen-specific incidence of diarrhoea with age, notably for Norovirus, Rotavirus, *C. difficile*, *Campylobacter*, ETEC and *Salmonella*. In the age group of 12- 23 months, the highest incidence belonged to norovirus (incidence 21.68/100 child-years, 95%CI: 16.57, 27.75), followed by *Salmonella* (incidence 16.45/100 child-years, 95%CI: 12.05, 21.80) and *C. difficile* (14.58/100 child-years, 95%CI: 10.47, 19.65).

Using the second approach, in which diarrhoeal episodes captured by the follow up time covered by biweekly SMS were counted, diarrhoeal disease due to *Salmonella* was most common with an incidence of 10.6/100 child-years of observation (95%CI: 8.34, 13.25), followed by norovirus with the incidence of 9.57/100 child-years of observation (95%CI: 7.43, 12.09). In the age group of 12- 23 months, the highest incidence belongs to norovirus (incidence 20.90/100 child-years, 95%CI: 13.48–, 30.64), followed by *C. difficile* (18.17/100 child-years, 95%CI: 11.32, 27.34) and *Salmonella* (incidence 16.35/100 child-years, 95%CI: 9.91, 21.11).

As shown in Table 3.16, using both approaches the incidence rates of diarrhoeal disease caused by viruses are higher than those caused by bacteria. In general, *C. difficile* had an incidence of 14.58 episodes (95%CI: 10.47, 19.65) per 100 CYO (approach 1) or 18.17 (95%CI: 11.32, 27.34) per 100 CYO (approach 2) in two-year olds, norovirus had an incidence of 21.68 (95%CI: 16.57, 27.75) per 100 CYO (approach 1) or 20.90 (95%CI: 13.48, 30.64) per 100 CYO (approach 2), *Salmonella* had an incidence of 16.45 (95%CI:

12.05, 21.80) per 100 CYO (approach 1) or 16.35 (95%CI: 9.91, 25.11) per 100 CYO (approach 2), *Campylobacter* had an incidence of 6.73 (95%CI: 4.08, 10.33) per 100 CYO (approach 1) or 9.99 (95%CI: 5.19, 17.11) per 100 CYO (approach 2). These pathogens were rarely identified in older children. While the incidence of most pathogens declined between the second and third years of life, the incidence of *Shigella*, though low overall, was similar in both two- (1.5 episodes per 100 CYO) and three-year olds (2.1 episodes per 100 CYO).

Table 3.16 Pathogen-specific incidence (pathogens were detected using Luminex).

		All	12–23 months		24–35 months		≥36 months	
Approach 1								
Follow-up (100 CY)		14.17		2.68		5.08		6.41
Pathogen	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)
Adenovirus	65	4.59 (3.56, 5.80)	21	7.85 (4.95, 11.70)	30	5.90 (4.03, 8.27)	14	2.18 (1.23, 3.54)
Norovirus	127	8.96 (7.49, 10.62)	58	21.68 (16.57, 27.75)	48	9.44 (7.02, 12.37)	21	3.28 (2.07, 4.89)
Rotavirus	58	4.09 (3.13, 5.24)	24	8.97 (5.84, 13.05)	23	4.52 (2.92, 6.63)	11	1.72 (0.89, 2.94)
<i>C. difficile</i>	69	4.87 (3.81, 6.11)	39	14.58 (10.47, 19.65)	25	4.92 (3.23, 7.11)	5	0.78 (0.28, 1.68)
<i>Campylobacter</i>	53	3.74 (2.82, 4.84)	18	6.73 (4.08, 10.33)	21	4.13 (2.61, 6.16)	14	2.18 (1.23, 3.54)
<i>E. coli</i> O157	9	0.64 (0.31, 1.15)	4	1.50 (0.46, 3.47)	4	0.79 (0.24, 1.83)	1	0.16 (0.01, 0.69)
ETEC	65	4.59 (3.56, 5.80)	23	8.60 (5.55, 12.61)	27	5.31 (3.55, 7.57)	15	2.34 (1.35, 3.73)
STEC	5	0.35 (0.13, 0.76)	2	0.75 (0.12, 2.31)	2	0.39 (0.07, 1.21)	1	0.16 (0.01, 0.69)
<i>Salmonella</i>	125	8.82 (7.37, 10.46)	44	16.45 (12.05, 21.80)	54	10.62 (8.04, 13.71)	27	4.21 (2.82, 6.01)
<i>Shigella</i>	25	1.76 (1.16, 2.55)	4	1.50 (0.46, 3.47)	13	2.56 (1.41, 4.21)	8	1.25 (0.57, 2.32)
<i>V. cholerae</i>	0	0	0	0	0	0	0	0
<i>Y. enterocolitica</i>	0	0	0	0	0	0	0	0
<i>Cryptosporidium</i>	33	2.33 (1.62, 3.22)	7	2.62 (1.12, 5.06)	15	2.95 (1.70, 4.71)	11	1.72 (0.89, 2.94)
<i>Entamoeba</i>	1	0.07 (0.00, 0.31)	0	0	1	0.20 (0.01, 0.87)	0	0
<i>Giardia lamblia</i>	9	0.64 (0.31, 1.15)	3	1.12 (0.28, 2.91)	6	1.18 (0.47, 2.39)	0	0
Approach 2								
Follow-up (100 CY)		6.79		1.10		2.48		3.21
Pathogen	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)
Adenovirus	38	5.60 (4.00, 7.57)	8	7.27 (3.32, 13.53)	20	8.06 (5.02, 12.12)	10	3.12 (1.56, 5.47)
Norovirus	65	9.57 (7.43, 12.09)	23	20.90 (13.48, 30.64)	28	11.28 (7.60, 15.99)	14	4.37 (2.46, 7.07)
Rotavirus	27	3.98 (2.66, 5.67)	10	9.09 (4.55, 15.93)	10	4.03 (2.02, 7.06)	7	2.18 (0.94, 4.22)
<i>C. difficile</i>	40	5.89 (4.25, 7.91)	20	18.17 (11.32, 27.34)	17	6.85 (4.09, 10.64)	3	0.94 (0.23, 2.43)
<i>Campylobacter</i>	33	4.86 (3.38, 6.71)	11	9.99 (5.19, 17.11)	11	4.43 (2.30, 7.59)	11	3.43 (1.78, 5.87)
<i>E. coli</i> O157	5	0.74 (0.26, 1.58)	1	0.91 (0.05, 4.00)	3	1.21 (0.30, 3.13)	1	0.31 (0.02, 1.37)
ETEC	40	5.89 (4.25, 7.91)	11	9.99 (5.19, 17.11)	18	7.25 (4.40, 11.13)	11	3.43 (1.78, 5.87)
STEC	2	0.29 (0.05, 0.91)	0	0	1	0.40 (0.02, 1.77)	1	0.31 (0.02, 1.37)
<i>Salmonella</i>	72	10.60 (8.34, 13.25)	18	16.35 (9.91, 25.11)	34	13.70 (9.59, 18.83)	20	6.24 (3.89, 9.38)
<i>Shigella</i>	13	1.91 (1.05, 3.15)	2	1.82 (0.30, 5.61)	6	2.42 (0.96, 4.90)	5	1.56 (0.56, 3.35)
<i>V. cholerae</i>	0	0	0	0	0	0	0	0
<i>Y. enterocolitica</i>	0	0	0	0	0	0	0	0
<i>Cryptosporidium</i>	18	2.65 (1.61, 4.07)	3	2.73 (0.68, 7.07)	9	3.63 (1.74, 6.53)	6	1.87 (0.74, 3.79)
<i>Entamoeba</i>	0	0	0	0	0	0	0	0
<i>Giardia lamblia</i>	7	1.03 (0.44, 1.99)	3	2.73 (0.68, 7.07)	4	1.61 (0.50, 3.74)	0	0

Approach 1: Follow-up time is the total follow-up time from study enrolment to study exit (all diarrhoea episodes were counted). Approach 2: Follow-up time is the total follow-up time covered by SMSs (only diarrhoeal episodes occurred within this period were counted). Incidence rates and 95% confidence intervals were estimated using univariable Poisson regression models. CY: child-years of observation.

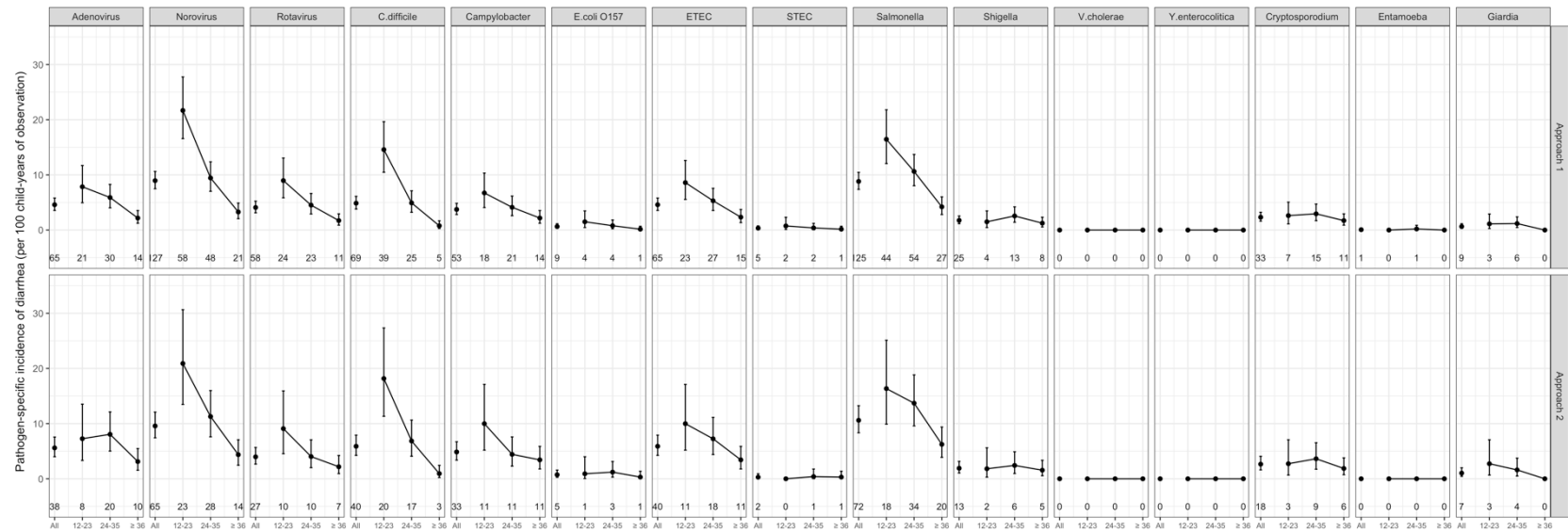


Figure 3.9 Pathogen-specific incidence of diarrhoea for all participants and for each age group by estimation approach.

Approach 1 is based on the total follow-up time, approach 2 is based on the follow-up time covered by SMS periods only.

Numbers in the bottom describes number of diarrhoea episodes.

3.3 Discussion

Diarrhoea remains a key burden in infectious diseases in Viet Nam. Several studies have focused on moderate to severe diseases in hospital-based studies in which the actual burden of diarrhoeal disease in the community is unknown^{29,45,141,187}. Some previous observational studies in Viet Nam have estimated the true burden of diarrhoea in the community. These studies have had various study designs, including being conducted in rural areas and using passive surveillance in a prospective cohort¹⁹⁰. They have also been performed in both rural and urban areas in the south of Viet Nam at an early age (under 1 year) using passive surveillance¹⁸⁴, and with a case control design study in the urban north¹⁸⁷. There have additionally been studies focusing on a specific pathogen using retrospective data^{191,197}, but there has been limited use of active surveillance in an urban community. The current study was a well-structured and well-organized prospective cohort in the community with a high rate of follow-up (748 enrollees and 94% follow-up rate after two years), and it was the first study of its type in Viet Nam using SMS messages for active surveillance in the community. With this study design we achieved a high rate of response. There was a large cohort in this area, and it provided a rich dataset for different analytical approaches. In the scope of this analysis I evaluated the probability of the disease in a specific period of time though the general incidence of diarrhoea, age-specific diarrhoeal, and additionally investigated a wide range of pathogens associated with diarrhoea among children age one to five years using both conventional microbiology and a multiplex molecular assay.

Diarrhoea contributes a major proportion of burden of the diseases among children under five all over the world, especially in LMICs⁵. Incidence of diarrhoeal disease among children under five from our prospective cohort study was 71.87/100 child-years of

observation (using the 2nd approach) or 53.29/100 child-years of observation (using the 1st approach). Our findings, in which the second approach likely provides more accuracy in incidence calculation, reveals a decrease of the overall burden of diarrhoeal disease over time compared to the rates (130/100 CYO) in the country over recent decades¹⁹⁰. This rate is substantially lower than the diarrhoea incidence rate in Southeast Asia in the early 1990s (240/100 CYO)¹², and in Cambodia in 2003 (532/100 CYO)¹⁹⁸. This finding reflects major improvements in clean water, hygiene, and sanitation¹⁹⁹. In addition, the general standard of living has improved, and consequently the burden of diarrhoea has decreased. A further explanation may be that the Vietnamese government passed a law regarding the prevention and control of infectious diseases in 2008. This law, which stipulates that diarrhoea is among the infectious diseases that need monitoring by reporting within 24 hours of the infection, appears to have been effective⁷⁸. However, the general diarrhoea incidence in this study is higher than in other locations in Viet Nam, such as in Nha Trang on the central coast (11.5/100 CYO)¹⁹¹. This discrepancy between incidences is likely due to the method used to capture diarrhoeal episodes. Our approach, which includes active surveillance, captured more cases than a passive study design. Home visits captured 50% of cases (217/885 diarrhoeal records from home visits and 262/885 diarrhoea records from SMS and phone calls) in addition to hospital visit cases (406/885 diarrhoeal records).

This study detected a wide range of etiological agents associated with mild and moderate diarrhoeal diseases in the community by using both microbiological culture and Luminex. Although rotavirus was considered the leading causes of diarrhoea globally^{15,27}, in hospitalized cases²⁰⁰, and in north Viet Nam¹⁸⁷, our findings did not show a consistent result for rotavirus in our cohort. One possible explanation for the lack of rotavirus

infections is the differing age groups of participants, who were recruited at a later age (12–36 months from enrolment) in our study. Our findings demonstrate a switch of the roles of rotavirus and norovirus in causing diarrhoeal disease in childhood populations in HCMC^{189,201}, with norovirus becoming more common. This result may be a consequence of improved diagnostic testing in our study. In contrast to a case-control study conducted in Hanoi in 2006¹⁸⁷, which found no stool samples positive with *Salmonella*, our study found norovirus and *Salmonella* to be the most prevalent (163/628 (26%) and 140/628 (22%), respectively). The high rate of *Salmonella* in our study, especially in hospital visits, is consistent with hospitalized diarrhoea cases in HCMC from a previous study¹⁴¹. Again, this high rate is likely due to differing microbiology testing used to detect the various pathogens. In this study we used Luminex xTAG GPP, which has been tested for diagnostic accuracy previously¹⁷⁸.

Of additional note is the low rate of *Shigella* infections in this study (30/628 [5%], with an incidence of 1.76/100 CYO [95%CI: 1.16, 2.55]) which defines a trend of decreasing rate of infection with this pathogen^{187,191} and shows a consistent pattern with recent disease trends in Viet Nam²⁰².

This study affirmed several common clinical characteristics of mild to moderate diarrhoeal diseases from the community among children aged less than five years. In this study, the majority of hospital visits associated with diarrhoea were more likely to be accompanied by mucous/bloody stool and vomiting, which is consistent with the characteristics of hospitalized diarrhoea in other studies worldwide and in Viet Nam¹⁸⁷. In addition, I found that diarrhoea incidence was significantly higher during the rainy season compared with the

dry season. This finding shows a similarity to most other studies around the world conducted where diarrhoea and malaria increase during the wet season²⁰³. A possible explanation is the contamination of water by human waste during the rainy season^{204,205}.

Regarding diarrhoeal management, among cases that required hospital visits in this study, antimicrobials were prescribed in 59% (179/301) of diarrhoeal episodes. Antimicrobials were more likely to be prescribed in diarrhoeal episodes associated with mucous stool than in diarrhoeal episodes with watery stool (71% [119/167] and 45% [60/134], respectively, Table 3.3). These proportions are lower than those in a recent finding in this setting, where 85% of paediatric diarrhoeal patients admitted to hospital were prescribed an antimicrobial¹⁴¹. The discrepancy might be because of the severity of hospitalized diarrhoea, in which participants came to the designated hospital due to mucous and bloody diarrhoea in the latter study while any diarrhoea (including watery and/or mucous) could allow the participant to attend the study hospital in this study. These findings are largely consistent with the management of diarrhoeal disease in Chapter 4; antimicrobial use in hospital was five to ten times higher than that from retail pharmacies. These findings highlight the issue of using antimicrobials in the hospital, which has been estimated to be inappropriate in one-third of antimicrobial prescriptions in Vietnamese hospitals²⁰⁶. The issue of inappropriate antimicrobial use may promote AMR in various bacterial pathogens, especially in settings with limited resources for diagnostic testing, such as Viet Nam. In combination with data from chapter 4, these findings highlight the necessity for diagnostic testing in hospitals to improve clinical decisions for antimicrobial use based on differentiating between a viral and a bacterial infection.

This prospective study has several caveats. Despite the best method for capturing diarrhoea in the community with the “prior 7 days” having been applied—it has been shown that frequent contact for diarrhoea surveillance in community may lead to underreporting—the biweekly SMS showed a gap of 7 days in the time of following up to all participants. In addition, the pathogen-specific incidence likely represents an underestimation in the true incidence of pathogens because it is likely that mild diarrhoeal events of short duration were more likely to be missed due to recall bias. Furthermore, it is less likely that a diarrhoeal episode of short-duration will provide a stool sample for pathogen analysis, thus leading to bias associated with pathogens that cause prolonged diarrhoea. Although these biases are problematic, pathogen-specific incidence is a reasonable estimation of the diarrhoeal disease incidence in hospital as all diarrhoeal events from hospital were sampled. A further bias that may be considered when interpreting incidence is the time to follow up. For example, using all diarrhoeal episodes during two years of follow-up but with the actual period of time covered by SMS only (as in the first approach), these diarrhoeal incidences likely underestimated the true incidences as active surveillance using SMSs only covered half of the follow-up time. Another limitation was that Luminex used for pathogen detecting method did not provide details of the subspecies of the bacteria, such as *Salmonella*. Besides, regarding the reinfection proportions, my method did not reveal the repeated infections of the same pathogens or the chronic carriage of the same pathogens over time.

3.4 Conclusion

This study was a large prospective population-based study in the south of Viet Nam to assess the burden of diarrhoea among children under five in the community in an urban

area. The results reveal a high burden of infectious diseases among children in Ho Chi Minh City and demonstrate a wide variety of pathogens detected during passive and active surveillance of diarrhoeal diseases. Norovirus and *Salmonella* were the most commonly detected pathogen and high pathogen-specific incidences were found in children of early age. Further studies should be conducted to better understand the immune response of children to specific pathogens detected during surveillance for diarrhoeal disease; this approach would provide essential data for vaccine development. Such cohort studies would also benefit from pathogen genome studies to investigate potential genetic associations with diarrhoeal disease aetiology among children in different settings around South and Southeast Asia.

4. Quantifying antimicrobial access and usage for paediatric diarrhoeal diseases in an urban community setting in Asia

AMR bacterial infections have become a major public health issue in LMICs. Ease of access and widespread use of antimicrobials are postulated to be two key drivers of this current AMR epidemic. However, accurately assessing antimicrobial access and usage for particular disease syndromes are hindered by the intrinsic complexity of unregulated antimicrobial sales in the community. In this chapter I aimed to assess antimicrobial access and usage for diarrhoeal disease among children under 5 in the community in HCMC. I used a mixed-method approach, in which the geospatial mapping (Methods section 2.2.3 Geospatial analysis) to assess antimicrobial access in the community; the simulated client's pharmacy survey (Methods section 2.1.3 Cross sectional studies in community), the community survey on parents and care givers about their behaviour (Methods section 2.1.3) and the diarrhoeal cohort (Methods section 2.1.2 Diarrhoea cohort) to investigate antimicrobial usage for paediatric diarrhoeal disease in the community.

4.1 Background

AMR is rapidly becoming a global public health issue¹¹¹. In the past decade, many of the key antimicrobials that we have come to rely in human medicine are in the process of losing, or have already lost, their effectiveness in treating infections caused bacterial pathogens^{114,115}. AMR is a complex scientific, political, economic, and social issue, and whilst developed countries are leading research efforts to tackle the problems, the greatest impact of AMR is currently being felt in LMICs^{126–128}. AMR is magnified in LMICs as antimicrobial access and usage, poor sanitation and limited access to clean water are

amongst the principal drivers. Therefore, unrestricted access to low-cost antimicrobials combined with a high burden of infectious disease, limited diagnostic capacity, and large urbanizing populations demanding better medical care has created a favourable environment for propagating AMR bacteria in LMICs in Asia^{131,132}.

One of the main issues influencing AMR is the indiscriminate usage of antimicrobials for treating common human diseases, of which diarrhoeal disease is likely a major contributor. It was estimated that the worldwide burden of diarrhoea was >1.7 billion episodes in 2010, with the vast majority of these episodes arising in LMICs²⁰⁷. The etiological agents of diarrhoea are broad (viruses, bacteria, and parasites) and can induce an array of symptoms, the more severe of which may necessitate an antimicrobial. Currently, the WHO recommends ORS, zinc, and an antimicrobial for those with more severe diarrhoea⁴⁶. However, a large disease burden, combined with a lack of financial and diagnostic resources means that the causative agent in LMICs is rarely or never identified²⁹. This lack of a confirmative diagnosis results in the empirical use of antimicrobials in the community or a healthcare facility. However, many of those receiving antimicrobials for diarrhoea may not require them¹⁴¹, as the disease is generally self-limiting, is most likely to be of viral aetiology^{29,184} and in LMICs in Asia many diarrhoeagenic bacteria are resistant to empirical antimicrobials.

Viet Nam is a rapidly developing LMIC in Southeast Asia with a current population of >90 million people, which is estimated to be >100 million by 2020¹. HCMC is the major financial centre in the south of the country and a typical urban, modernizing Asia megacity. An increasing population (>8 million people), a high infectious disease burden, and easy

antimicrobial access represents an unparalleled opportunity for investigating AMR and antimicrobial usage. Diarrhoeal disease is common in HCMC, with the two main paediatric hospitals jointly admitting >20,000 children with diarrhoea per annum¹⁹⁶. Here, we aimed to assess access and practices related to antimicrobial sales by mapping pharmacy locations and using a simulated client method in an urban district of HCMC. Further, hypothesizing that antimicrobial usage for diarrhoeal disease is excessive in this setting, we measured the primary choices of healthcare services for the parents or caregivers of children with diarrhoeal disease in the community. By combining denominators derived from parents and caregivers interviews we were able to estimate the annual antimicrobial use for watery and mucous diarrhoea in children aged 1 to <5 years living in central HCMC.

4.2 Results

4.2.1 The density and distribution of outlets selling antimicrobials in District 8 in HCMC

In September 2016 we mapped a total of 301 pharmacies and 243 private or public healthcare clinics in District 8 (Figure 4.1A). The distribution of pharmacies was not uniform and ranged from 0 to 61 pharmacies/km², with a mean density of 15.7 pharmacies/km². The majority of outlets were located on main thoroughfares in the northern portion of the district (Figure 4.1A). The overall number of pharmacy outlets equated with 7.59 pharmacies/10,000 people, which corresponded with a pharmacy for every 1,316 people. The number of pharmacies within the population varied from 0 to 38.2 pharmacies/10,000 people, with four high density (>30 outlets/10,000 people) areas; two in the north and two in the south of the district (Figure 4.1B). The high density of pharmacy shops exemplified routine access to antimicrobials in this community. The high density of

pharmacy shops epitomized routine access to antimicrobials in this community and equated with >75% of the study area population living within 250m of an outlet selling antimicrobials (Figure 4.1C).

Table 4.1 Pharmacies and healthcare clinics in the various wards of district 8 in Ho Chi Minh City.

Ward in district 8	Healthcare facilities	Pharmacies	Population ^a		Pharmacy density (per 10,000 people)		Acreage (km ²)	Pharmacy density
			All	1 to <5 years	All	1 to <5 years		
1	7	10	25,782	944	3.88	105.93	0.48653	14
2	20	29	20,694	843	14.01	344.01	0.50162	40
3	15	16	24,803	1,139	6.45	140.47	0.50999	29
4	51	36	42,200	2,284	8.53	157.62	1.44745	35
5	46	48	40,177	2,538	11.95	189.13	1.62052	28
6	15	34	30,144	1,353	11.28	251.29	1.46857	10
7	12	28	31,685	1,414	8.84	198.02	5.67584	2
8	4	5	9,024	444	5.54	112.61	0.30342	13
9	8	14	21,564	1,194	6.49	117.25	0.44648	18
10	15	14	15,841	836	8.84	167.46	0.2524	59
11	7	5	7,591	294	6.59	170.07	0.26942	26
12	7	10	16,653	816	6.00	122.55	0.30182	23
13	12	7	8,809	480	7.95	145.83	0.25577	47
14	4	6	20,575	1,144	2.92	52.45	0.55483	7
15	10	13	39,623	2,354	3.28	55.23	1.53309	7
16	6	26	41,010	1,243	6.34	209.17	3.54709	2
Total	239	301	396,175	19,320	7.59	142	19	12

^a Data from HCMC Demographic Department 30/06/17

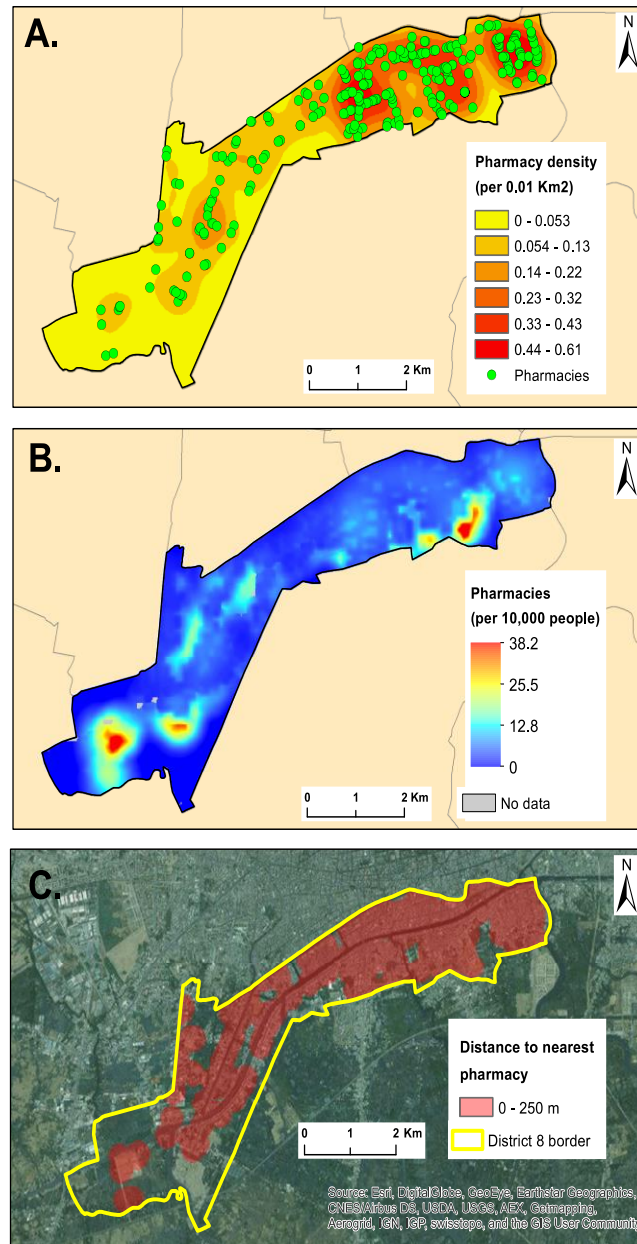


Figure 4.1 The geographical distribution of pharmacies in district 8 of Ho Chi Minh City

Maps of district 8 in Ho Chi Minh City showing; A) the point locations of pharmacies and the kernel distribution of pharmacy shop density. Green points identify the pharmacy shops and pharmacy density is determined by colour intensity (see key). B) The distribution of pharmacy shops density per 10,000 inhabitants. The greater the intensity of red the higher the numbers of pharmacy shops per 10,000 people (see key, areas in grey are those with no data due to missing data in the population raster). C) The geographical areas in district 8 that are within 250m of a pharmacy. Red shaded areas are locations in with residents live with 250m of a location selling antimicrobials.

4.2.2 Pharmacy prescribing practices for diarrhoeal diseases in the community

There were 48 pharmacy shops (37 were open upon visitation) in a ward within the study area (Figure 4.1) and with two different scenarios for paediatric diarrhoea surveyed. In the first scenario (watery diarrhoea), the number of prescribed medications ranged from one to five, with the majority (65%; 24/37) of pharmacies prescribing two different medications (Figure 4. 2A). The median total cost for each pharmacy visit was 17,000 Vietnamese Dong (VND) (1 United States Dollar (USD) = ~23,000VND); range 7,000 to 54,000VND. A total of 46 different types and brands of medications were sold, which could be broadly categorized into 11 classes; anti-motility, zinc, anti-emetics, anti-secretory, probiotics, ORS, antimicrobial, antipyretic, adsorbent, unlabelled, and others. Unlabelled treatments were prescribed in 16% (6/37) of pharmacy visits, with probiotics (81%; 30/37) and adsorbents (68%; 25/37) being the most commonly sold treatments. Antimicrobials were prescribed by 3/37 (8%) of pharmacies for watery diarrhoea (Figure 4.2 B).

In the second scenario (mucous diarrhoea with fever), 53 different medications were sold by the 37 pharmacies; undefined/unlabelled medications were sold in 49% (18/37) of pharmacy visits (Figure 4.2B). Probiotics (81%; 30/37) and adsorbents (51%; 19/37) remained the most common medications prescribed, but the proportion of pharmacies prescribing an antimicrobial increased to 22% (8/37). There was also an increase in the number of medications sold for mucous diarrhoea in comparison to watery diarrhoea (Figure 4.3A), with a higher proportion of pharmacies selling a combination of three (41%; 15/37), four (14%; 5/37), or five (5%; 2/37) medications. Despite there being a greater number of medications sold, the cost to caregivers in this scenario was not substantially greater than with watery diarrhoea (median 18,000 VND; range 3,000–31,000 VND).

There was considerable disparity in the type of antimicrobials sold in the 37 sampled pharmacies, with pharmacies prescribing antimicrobials from five different classes: trimethoprim-sulphate, sulphonamides, fluoroquinolones, macrolides, and cephalosporins (1st, 2nd, and 3rd generation). When the mystery shoppers returned to the same pharmacy shops a week later and asked to buy ciprofloxacin, 100% (37/37) of the vendors sold the requested antimicrobial. This medication was reliably sold without dosing advice and without the caregiver having to provide information as to why the antimicrobial was required. Generic ciprofloxacin was sold in a range of different packaging from 14 different manufacturers in two different concentrations.

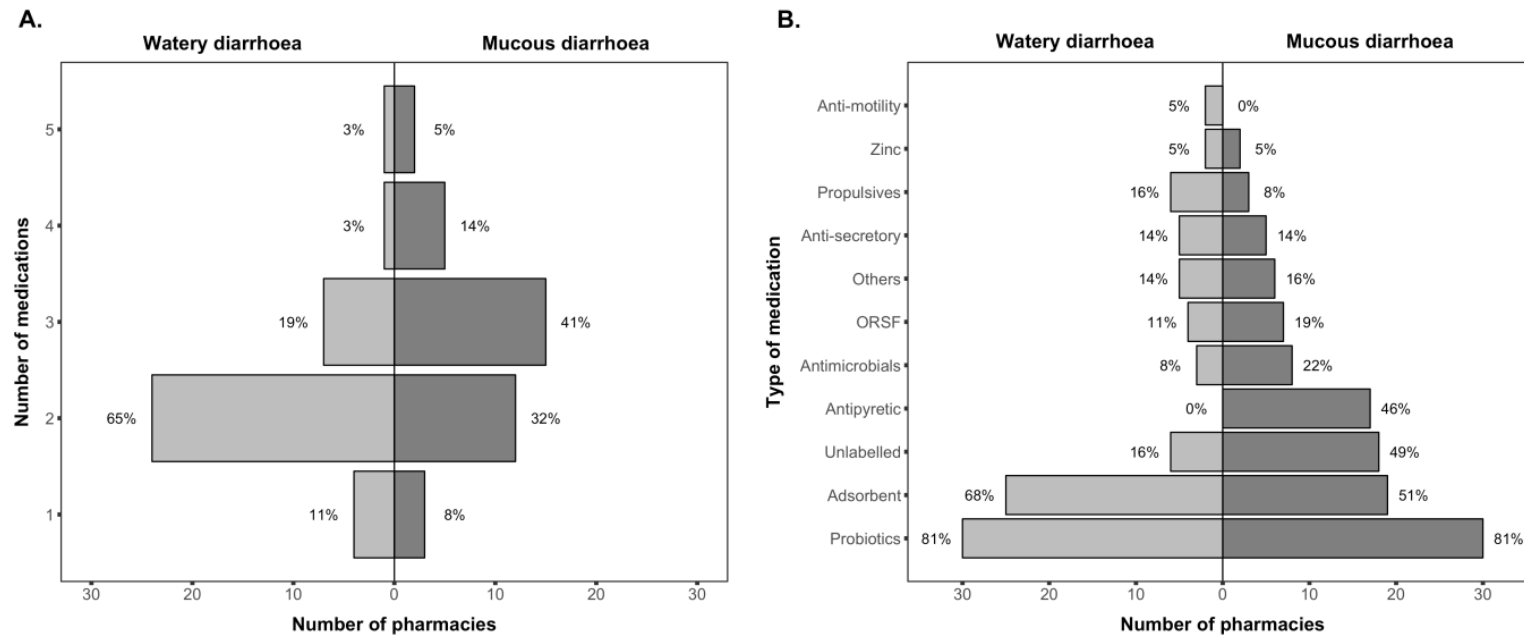


Figure 4.2 Medications sold by pharmacies in Ward 5 in District 8 for paediatric diarrhoea

A) Rotated histogram showing the number and proportion (x-axis) of the 37 pharmacies visited during the “mystery shopper” simulated clients method selling different mediations (y-axis) for watery and mucous diarrhoea, respectively. B) Rotated histogram showing the number and proportion (x-axis) of the 37 pharmacies visited during the “mystery shopper” simulated clients method selling between 1 and 5 medications (y-axis) for watery and mucous diarrhoea, respectively.

4.2.3 Knowledge, attitudes, and practices regarding antimicrobial usage in caregivers

I then aimed to evaluate how the community in district 8 access antimicrobials and understand their knowledge, attitudes, and practices regarding antimicrobial usage. There were 396 individuals that were either caregivers or parents of children aged <5 years residing in district 8 interviewed. The demographic characteristics of these participants are summarized in Table 4.2. The majority (82%; 325/396) of those interviewed were women and the median age was 39 years (range 19 to 87 years). A small fraction of those interviewed (10%; 39/381) indicated they had some background in healthcare. The majority (69%; 243/351) of interviewees had average household income ($\geq 1,300,000 \leq 9,000,000$ VND/household/month), with 21% (74/351) reported living in a poor household ($< 1,300,000$ VND/household/month). Most caregivers/parents (60%; 232/386) had ≥ 2 children living in the household. Many (59%; 231/394) reported that their children had received medication for a medical condition within the last month, of which approximately half (47%; 109/231) were reported to be an antimicrobial.

Table 4.2 The demographic characteristics of parents and caregivers in the community behaviour survey.

Characteristics	Number of responders	%
Total	396	100
Female sex	325/396	82.1
Role		
Parents	215/394	54.6
Caregiver	179/394	45.4
Age (years)	39 (31–54)	
<41	209/384	54.4
41–60	131/384	34.1
>60	44/384	11.5
Healthcare trained	39/381	10.2
Employment		
Worker	50/396	12.6
Officer	35/396	8.8
Health staff	12/396	3.0
Business owner	70/396	17.7
Housewife	157/396	39.6
Others (retired)	76/396	19.2
Education (years)		
≤ 12	304/393	77.2
>12	89/393	22.6
Monthly income ^a		
Below average	74/381	19.4
Average	243/381	63.8
Above average	34/381	8.9
Number of children		
1	154/386	39.9
≥ 2	232/386	60.1
Children <5 years		
1	300/386	77.7
≥ 2	86/386	22.3
Age of child (years)	3 (2–4)	
Infections in previous month	218/396	55.1
Medication last month	231/396	58.3
Current treatment		
Respiratory	136/396	34.3
Fever	41/396	10.4
Flu	13/396	3.3
Diarrhoea	5/396	1.3
Others	9/396	2.3
Antimicrobials in previous month	109/305	35.7

a) Monthly income <1,300,000VND/month is determined to be poor and below average (as suggest by the Vietnamese government)²⁰⁸; we considered ≥9,000,000VND/month a good monthly income and above average.

Most of parents and caregivers had limited knowledge about antimicrobials. Many assumed that antimicrobials could be generically used to treat coughing (46%; 181/396), fever (35%; 137/396), colds (30%; 118/396), headaches (21%; 81/396), and diarrhoea (19%; 76/396). Approximately half (51%; 203/396) of the parents and caregivers agreed with the statement “some infections can be difficult to treat if there is antimicrobial resistance” and a 59% (233/396) agreed with the statement “resistance can occur if they do not take a sufficient dose”. In self-reported prescribing, more than half of parents and caregivers (54%; 213/394) said they regularly purchased antimicrobials from pharmacies. Among people who ever bought antimicrobials from pharmacies, many of them (85%; 182/213) bought antimicrobials within the 30 days prior to the interview (Table 4.3).

Table 4.3 Knowledge, attitudes, and practice of parents and caregivers toward antimicrobials and antimicrobial resistance.

Feature		Variable	Positive response	Proportion (%)
Knowledge	Perceptions of antimicrobials treatment	Can be used for treating headaches	81/396	20.5
		Can be used for treating diarrhoea	76/396	19.2
		Can be used for treating teething	36/396	9.1
		Can be used for treating fever	137/396	34.6
		Can be used for treating colds	118/396	29.8
		Can be used for treating coughing	181/396	45.7
	Knowledge on prescription antimicrobials	Knowing that antimicrobials could be bought from doctors' prescription	305/396	77.0
		Knowing that antimicrobials could be got on request to doctor	17/396	4.3
		Knowing that antimicrobials could be got on request to drug seller	218/396	55.1
		Do not know how to have antimicrobials	22/396	5.6
Attitude	Some infections can be difficult to treat if there is antimicrobial resistance	Agree	203/396	51.3
		Disagree	23/396	5.8
		Do not know	167/396	42.1
		Other	3/396	0.8
	Resistance can happen if not taking enough doses	Agree	233/396	58.8
		Disagree	21/396	5.3
		Do not know	139/396	35.1
		Other	3/396	0.8
Practice	Practice of buying drugs from pharmacies	Ever bought drugs from pharmacies	357/395	90.4
		Ever bought antimicrobials from pharmacies	213/394	54.1
	Time of buying antimicrobials	Ever bought antimicrobials during the last 30 days	182/305	59.7
		Ever bought antimicrobials more than 6 months	74/305	24.3
		Don't remember the latest time of buying antimicrobials	49/305	16.1

4.2.4 Diarrhoeal treatment seeking behaviour by caregivers in the community

Figure 4. 3 describes the top three preferences for places where parents and caregivers would seek healthcare advice if their children had either of the two previously described diarrhoeal diseases scenarios. In both scenarios, the most common first choice was to treat their children at home. However, a specialized hospital was the most common option, with 81% (319/396) and 88% (347/396) of responders selecting this as one of their top three preferences for watery and mucous diarrhoea, respectively. Visiting a local pharmacy as a first choice ranked third for watery diarrhoea and second for mucous diarrhoea. Overall, visiting a pharmacy was selected as one of the top three preferences by 45% (180/396) and 34% (134/396) of caregivers for watery and mucous diarrhoea, respectively (Figure 4.4). Parents (rather than a caregivers) and those of younger age were more likely to take their children to a pharmacy than a hospital for watery diarrhoea ($p=0.015$ and $p=0.051$, respectively). Similarly, parents (rather than caregivers) were significantly more likely to take their children to a pharmacy than a hospital for mucous diarrhoea (Table 4.4)

Table 4.4 The demographic characteristics of parents and caregivers who chose a pharmacy or a hospital as first choice for a child with diarrhoea.

	Watery diarrhoea			Mucous diarrhoea		
	Pharmacy first	Hospital first	<i>p</i> value ^a	Pharmacy first	Hospital first	<i>p</i> value ^a
	choice (n=87)	choice (n=89)		choice (n=65)	choice (n=142)	
Female sex	73/87 (84%)	72/89 (81%)	0.693	55/65 (85%)	113/142 (80%)	0.448
Role of parent (not caregivers)	54/86 (63%)	39/89 (44%)	0.015	43/64 (67%)	69/142 (49%)	0.015
Age in years (IQR) ears)	38/81 (30, 50)	40/89 (32, 57)	0.051	38/60 (31, 50)	40/139 (32, 56)	0.198
Healthcare trained	7/83 (8%)	11/83 (12%)	0.461	4/63 (6%)	16/138 (12%)	0.316
Employment	87/87 (100%)	89/89 (100%)	0.661	65/65 (100%)	142/142 (100%)	0.943
Worker	10/87 (11%)	10/89 (11%)		6/65 (9%)	16/142 (11%)	
Officer	6/87 (7%)	6/89 (7%)		5/65 (8%)	10/142 (7%)	
Health staff	2/87 (2%)	6/89 (7%)		1/65 (2%)	6/142 (4%)	
Business owner	15/87 (17%)	10/89 (11%)		11/65 (17%)	23/142 (16%)	
Housewife	35/87 (40%)	34/89 (38%)		29/65 (45%)	56/142 (39%)	
Other (retired)	19/87 (22%)	23/89 (26%)		13/65 (20%)	31/142 (22%)	
Education ≤12 years	75/87 (86%)	68/88 (77%)	0.171	55/65 (85%)	115/141 (82%)	0.695
Monthly income ^b			0.406			0.739
≤1.3M VND	15/78 (19%)	14/80 (18%)		11/57 (19%)	20/127 (16%)	
1.3–9M VND	59/78 (76%)	57/80 (71%)		42/57 (74%)	95/127 (75%)	
>9M VND	4/78 (5%)	9/80 (11%)		4/57 (7%)	12/127 (9%)	
Only 1 child	36/84 (43%)	37/84 (44%)	1.000	21/65 (32%)	61/134 (46%)	0.091
Only 1 child aged <5 years	68/85 (80%)	71/86 (83%)	0.699	49/65 (75%)	105/134 (78%)	0.718
Age of first child (years)	3/87 (2, 4)	2/87 (2, 4)	0.486	3/65 (2, 4)	3/140 (2, 4)	0.553
Infections in previous month	57/86 (66%)	44/86 (51%)	0.063	43/65 (66%)	73/139 (53%)	0.071
Medication in previous month	60/87 (69%)	51/87 (59%)	0.207	45/65 (69%)	83/141 (59%)	0.167
Antimicrobials in previous month	24/60 (40%)	26/70 (37%)	0.857	18/41 (44%)	46/118 (39%)	0.585

a) *p*-values calculated using Fisher's exact test for categorical variables and the Mann-Whitney U-test for continuous variables. Significant *p*-values are highlighted in bold.

b) Monthly income <1,300,000VND/month is determined to be poor and below average (as suggest by the Vietnamese government)²⁰⁸; while income ≥9,000,000VND/month a good monthly income and above average.

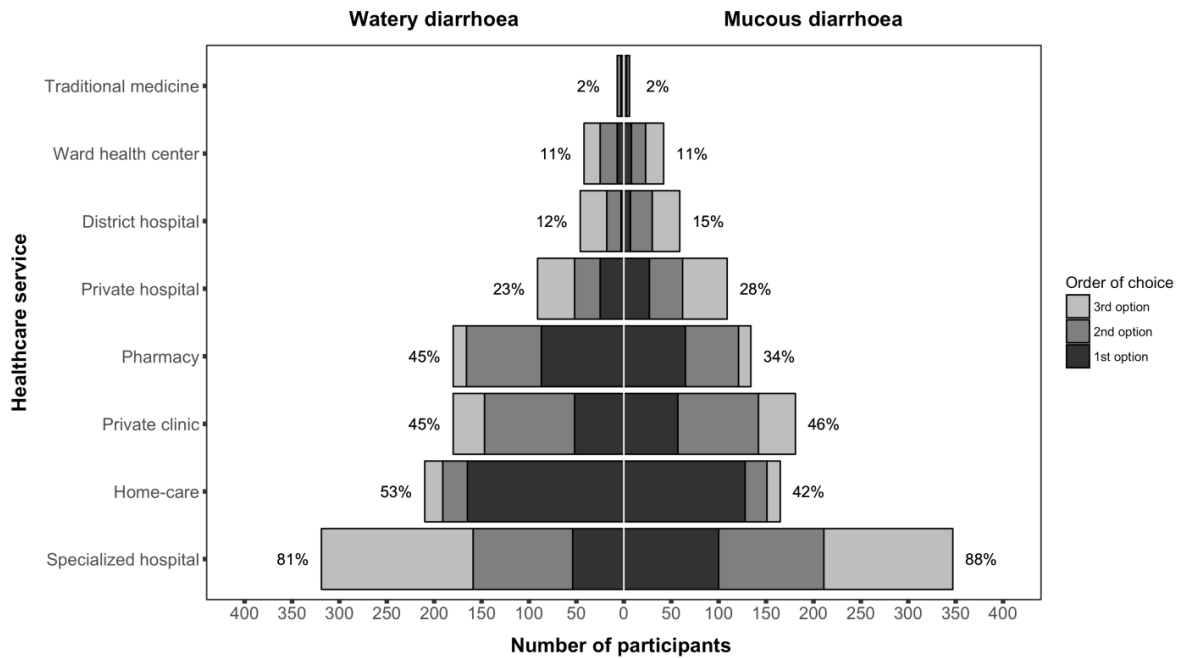


Figure 4.3 Healthcare utilization for paediatric diarrhoea by caregivers in District 8

Pyramid plot showing the number and proportions of 396 individuals, caregivers, or parents of children aged <5 years residing in District 8 (x-axis) selecting the various healthcare services (y-axis) as first, second, or third choices (shaded, see key) for watery and mucous diarrhoea.

4.2.5 Quantifying antimicrobial use for diarrhoeal treatment

Lastly, I aimed to calculate antimicrobial usage in the community and in hospitals for diarrhoeal disease in children aged between 1 and 5 years (Figure 4.4). From following 748 children and quantifying episodes of diarrhoea over a two-year period in the diarrhoea cohort, I estimated the total annual incidence of watery and mucous diarrhoea to be 536 (95%CI 463–609) and 210 (95%CI 154–267)/1,000 child-years, respectively (B). The annual incidence of watery and mucous diarrhoea that attended hospital were 112 (95%CI 83–141) and 116 (95%CI 83–149)/1,000 child-years, respectively (C). Among the diarrhoeal episodes attending hospital, 66/159 (42%) watery episodes and 107/164 (65%) mucous episodes received antimicrobial treatment in hospital (D). I therefore estimated the total number of diarrhoeal episodes within the study area in this age group as 10,355 (95%CI 8,945–11,765) and 4,057 (95%CI 2,975–5,158) per year for watery and mucous diarrhoea, respectively (Table 4.5).

Combining data from the surveys of the self-reported behaviour and the pharmacy practice, I estimated that the annual number of diarrhoeal episodes in children aged 1–5 years that would receive antimicrobial treatment at pharmacies were 184 (95%CI 159–209) for watery diarrhoea and 144 (95%CI 105–183) for mucous diarrhoea (Table 4.5). However, data from the diarrhoea cohort indicated that the number of paediatric diarrhoeal cases that received an antimicrobial attending in hospital were approximately five (898 (95%CI 673–1,130)) and ten times higher (1,462 (95%CI 1,046–1,878)), for watery and mucous diarrhoea, respectively (Table 4.5). In total, I estimated that at least 19% (2,688/14,427) (a total from 2,359 and 329) of diarrhoea episodes in children aged 1–5 years in the study area would receive an antimicrobial annually.

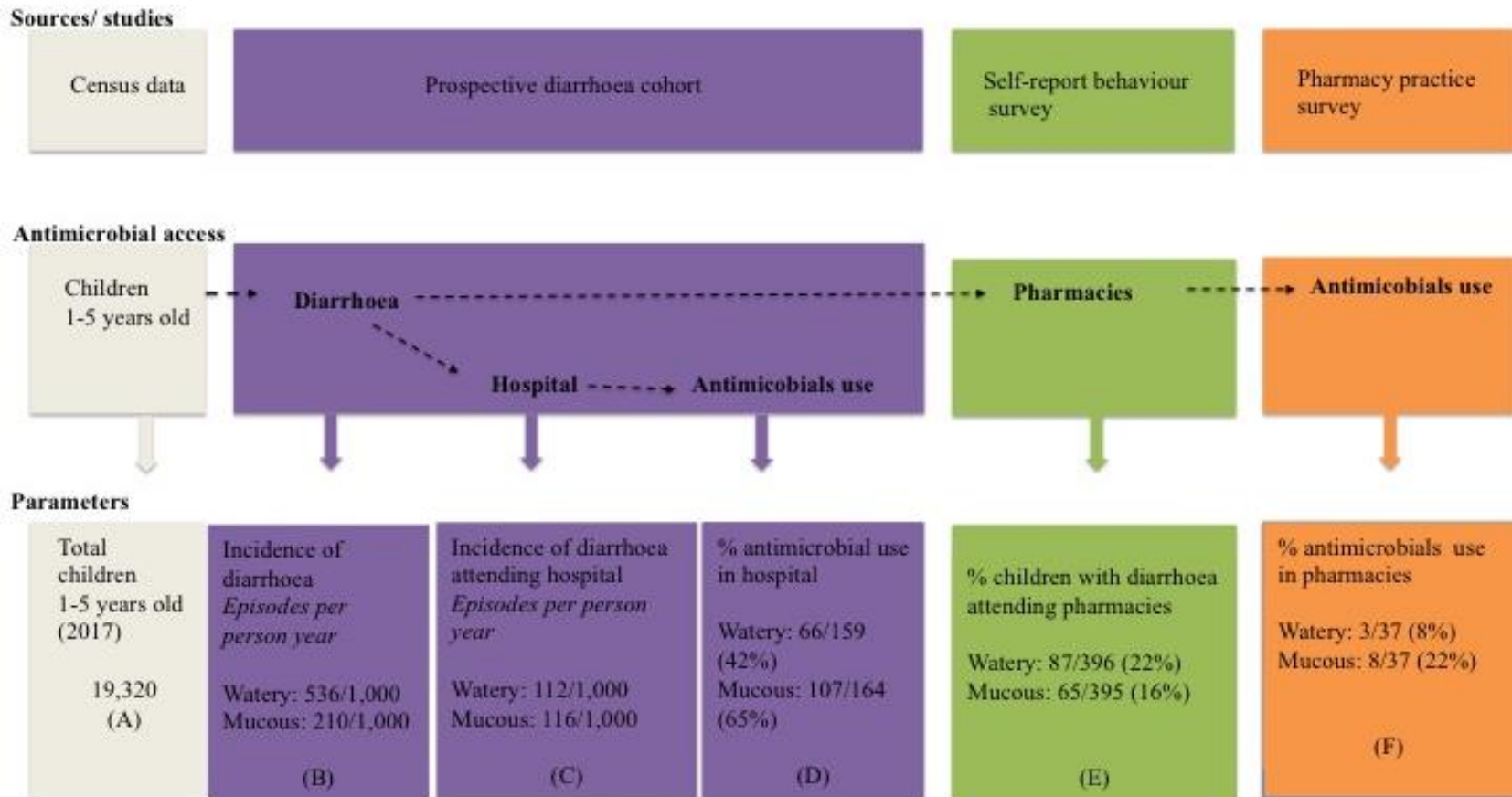


Figure 4.4 Estimating antimicrobial usage for diarrhoea in children aged 1 to 5 years in pharmacies and hospital. Diagram outlining the sources of data, parameters, and calculations used to quantify antimicrobial usage for diarrhoea in children aged 1–5 years in this population. Note each of the parameters have been calculated from the studies (A–F).

Table 4.5 The burden of diarrhoea and antimicrobial treatment for diarrhoea in children aged 1 to 5 years.

Diarrhoea type	Total number of children with diarrhoea in District 8 per year (95% CI)	Number of children with diarrhoea episodes (95% CI)			
		Attending hospital	Receiving an antimicrobial in hospital	Attending a pharmacy	Receiving an antimicrobial at a pharmacy
Calculation ^a	(A x B)	(A x C)	(A x C x D)	(A x B x E)	(A x B x E x F)
Watery	10,355 (8,945–11,765)	2,163 (1,622–2,724)	898 (673–1,130)	2,275 (1,965–2,584)	184 (159–209)
Mucous	4,057 (2,975–5,158)	2,241 (1,603–2,878)	1,462 (1,046–1,878)	667 (489–848)	144 (105–183)
Total	14,427 (12,636–16,219)	4,404 (3,560–5,249)	2,359 (1,884–2,834)	2,945 (2,585–3,305)	329 (282–375)

Calculation derived from parameters described in Figure 2.8. Parameters include: (A) Total children ages 1–5 years by 2017 was 19,320; (B) Incidences of watery and mucous diarrheal were 536/1000 CYO and 210/1000 CYO respectively; (C) Incidences of watery and mucous diarrhoea attending hospital were 112/1000 CYO and 116/1000 CYO; (D) % of antimicrobial uses in hospital for watery and mucous diarrhoea were 42% (66/159) and 65% (107/164) respectively.

4.3 Discussion

Antimicrobial resistant infections are increasing throughout Asia, and are likely being driven in part by ease of access to antimicrobials. This factor is likely more relevant in a setting with a high burden of infectious diseases, low diagnostic capacity, and a large urbanizing population^{8,209,210}. Viet Nam is the world's fifteenth most populous country, with >96 million people in 2017 and urbanization increased from 28% in 2014 to 34.9% in 2017¹⁹⁴. Antimicrobial dispensing in Viet Nam is known to be poorly regulated, leading to inappropriate prescribing and self-medication for a number of common infections, including acute respiratory infections^{211,212}. It is also known that antimicrobials usage is also excessive for paediatric diarrhoeal disease, which is common in Viet Nam, and among the top motives for seeking healthcare facilities in HCMC^{29,184,191,195}. However, it was unknown whether antimicrobial usage for treating paediatric diarrhoeal disease was more prevalent in the community or a hospital setting. Here, we report simple access to community outlets selling antimicrobials in an urban district of HCMC. My results from this study suggest that antimicrobial usage for paediatric diarrhoeal disease is excessive in this setting, but surprisingly, antimicrobial usage for paediatric diarrhoeal disease is almost ten times more common in hospitals than in the community.

The general density of pharmacies in the community in HCMC is high (7.59/10,000 people) and outnumbers the number of pharmacy staff that have been trained at any level (assistant or university degree) in this city (5.98/10,000 people)²¹³. This density of pharmacies in HCMC is reflective of the general pattern in LMICs, for example Sade *et al.* reported a comparative distribution in an urban of India (5.84/10,000 people)²¹⁴. Alarming, the number of pharmacy outlets in HCMC is more than double the mean

density of pharmacies in other areas in the world, such as Europe (3.06/10,000 people)²¹⁵, Southeast Asia (3.02/10,000 people)²¹⁶, three times higher than that of some countries in Western Pacific (2.28/10,000 people)²¹⁶, and the USA (2.11/10,000 people)²¹⁷, and six times higher than that in parts of Africa²¹⁶. Ease of access to retail pharmacies may improve equity in the use of medication, but also allows access to drugs that should be better controlled, especially when pharmacy staffs are under qualified and/or fail to follow appropriate guidelines.

My study demonstrated the common vending of antimicrobials without prescription for paediatric diarrhoeal disease in the community in HCMC. However, this practice varied according to the reported severity of the disease, in which less severe symptoms were associated with fewer dispensed antimicrobials. Notably, I estimated that antimicrobials sales for paediatric diarrhoeal disease in the community in HCMC was lower than that in Ethiopia (26%; 58/223)¹⁷⁵, and other Asian countries, such as Pakistan (14%)²¹⁸, and Thailand (52%)²¹⁹. The low proportion of antimicrobials dispensed for acute watery diarrhoea in HCMC (8%, 3/37) complements a declining trend in antimicrobials use for watery diarrhoea in Viet Nam. Available data suggests that antimicrobials dispensed for acute watery disease has decreased over the last 20 years, from 45% in 1997²²⁰, to 14% in 2013²²¹, to 8% (3/37) in our study. This trend may result from a substantial improvement in practices in pharmacies to alleviate antimicrobial prescribing and the impact of resistance. However, despite an array of public health engagement and intervention projects, antimicrobials still remain the most inappropriate drugs dispensed from retail pharmacies in Viet Nam¹⁴⁰. It is additionally worth noting that previous studies suggested that antimicrobial usage is much greater in rural areas than in urban areas²¹². Therefore, my

results from an urban setting may underestimate the extent of the issue from other locations in and around HCMC and across the country.

The major finding in this study was that antimicrobials usage for paediatric diarrhoea in HCMC is more common in hospitals than in the community. I predicted that the diarrhoeal disease management, including antimicrobials dispensing, in the community is poor in HCMC, which is similar to a number of other areas in the world^{218–220,222,223}. I found that the total number of diarrhoeal episodes that would receive an antimicrobial in the hospital was five to ten times higher than that from retail pharmacies; this observation was contrary to our hypothesis. My findings are consistent with a recent finding in this setting, where 85% of bloody and mucous paediatric diarrhoeal patients admitted to hospital were prescribed antimicrobials¹⁴¹. While one-third of antimicrobials prescribed in hospitals may be considered inappropriate²⁰⁶, this practice could enhance associated with AMR. A potential explanation for our finding is that children with diarrhoeal disease attending a specified hospital are generally considered to be the more serious cases. Therefore, doctors are inclined to prescribe first-line therapies to prevent disease progression and to satisfy parent expectations for treating the infection with the best available medication. It is apparent that, in a setting with limited-to-no diagnostic testing, that this clinical approach is thought to be optimal for preventing complication and saving lives. I suggest that antimicrobials are used as an “insurance policy” rather than as a therapeutic treatment option. There is a large demand for hospital care in HCMC and doctors have limited time to make clinical decisions and this approach may be more effective and less time consuming. Therefore, there is a clear requirement for tests that can identify children at the

more severe end of the disease spectrum and that can simply identify between a viral and a bacterial infection.

This study has limitations. I likely underestimated the overall antimicrobial use as our calculation did not take into account antimicrobial use in facilities other than specialized hospitals. Additionally, relying on the choice of attending pharmacy as the first option only and the practice of selling antimicrobials in a small number of pharmacies from two scenarios may underestimate antimicrobial sales in pharmacies. Even though I employed a thorough process to quantify antimicrobial use for diarrhoea, our data extrapolation may contain errors as we compiled data from several sources. In addition, aiming to assess antimicrobials dispensing only, I did not record or evaluate the retailer's consulting abilities for using these treatments, which may reflect local antimicrobial handling practices. Lastly, my combined studies focused on diarrhoeal disease in young children only, I recognize that this approach limited the understanding of antimicrobial usage in other populations and other presentations.

4.4 Conclusion

I conclude that the pharmacy retailer density is extremely high in urban HCMC, which is associated with straightforward antimicrobial access in the community. However, antimicrobial usage for diarrhoeal diseases in children is more common in hospitals than through community pharmacies. Given these findings, combined with a general low level of knowledge regarding antimicrobial and the self-reporting behaviour parents and caregivers, I predict a high burden of antimicrobial overuse in the community in Viet Nam. I propose further applied research to improve the knowledge, attitudes, and practice of

parents and caregivers in terms of antimicrobial prescribing and AMR. I additionally advocate better education, training, and guidelines for antimicrobial usage and AMR for all health professionals with the authority to prescribe antimicrobials in Viet Nam. Lastly, rapid diagnostic tests at the first point of care contact are essential for optimizing antimicrobial therapy in the community.

5. Obesity and age associated with the carriage of fluoroquinolones and third generation cephalosporins resistance genes in commensal *E. coli* from a cohort of urban Vietnamese children

AMR bacterial infections in LMICs are well-established global health issue. Antimicrobials are commonly used worldwide in agriculture in both developed and developing countries for the purpose of growth and nutrition supplements in livestock²²⁴. It was noted for more than 35 years that humans can be transferred antimicrobial resistant bacteria by farm animals because the antimicrobials used in livestock can be ingested by humans when people consume food²²⁵. The consequences are first susceptible bacteria among human intestinal system can be killed or suppressed by the antimicrobials in food, allowing resistant bacteria to thrive then these bacteria can cause bacterial resistance infection¹⁰⁸. Recent evidence shows that infants who have not been exposed to antimicrobials have similar faecal ARGs to their mothers¹³⁵. I hypothesize that commensal AROs in the community in LMICs are a major reservoir of ARGs that can be transferred to pathogens.

Therefore, in this chapter I aimed to assess the prevalence and epidemiological factors associated with the carriage of ciprofloxacin and ceftriaxone resistant *Escherichia coli* and associated resistance genes in a cohort of healthy children residing in urban HCMC, Viet Nam. Using rectal swabs from 498 children cultured onto MCA independently supplemented with resistant concentrations of ciprofloxacin and ceftriaxone, I aim to detect resistant *E. coli* to these two commonly used antimicrobials. Screening the individual *E. coli* populations by conventional PCR from samples, I aim to detect a panel of PMQR and

ESBL encoding genes. Using univariable and multivariable logistic regression, I investigate the associations between phenotypic/genotypic resistance and demographic characteristics.

5.1 Background

AMR has become an increasingly recognised global health problem. The social complexities associated with AMR are multifaceted; however, a major driver of AMR is thought to be the unsolicited use of antimicrobials in the community, which is particularly common in many LMICs^{95,226}. It is generally accepted that the more a community is exposed to antimicrobials, the more likely AMR bacteria will emerge and be maintained²²⁷. Multiple studies have demonstrated a recent dramatic rise in pathogenic bacteria exhibiting resistance against fluoroquinolones and third-generation cephalosporins^{228–230}; two of the most commonly used classes of antimicrobials globally²³¹. Consistently, cephalosporins and fluoroquinolones are the most consumed antimicrobials classes in Viet Nam, with prescribed consumption from hospitals equal to that from retail pharmacies^{179,226}.

E. coli is a Gram-negative bacterium, which is abundant in the intestine of healthy people and can be used to assess the composition of AMR in the human gut^{143,232,233}. *E. coli* are proficient vehicles of AMR gene circulation, and are commonly co-resistant to several clinically important antimicrobials including cephalosporins, fluoroquinolones, and sulfamethoxazole/trimethoprim^{234,235}. Therefore, commensal *E. coli* are sentinel Gram-negative organisms that play a principal role in the maintenance and spread of plasmid associated AMR genes within the Enterobacteriaceae^{182,235}. Specifically, *E. coli* are known to be a major reservoir of plasmids carrying both ESBLs and PMQR genes^{73,179}.

The expression of ESBLs genes is a key resistance mechanism against later generation beta-lactams; the circulation of genes encoding these enzymes is of critical importance in human health⁷³. ESBLs, which can hydrolyse a wide variety of β -lactamases, are presently classified into several sub groups, the most frequently identified include TEM, SHV, and CTX-M^{236,237}. TEM and SHV are historic β -lactamase genes that have evolved to exhibit ESBL activity; TEM and SHV variants that have ESBL activity can be commonly found in members of the Enterobacteriaceae associated with infection. CTX-M genes were first described in the 1980s and found to have greater activity for reducing cefotaxime susceptibility than existing ESBL genes. The *bla*_{CTX-M} genes have been extremely successful, becoming globally ubiquitous and undergoing extensive diversification (>100 variants have been identified). This important group of ESBL genes is broadly divided into five phylogroups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. The most dominant global lineage is *bla*_{CTX-M-1}, which incorporates *bla*_{CTX-M-15}, a particularly successful and persistent variant found commonly in Asia²³⁶.

Fluoroquinolone resistance generally occurs as a result of mutations in the chromosomal drug target genes encoding the DNA gyrase (*gyrA* and *gyrB*) and the topoisomerase IV (*parC* and *parD*)²³⁸. However, fluoroquinolone resistance can also be induced and enhanced by the PMQR genes. The most common PMQR genes are the *qnr* family (*qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*), which act by altering the DNA gyrase and topoisomerase IV, thus reducing the interaction between drug and target^{107,239}. The *qnr* genes have been found on a broad range of plasmid structures in various pathogens within the Enterobacteriaceae. Alternative PMQRs include *aac(6')-Ib-cr*, which encodes a modified aminoglycoside acetyltransferase that inactivates specific fluoroquinolones, and the efflux pump encoding

genes, *qepA* and *qqxAB*^{238,240}. Notably, PMQR genes frequently co-exist on the same plasmid backbones as ESBL genes, which can be co-transferred during horizontal gene exchange^{107,241,242}.

Several studies have focused on characterising the carriage of AMR organisms in hospitalized patients and healthy individuals in the community in LMICs^{229,235,243}. The majority of these investigations have isolated *E. coli* and then performed conventional antimicrobial susceptibility testing²⁴⁴. In addition, various studies have measured the prevalence of ESBL producing commensal *E. coli* in Vietnamese individuals^{245,246}. However, less is known about the prevalence of AMR bacteria and the diversity of associated AMR genes in the commensal microbiota of healthy children living in an urban community. Furthermore, the potential risk factors associated with the carriage of AMR organisms of AMR genes have not been defined. Here, I use a direct plating method on selective media to measure phenotypic and genotypic resistance in *E. coli* against fluoroquinolones and third generation cephalosporins among a cohort of 498 healthy children resident in HCMC²⁴⁷. Further, I aim to identify epidemiological risk factors associated with the carriage of phenotypic and genotypic resistance in these urban children. Such data from an urbanizing LMIC is important for a better understanding the circulation of AMR bacteria in the community and to quantify the key populations associated with the global AMR crisis.

5.2 Results

5.2.1 Baseline characteristics

Between March and August 2016, rectal swabs were collected from 498 children without diarrhoea attending a routine health check at the designated healthcare facility. All children were from a single district (eight) in HCMC (the same study area assessed for access to pharmacies in this age group, which has been described in Chapter 4) and enrolled in a longitudinal cohort study of 748 individuals (the diarrhoea cohort, described in Method section 2.1.2 Diarrhoea cohort). This population was considered to be generally representative of the child population in HCMC. The baseline characteristics of those sampled are in Table 5.1; 269/498 (54%) were male, 211/498 (42.4%) were born by Caesarean section, and 64/498 (12.9%) were preterm deliveries (Table 5.1). The median age of the children at the point of sampling was 46 months. At the time of the health check in which they were sampled, the majority of children were attending nursery/school (67.9%; 338/498), and only a small fraction of children were underweight, stunted, or wasted. However, more than a quarter of children were overweight (27.5%; 137/498), with a similar proportion (136/498; 27.3%) having a minor non-diarrhoeal illness at the time of visit. A substantial proportion (42.8%; 213/498) of children had received antimicrobials in the six months prior to sampling and 26.9% (134/498) regularly consumed some form of probiotic as part of their diet (Table 5.1). A third (29.1%; 145/498) of the children had taken oral vitamin A supplementation, provided via a government public health nutrition program. The children were a broad cross-section of the community in this urban district of HCMC and had correspondingly consumed a wide variety of animal products in the three

days prior to sampling, which included pork (89.6%, 446/498), shrimp (85.1%; 424/498), eggs (77.7%; 387/498), beef (75.9%; 378/498), and poultry (64.5%; 321/498) (Table 5.1).

5.2.2 Ciprofloxacin and ceftriaxone resistant *E. coli*

Aiming to assess the prevalence of antimicrobial resistant *E. coli* carriage in this population, rectal swabs were independently plated onto MCA supplemented with ceftriaxone and ciprofloxacin to isolate resistant organisms. *E. coli* colonies that were resistant to ceftriaxone (according to current CLSI guidelines²⁴⁸) were cultured from 80.3% (400/498) of the rectal swabs and *E. coli* colonies that were resistant to ciprofloxacin (according to current CLSI guidelines²⁴⁸) from 67.7% (337/498) of the rectal swabs. As shown in Figure 5.1, resistant *E. coli* colonies were cultured from at least one of the antimicrobial supplemented plates (i.e. either ciprofloxacin or ceftriaxone) in 84.7% (422/498) samples; no antimicrobial resistant *E. coli* colonies were isolated on either plate from the remainder of samples (76/498; 15.3%). From the 422 samples from which we isolated resistant *E. coli* colonies on at least one plate, 315 produced *E. coli* colonies on both the ceftriaxone supplemented media and the ciprofloxacin supplemented media. Figure 5.2 provides a more detailed perspective at the prevalence of *E. coli* colonies that were resistant to any of the two antimicrobials tested. More samples contained ceftriaxone resistant *E. coli* colonies than ciprofloxacin resistant *E. coli* colonies.

Table 5.1 Baseline characteristics of the 498 study participants.

Characteristics	Frequency (%) or Median (IQR)
Male	269 (54.0%)
Age at visit (month)	46.4 (35.6, 52.5)
Preterm at birth	64 (12.9%)
Low birth weight	20 (4.0%)
Caesarean section	211 (42.4%)
The only child in the family	271 (54.4%)
Early breastfeeding at birth	340 (68.3%)
Feeding at birth	
Only formula	42 (8.4%)
Exclusive breastfeeding	20 (4.0%)
Breast milk plus formula	436 (87.6%)
Breastfeeding duration (month)	4.0 (3.0, 6.0)
Breastfeeding up to 12 month	60 (12.0%)
Attending school	338 (67.9%)
Nutritional status *	
Underweight (weight for age)	2 (0.4%)
Stunting (height for age)	10 (2.0%)
Overweight (weight for height)	137 (27.5%)
Wasting (weight for height)	1 (0.2%)
Probiotic using prior 6 months	134 (26.9%)
Antimicrobials using prior 6 months	213 (42.8%)
Oral vitamin A supplementation prior 6 months	145 (29.1%)
Food consumption prior 3 days	
Pork	446 (89.6%)
Beef	378 (75.9%)
Shrimp	424 (85.1%)
Fish	339 (68.1%)
Egg	387 (77.7%)
Shellfish	108 (21.7%)
Pate (made from live liver)	53 (10.6%)
Current sickness	136 (27.3%)
Hospital admission prior 6 months	19 (3.8%)
Low maternal education (< = 9yrs)	302 (60.6%)
In-paid employment mothers	342 (68.7%)
Household crowding **	348 (69.9%)
Main water use at home	
Piped to residence	331 (66.5%)
Bottled water	163 (32.7%)
Others	4 (0.8%)
Ice using at home	82 (16.5%)
Exposure to flooding	107 (21.5%)
Exposure to animal around home	300 (60.2%)
Presence of toilet soap at home	498 (100.0%)
Handwashing behaviour after toilet	
Always	269 (54.0%)
Sometimes	227 (45.6%)
Never	2 (0.4%)
Handwashing after handling diaper	
Always	420 (84.3%)
Sometimes	78 (15.7%)
Never	0 (0.0%)

* Based on the WHO Child Growth Standards: Underweight: weight for age < -2 standard deviations (SD); Stunting: height for age < - 2SD; Wasting: weight for height < - 2SD; Overweight: weight for height >+2SD; **Two or more people per room

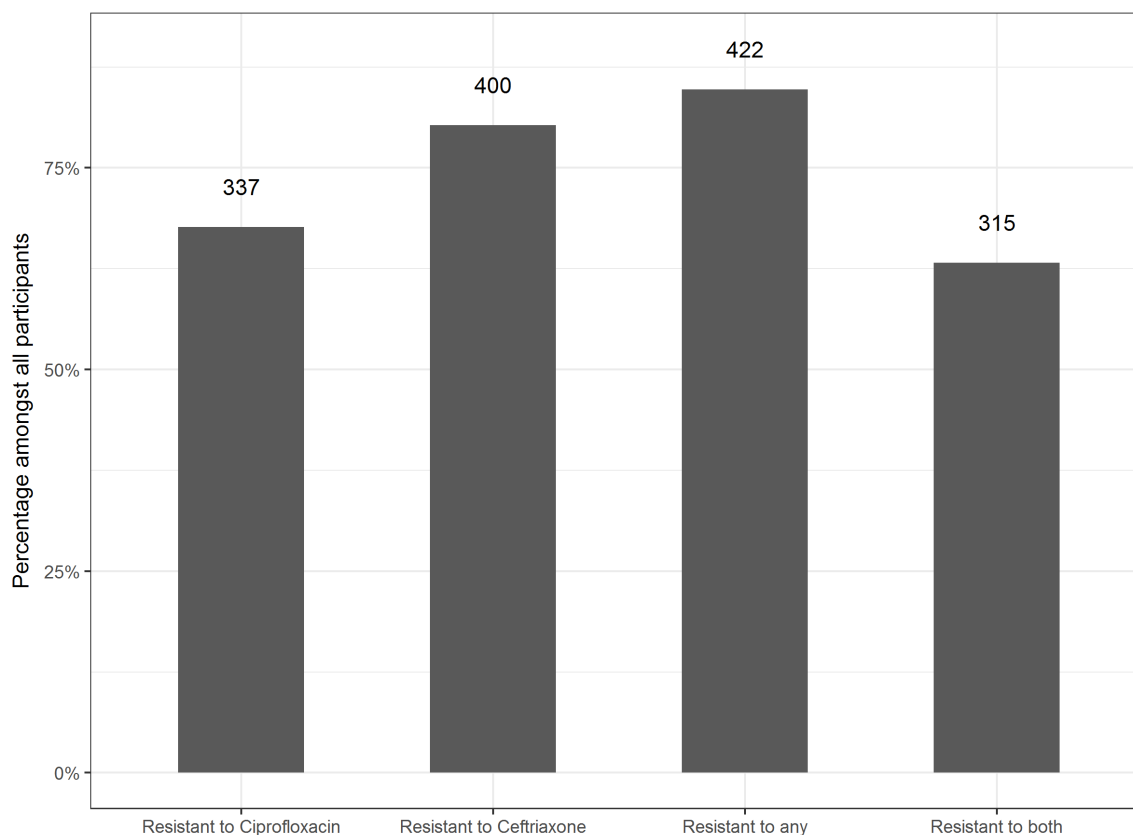


Figure 5.1 The prevalence of antimicrobial resistant commensal *E. coli* in a cohort of 498 Vietnamese children. Bar chart showing the prevalence of faecal samples from rectal swabs from which *E. coli* exhibiting resistance to flouroquinolones (ciprofloxacin), third generation cephalosporins (ceftriaxone), or both could be isolated.

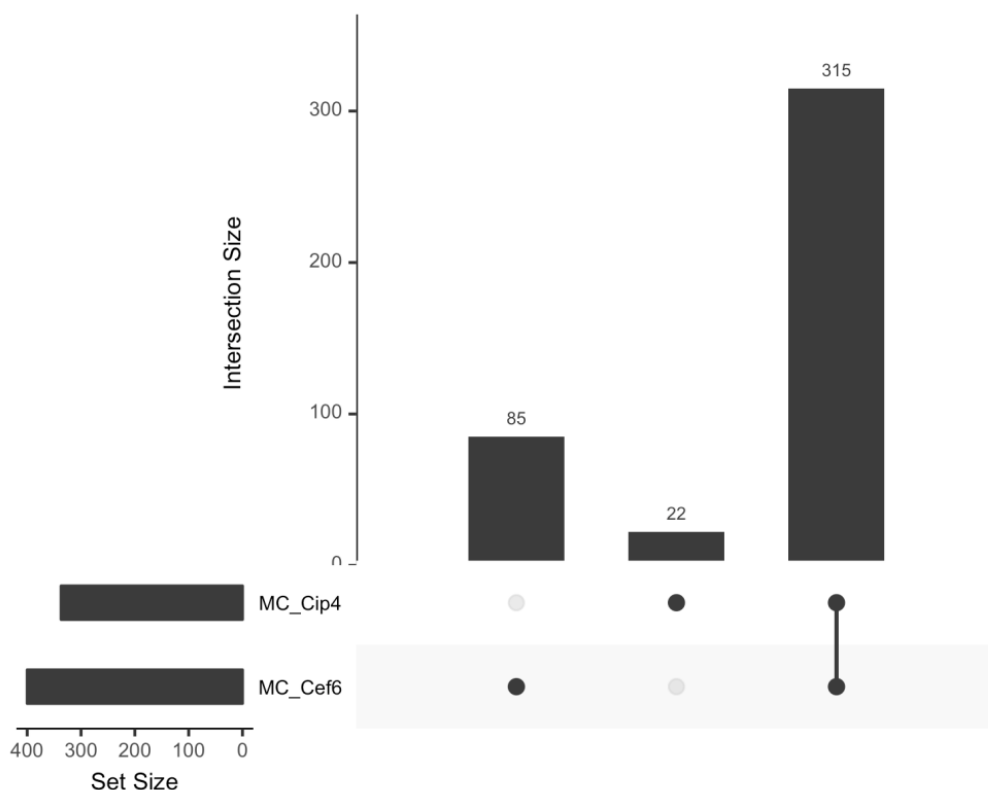


Figure 5.2 The prevalence of fluoroquinolones (ciprofloxacin) and third generation cephalosporins (ceftriaxone) resistant *E. coli*. Horizontal bar chart at bottom-left represents frequencies of sample with *E. coli* resistant to ciprofloxacin (MC_Cip4) and ceftriaxone (MC_Cef6). Top-right bar chart represents frequencies of sample with *E. coli* resistant to ceftriaxone only, sample with *E. coli* resistant to ciprofloxacin only, and sample with both resistances.

5.2.3 Risk factors for ciprofloxacin and ceftriaxone commensal *E. coli* phenotype

I aimed to identify epidemiological characteristics associated with the detection of fluoroquinolones and cephalosporin resistant *E. coli* in the rectal swabs. However, after investigating multiple factors including age, sex, antimicrobial usage, and other household variables, I found that the antimicrobial resistant *E. coli* were so ubiquitous that no factors were significantly associated with their carriage (Table 5.2).

Table 5.2 Associations between the presence of *E. coli* resistant to any antimicrobials and characteristics of the study participants.

Characteristic	Presence of suspected antimicrobial resistant <i>E. coli</i>		Univariable analysis			Multivariable analysis		
	Yes (N=422)	No (N=76)	OR	(95% CI)	p value	OR	(95% CI)	P value
Low maternal education	256 (60.7%)	46 (60.5%)	1.01	(0.61, 1.65)	0.982	0.93	(0.55, 1.56)	0.782
In-paid employment mother	287 (68.0%)	55 (72.4%)	0.81	(0.46, 1.38)	0.446	0.75	(0.42, 1.33)	0.336
Male	228 (54.0%)	41 (53.9%)	1.00	(0.61, 1.64)	0.990	0.94	(0.56, 1.57)	0.823
Age * (month)	43.3 (35.6, 52.4)	47.3 (35.7, 53.3)	0.97	(0.94, 1.00)	0.044	0.97	(0.94, 1.00)	0.088
Preterm at birth	55 (13.0%)	9 (11.8%)	1.12	(0.55, 2.51)	0.773	1.12	(0.54, 2.57)	0.765
Caesarean section	183 (43.4%)	28 (36.8%)	1.31	(0.80, 2.19)	0.287	1.28	(0.76, 2.18)	0.360
Breastfeeding duration * (month)	4.0 (3.0, 6.0)	4.0 (3.0, 6.0)	0.99	(0.95, 1.04)	0.765	0.99	(0.95, 1.04)	0.812
Weight-for-height z score *	1.0 (-0.2, 2.2)	0.7 (-0.3, 1.8)	1.08	(0.93, 1.26)	0.342	1.04	(0.87, 1.24)	0.677
Antimicrobials use in the prior 6 months	183 (43.4%)	30 (39.5%)	1.17	(0.72, 1.95)	0.527	1.23	(0.72, 2.13)	0.449
Probiotic use in the prior 6 months	110 (26.1%)	24 (31.6%)	0.76	(0.45, 1.32)	0.325	0.67	(0.38, 1.19)	0.169
Pork or beef consumption in the prior 3 days	416 (98.6%)	75 (98.7%)	0.92	(0.05, 5.52)	0.942	1.02	(0.05, 6.67)	0.985
Poultry or egg consumption in the prior 3 days	383 (90.8%)	72 (94.7%)	0.55	(0.16, 1.41)	0.228	0.51	(0.15, 1.35)	0.188
Seafood consumption in the prior 3 days	412 (97.6%)	74 (97.4%)	1.11	(0.17, 4.33)	0.892	1.09	(0.16, 4.55)	0.919
Ice using in the prior 6 months	68 (16.1%)	14 (18.4%)	0.85	(0.46, 1.66)	0.622	0.86	(0.45, 1.73)	0.661
Piped water to residence	284 (67.3%)	47 (61.8%)	1.27	(0.76, 2.09)	0.358	1.20	(0.70, 2.05)	0.504
Exposure to flooding	89 (21.1%)	18 (23.7%)	0.86	(0.49, 1.57)	0.616	0.88	(0.48, 1.66)	0.692
Exposure to animal	251 (59.5%)	49 (64.5%)	0.81	(0.48, 1.34)	0.410	0.80	(0.46, 1.34)	0.396
Attending school	283 (67.1%)	55 (72.4%)	0.78	(0.44, 1.32)	0.356	0.93	(0.50, 1.70)	0.814
Household crowding	292 (69.2%)	56 (73.7%)	0.80	(0.45, 1.37)	0.427	0.76	(0.42, 1.33)	0.345
Hospital admission in the prior 6 months	16 (3.8%)	3 (3.9%)	0.96	(0.31, 4.20)	0.948	0.84	(0.25, 3.86)	0.801
Current sickness	120 (28.4%)	16 (21.1%)	1.49	(0.84, 2.77)	0.174	1.58	(0.83, 3.16)	0.164

*Median (IQR), OR: odds ratio of presence of suspected antimicrobial resistant *E. coli*; CI: confidence interval

5.2.4 The prevalence of antimicrobial resistant genes in rectal swabs from healthy children

The 498 paired MCA plates were scraped and aliquoted; DNA was extracted from the mixed *E. coli* populations, which means all types of detectable *E. coli*, and all were subjected to PCR amplification to identify a panel of genes associated with resistance to fluoroquinolones and cephalosporins. As shown in Figure 5.3, the prevalence of any resistant genes, including PMQR and β -lactamase genes, was 82.7% (412/498), among which only resistant to ceftriaxone was 78.3% (39/498) and only resistant to ciprofloxacin was 43.1% (20/498). The overall prevalence of PMQR genes (*aac* (6')-Ib-cr, *qepA*, *qnrA*, *qnrB*, and *qnrS*) in the samples was 86.7% (432/498); the corresponding prevalence of type β -lactamase genes was 90.6% (451/498). Notably, individual *qnr*-determinants were the most commonly detected (83.9%; 418/498) resistance genes, especially *qnrB* (67.3%; 335/498) and *qnrS* (61.4%; 306/498) (Figure 5.4A). The prevalence of ESBL genes (*bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, and *bla*_{OXA}) and AmpC genes (MOX, DHA, EBC, FOX, ACC, CIT) were 83.7% (417/498) and 67.5% (336/498), respectively. Alarming, the CTX-M class of ESBL gene was the most commonly detected (75.1%; 374/498), followed by *bla*_{TEM} (36.9%; 184/498) (Figure 5.4B).

Generally, *qnrB*, *qnrS*, *bla*_{CTX-M9}, and DHA were sequentially the most frequently detected genes, and typically identified in the same sample (Figure 5.5A). More than half (52.2%; 260/498) of samples had a combination of at least one *qnr*, one AmpC, and one *bla*_{CTX-M} (Figure 5.5B).

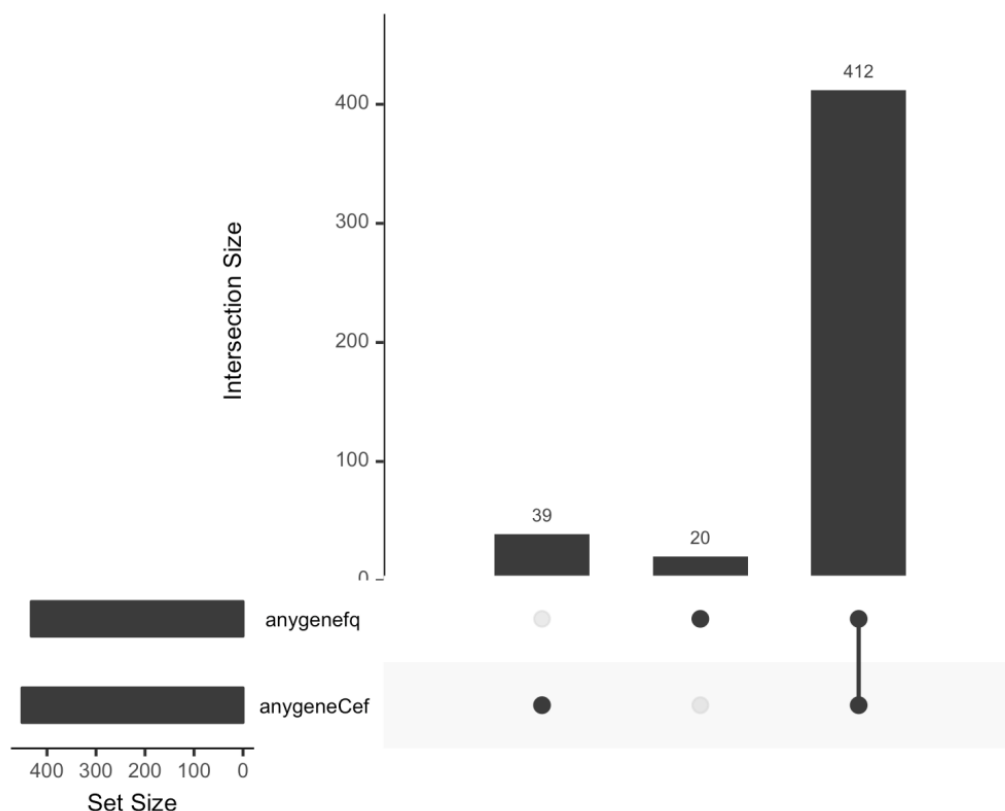


Figure 5.3 Frequency of resistant genes to fluoroquinolones (ciprofloxacin) and third generation cephalosporins (ceftriaxone). Horizontal bar chart at bottom-left represents frequencies of sample with any gene resistant to ciprofloxacin (anygenefq) and any gene resistant to ceftriaxone (anygeneCef). Top-right bar chart represents frequencies of sample having any gene resistant to ceftriaxone only, sample having any gene resistant to ciprofloxacin only, and sample having both resistant genes.

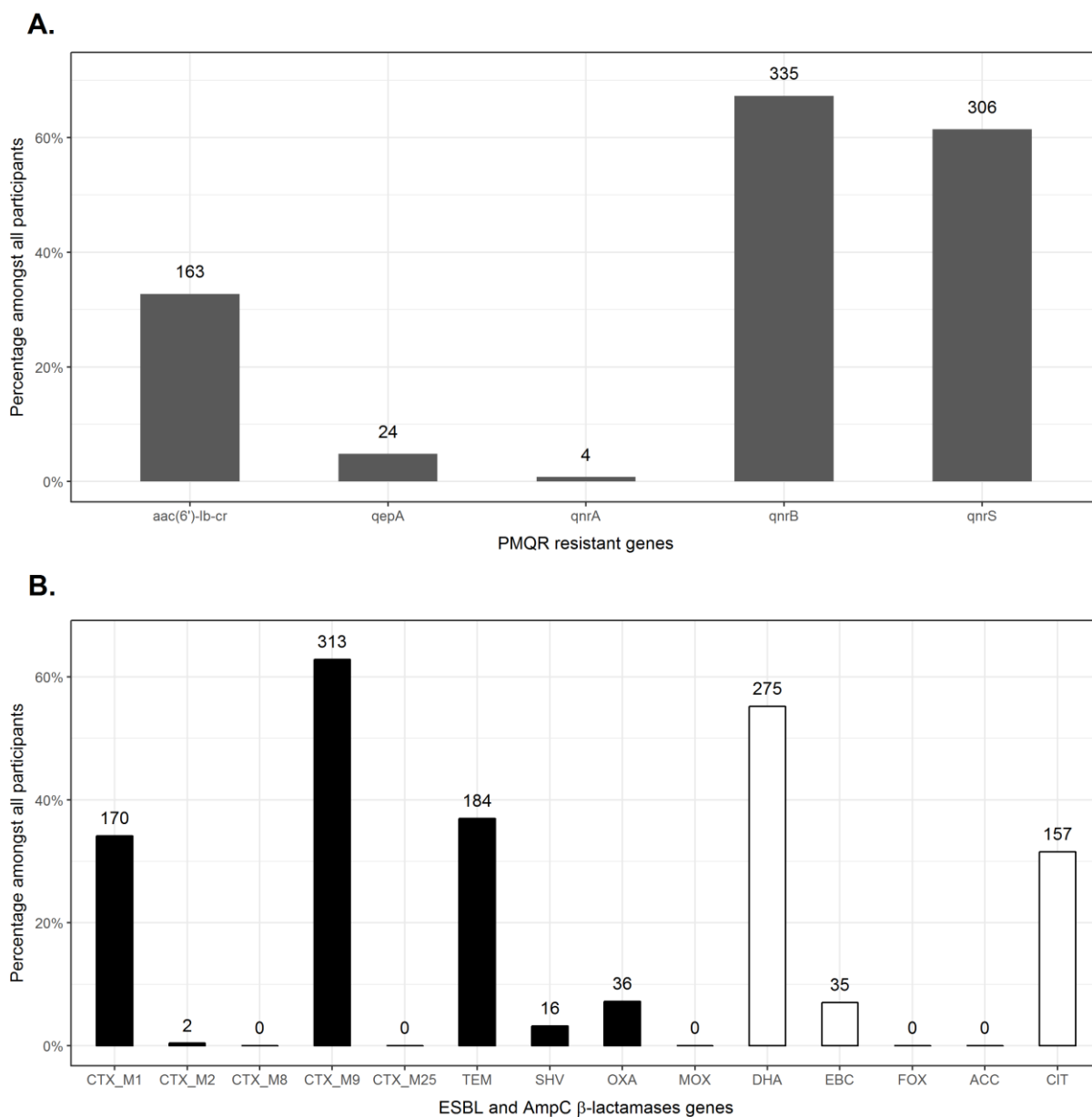


Figure 5.4 The prevalence of selected antimicrobial resistance genes in commensal *E. coli* in a cohort of 498 Vietnamese children.

Bar charts showing the prevalence of PMQR genes (A) and β -lactamase genes (B) in commensal *E. coli* isolated from faecal samples of Vietnamese children.

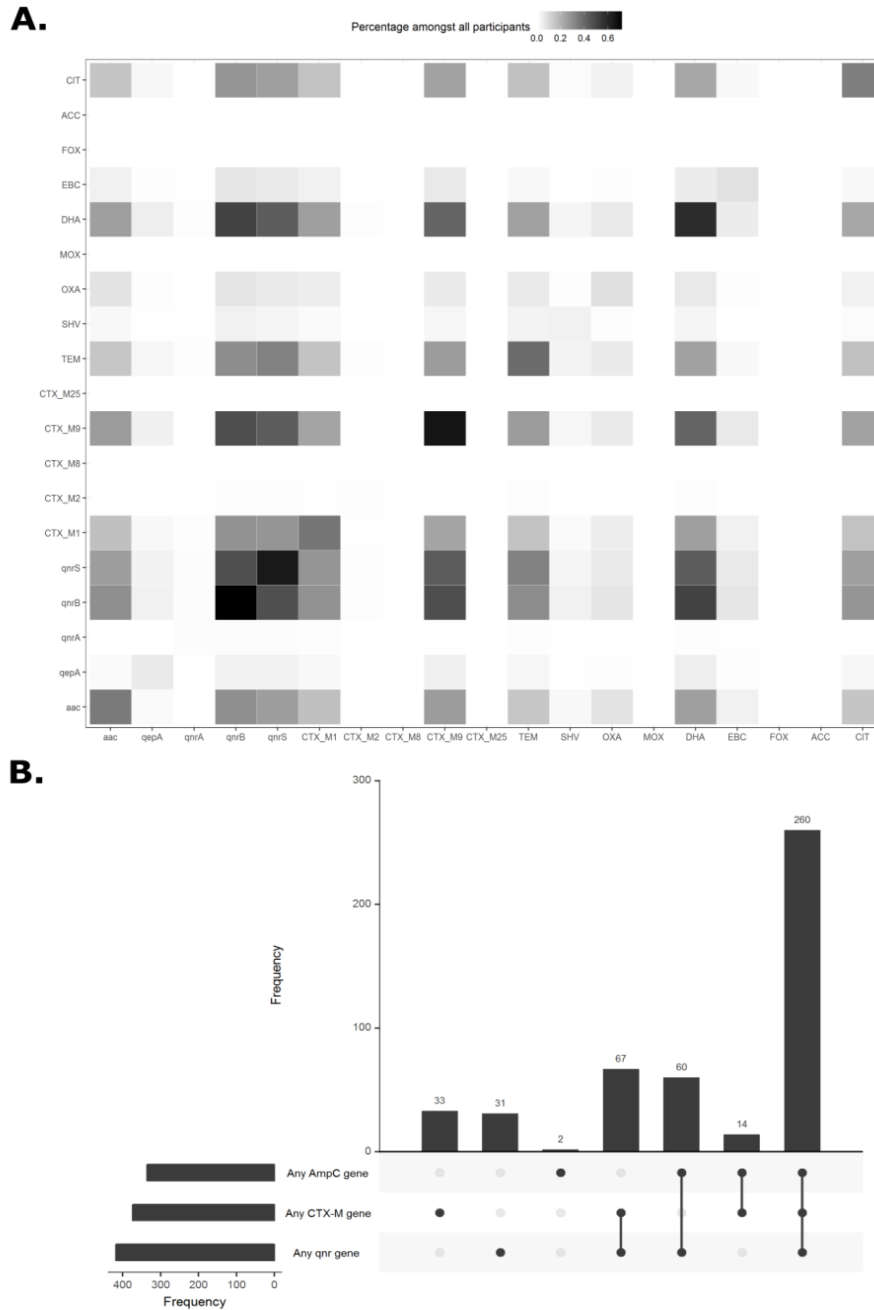


Figure 5.5 The co-selection of specific antimicrobial resistant genes in commensal *E. coli* in a cohort of 498 Vietnamese children.

(A) Heat-map showing prevalence of co-selection screened antimicrobial resistant genes; the greater the intensity the more common the combination (see key). (B) Two-dimensional bar plots showing the prevalence of the co-existence of various combinations *qnr*, AmpC, and CTX-M genes.

Not all antimicrobial resistant samples were associated with a corresponding antimicrobial resistance gene marker. From the ciprofloxacin resistant *E. coli*, 37/337 (11%) samples did not generate an amplicon associated with a PMQR genes; potentially explained by chromosomal DNA gyrase mutations. Correspondingly, from the ceftriaxone resistant *E. coli*, 20/400 (5%) did not produce an ESBL or AmpC amplicon. Conversely, from the 161 samples with no ciprofloxacin resistant *E. coli*, 73% (118/161) generated an amplicon for at least one *qnr* gene. From the 98 rectal swabs containing no *E. coli* exhibiting phenotypic ceftriaxone resistance, a large proportion (72%; 71/98) generated at least one ESBL or an AmpC amplicon.

5.2.5 Risk factors for PMQR and β -lactamase genes carriage

I then explored potential epidemiological associations between the participants' characteristics/behaviour and the most common PMQR genes (*qnr*-family: 83.9%; 418/498) (Table 5.3) and β -lactamase genes (*bla*_{CTX-M} (75.1%; 374/498) (Table 5. 4). The detection of *qnr* genes in commensal *E. coli* were significantly more common in children with higher weight-for-height z-scores (OR: 1.24, 95%CI: 1.05–1.48, $p=0.01$). However, no significant association was found between the age of the children when sampled and the presence of any *qnr* gene ($p>0.05$). Conversely, a relationship between weight-for-height z-score and the presence of *bla*_{CTX-M} genes were not identified ($p>0.05$). However, *bla*_{CTX-M} genes were more commonly detected in the commensal *E. coli* in children of a younger age (OR: 0.97, 95%CI: 0.94–0.99, $p=0.01$). Additionally, potential epidemiological associations between participants' features and the presence of the most common genes in combination (*bla*_{CTX-M} and *qnr*), were investigated. There was a consistent relationship,

whereby the co-existence of these genes was significantly higher among younger children (OR: 0.95, 95%CI: 0.9–1.00, $p=0.03$) (Table 5.5).

Table 5.3 Associations between the presence of *qnr* genes and characteristics of the study participants.

Characteristic	Presence of any <i>qnr</i> gene		Univariable analysis			Multivariable analysis		
	Yes (N=418)	No (N=80)	OR	(95% CI)	P value	OR	(95% CI)	P value
Low maternal education	249 (59.6%)	53 (66.2%)	0.75	(0.45, 1.23)	0.259	0.77	(0.45, 1.29)	0.320
In-paid employment mother	286 (68.4%)	56 (70.0%)	0.93	(0.54, 1.55)	0.780	0.74	(0.42, 1.29)	0.292
Male	229 (54.8%)	40 (50.0%)	1.21	(0.75, 1.96)	0.432	1.10	(0.66, 1.83)	0.700
Age * (month)	46.4 (35.6, 52.4)	47.2 (35.8, 53.1)	0.99	(0.96, 1.02)	0.512	0.99	(0.96, 1.02)	0.419
Preterm at birth	54 (12.9%)	10 (12.5%)	1.04	(0.52, 2.25)	0.918	0.99	(0.49, 2.20)	0.988
Caesarean section	184 (44.0%)	27 (33.8%)	1.54	(0.94, 2.58)	0.086	1.48	(0.88, 2.53)	0.138
Breastfeeding duration * (month)	4.0 (3.0, 6.0)	4.0 (2.8, 6.0)	1.04	(0.99, 1.09)	0.162	1.04	(0.99, 1.10)	0.117
Weight-for-height z score *	1.0 (-0.1, 2.3)	0.4 (-0.4, 1.5)	1.22	(1.04, 1.44)	0.012	1.24	(1.05, 1.48)	0.011
Antimicrobials use in the prior 6 months	180 (43.1%)	33 (41.2%)	1.08	(0.67, 1.76)	0.764	0.92	(0.54, 1.57)	0.753
Probiotic use in the prior 6 months	116 (27.8%)	18 (22.5%)	1.32	(0.76, 2.39)	0.324	1.30	(0.72, 2.41)	0.388
Pork or beef consumption in the prior 3 days	412 (98.6%)	79 (98.8%)	0.87	(0.05, 5.18)	0.896	0.90	(0.05, 6.13)	0.929
Poultry or egg consumption in the prior 3 days	385 (92.1%)	70 (87.5%)	1.67	(0.75, 3.42)	0.200	1.72	(0.75, 3.68)	0.193
Ice using in the prior 6 months	65 (15.6%)	17 (21.2%)	0.68	(0.38, 1.27)	0.221	0.69	(0.37, 1.32)	0.250
Piped water to residence	278 (66.5%)	53 (66.2%)	1.01	(0.60, 1.66)	0.964	0.92	(0.53, 1.58)	0.769
Exposure to flooding	89 (21.3%)	18 (22.5%)	0.93	(0.53, 1.69)	0.810	0.91	(0.50, 1.72)	0.773
Exposure to animal	251 (60.0%)	49 (61.3%)	0.95	(0.58, 1.55)	0.840	1.03	(0.61, 1.72)	0.917
Attending school	286 (68.4%)	52 (65.0%)	1.17	(0.70, 1.92)	0.551	1.30	(0.72, 2.30)	0.383
Household crowding	293 (70.1%)	55 (68.8%)	1.07	(0.63, 1.77)	0.811	1.03	(0.59, 1.75)	0.922
Hospital admission in the prior 6 months	18 (4.3%)	1 (1.2%)	3.55	(0.72, 64.42)	0.139	3.47	(0.66, 64.31)	0.165
Current sickness	117 (28.0%)	19 (23.8%)	1.25	(0.73, 2.23)	0.430	0.99	(0.54, 1.85)	0.970

*Median (IQR); OR: odds ratio of presence of any *qnr* gene; CI: confidence interval

Table 5.4 Associations between the presence of CTX-M genes and characteristics of the study participants.

Characteristic	Presence of any CTX-M resistant genes		Univariable analysis			Multivariable analysis		
	Any CTX-M genes(N=374)	None CTX_M genes (N=124)	OR	(95% CI)	P value	OR	(95% CI)	P value
Low maternal education	224 (59.9%)	78 (62.9%)	0.88	(0.58, 1.33)	0.551	0.92	(0.59, 1.42)	0.694
In-paid employment mother	259 (69.3%)	83 (66.9%)	1.11	(0.72, 1.71)	0.631	1.12	(0.70, 1.77)	0.643
Male	204 (54.5%)	65 (52.4%)	1.09	(0.72, 1.64)	0.681	1.07	(0.70, 1.64)	0.749
Age * (month)	41.8 (35.6, 52.3)	47.3 (36.0, 53.3)	0.97	(0.94, 0.99)	0.006	0.97	(0.94, 0.99)	0.014
Preterm at birth	45 (12.0%)	19 (15.3%)	0.76	(0.43, 1.38)	0.351	0.70	(0.39, 1.30)	0.253
Caesarean section	158 (42.2%)	53 (42.7%)	0.98	(0.65, 1.48)	0.923	0.98	(0.64, 1.51)	0.937
Breastfeeding duration * (month)	4.0 (2.0, 6.0)	4.0 (3.0, 6.0)	1.01	(0.98, 1.06)	0.499	1.01	(0.97, 1.05)	0.704
Weight for height z-score	0.8 (-0.2, 2.1)	1.0 (-0.2, 2.2)	0.96	(0.85, 1.08)	0.480	0.95	(0.82, 1.09)	0.463
Antimicrobials use in the prior 6 months	167 (44.7%)	46 (37.1%)	1.37	(0.90, 2.09)	0.139	1.26	(0.81, 1.98)	0.314
Probiotic use in the prior 6 months	102 (27.3%)	32 (25.8%)	1.08	(0.68, 1.73)	0.749	0.94	(0.58, 1.54)	0.789
Pork or beef consumption in the prior 3 days	369 (98.7%)	122 (98.4%)	1.21	(0.17, 5.69)	0.824	1.17	(0.16, 5.73)	0.854
Poultry or egg consumption in the prior 3 days	342 (91.4%)	113 (91.1%)	1.04	(0.49, 2.07)	0.914	1.04	(0.48, 2.13)	0.914
Ice using in the prior 6 months	63 (16.8%)	19 (15.3%)	1.12	(0.65, 2.00)	0.690	1.18	(0.67, 2.15)	0.581
Piped water to residence	247 (66.0%)	84 (67.7%)	0.93	(0.60, 1.42)	0.728	0.85	(0.53, 1.33)	0.473
Exposure to flooding	76 (20.3%)	31 (25.0%)	0.77	(0.48, 1.25)	0.277	0.70	(0.42, 1.18)	0.181
Exposure to animal	225 (60.2%)	75 (60.5%)	0.99	(0.65, 1.49)	0.949	1.03	(0.67, 1.60)	0.883
Attending school	251 (67.1%)	87 (70.2%)	0.87	(0.55, 1.34)	0.527	0.97	(0.59, 1.59)	0.899
Household crowding	260 (69.5%)	88 (71.0%)	0.93	(0.59, 1.45)	0.760	0.93	(0.58, 1.47)	0.757
Hospital admission in the prior 6 months	16 (4.3%)	3 (2.4%)	1.80	(0.59, 7.84)	0.326	1.48	(0.46, 6.67)	0.535
Current sickness	105 (28.1%)	31 (25.0%)	1.17	(0.74, 1.88)	0.503	1.28	(0.76, 2.22)	0.354

*Median (IQR); OR: odds ratio of presence of any CTX-M gene; CI: confidence interval

Table 5.5 Association between the co-existence of CTX-M and *qnr* genes and characteristics of the study participants.

Characteristic	Presence of both CTX-M and <i>qnr</i> family genes		Univariable analysis			Multivariable analysis		
	Yes (N=327)	No** (N=33)	OR	(95%CI)	p-value	OR	(95%CI)	p-value
Low maternal education	195 (59.6%)	24 (72.7%)	0.55	(0.24, 1.19)	0.133	0.57	(0.23, 1.28)	0.177
In-paid employment mother	222 (67.9%)	19 (57.6%)	1.56	(0.74, 3.21)	0.238	1.45	(0.65, 3.18)	0.360
Male sex	179 (54.7%)	15 (45.5%)	1.45	(0.71, 3.02)	0.309	1.36	(0.63, 2.95)	0.431
Age* (month)	41.9 (35.6, 52.4)	47.5 (36.0, 53.6)	0.95	(0.91, 0.99)	0.016	0.95	(0.91, 1.00)	0.033
Preterm at birth	42 (12.8%)	7 (21.2%)	0.55	(0.23, 1.44)	0.208	0.57	(0.23, 1.56)	0.258
Caesarean section	143 (43.7%)	12 (36.4%)	1.36	(0.66, 2.94)	0.412	1.24	(0.57, 2.83)	0.591
Breastfeeding duration* (month)	4.0 (2.0, 6.0)	4.0 (3.0, 9.0)	1.02	(0.96, 1.09)	0.647	1.01	(0.94, 1.09)	0.804
Weight-for-height z score*	0.9 (-0.2, 2.2)	0.5 (-0.5, 1.6)	1.11	(0.89, 1.40)	0.359	1.05	(0.82, 1.37)	0.683
Antimicrobials use in the prior 6 months	148 (45.3%)	14 (42.4%)	1.12	(0.55, 2.35)	0.755	0.94	(0.43, 2.07)	0.866
Probiotic use in the prior 6 months	91 (27.8%)	7 (21.2%)	1.43	(0.63, 3.68)	0.405	1.21	(0.50, 3.22)	0.686
Ice using in the prior 6 months	53 (16.2%)	7 (21.2%)	0.72	(0.31, 1.87)	0.475	0.84	(0.34, 2.35)	0.732
Piped water to residence	216 (66.1%)	22 (66.7%)	0.97	(0.44, 2.04)	0.944	0.82	(0.35, 1.81)	0.625
Exposure to flooding	67 (20.5%)	9 (27.3%)	0.69	(0.31, 1.62)	0.376	0.67	(0.28, 1.69)	0.382
Exposure to animal	197 (60.2%)	21 (63.6%)	0.87	(0.40, 1.79)	0.703	1.03	(0.46, 2.27)	0.938
Attending school	223 (68.2%)	24 (72.7%)	0.80	(0.34, 1.73)	0.589	0.91	(0.35, 2.17)	0.829
Household crowding	225 (68.8%)	20 (60.6%)	1.43	(0.67, 2.97)	0.343	1.39	(0.63, 3.01)	0.408
Current sickness	92 (28.1%)	6 (18.2%)	1.76	(0.75, 4.84)	0.204	1.51	(0.58, 4.57)	0.414

*Median (IQR); ** No: no CTX-M and *qnr* genes was identified; OR: odds ratio of presence of both CTX-M and *qnr* family genes; CI: confidence interval

5.3 Discussion

Commensal *E. coli* are important vehicles for the transmission of antimicrobial resistance determinants^{143,182,235}. Here, both the phenotypic and genotypic characteristics of AMR in a cohort of urban children in Viet Nam were investigated using direct plating and conventional PCR, a method which is sensitive, specific, inexpensive, and suitable for screening in LMICs^{245,246}. This study outlines several new insights into this potential reservoir of AMR genes and their association with demographic characteristics. An exceptionally high prevalence of fluoroquinolones and third generation cephalosporin resistant *E. coli* and their associated resistance genes in young children in this location were found. The most commonly identified PMQR genes were within the *qnr* family (including *qnrB* and *qnrS*) and the most commonly detected class of ESBL gene was *bla*_{CTX-M}. Additionally, the carriage of *qnr* genes was associated with higher weight for height z-scores and that younger children were more likely to carry *bla*_{CTX-M} ESBL genes in combination with *qnr* PMQR genes.

Previous comparable studies in Viet Nam demonstrated a low prevalence of quinolone resistant commensal *E. coli*^{245,249}. These two studies, conducted in Northern Viet Nam, suggested that even though the overall prevalence of AMR *E. coli* was low, the isolation of ciprofloxacin resistant organisms was higher in the urban area than in the rural area. Here a trend towards an increasing prevalence of ciprofloxacin resistant commensal *E. coli* was shown. This trend may reflect a generic higher degree of resistance to this group of antimicrobials over time in combination with dramatic increases in antimicrobial usage within Viet Nam, principally in urban areas. Results from direct plating showed a higher prevalence of ciprofloxacin resistant *E. coli* in the commensal microbiota of children in

Viet Nam compared to those described in Bolivia and Peru (18% in 2002 and 33% in 2005)^{250,251}. Additionally, in comparison to a recent study conducted in HCMC among healthy adults²⁵², these results from this study also show a higher prevalence of ciprofloxacin resistant *E. coli* (15.5%, 16/103 *E. coli* isolates vs. 67.7% 337/498 rectal swab samples). However, these differences may be derived from the method of resistance detection; the previous study exploited routine bacterial culture and antimicrobial susceptibility testing, or the population sampled.

Additionally, a substantially higher prevalence of PMQR and ESBL genes were detected than observed in previous investigations in human populations. My data illustrate a much higher prevalence of PMQR genes among the healthy paediatric population (86.7%; 432/498), than that have been described in other countries. For example, in Mexico in 2013, the prevalence of PMQR genes among Enterobacteriaceae isolates from children was 32%²⁵³. The global meta-analysis estimated that ESBL positivity rate for Enterobacteriaceae among healthy community reported in 2016 was 14%²⁵⁴. The ESBL producing Enterobacteriaceae among paediatric community varied according to level of development; 2.9% (Sweden)²⁵⁵, 11.6% (Tanzania)²⁵⁶, 23% (Lao)²⁵⁷, 24.8% (Lebanon)²⁵⁸ and 59% (Central African Republic). Potential explanations for this discrepancy may include the use of differing methods for detecting PMQR and ESBL determinants (i.e. PCR methods) and a temporal difference of five years, which may reflect a natural increase in the circulation of organisms and people carrying such AMR genes. Results from my study indicate that a high proportion of children in urban Viet Nam carry multiple AMR genes, implying high exposure to a range of antimicrobials in this local setting¹⁴², and is comparable to other reports in Southeast Asia^{257,259–261}.

Notably, the prevalence of carrying the ESBL *bla*_{CTX-M} gene was 75.1% (374/498), of which the majority (62.9%; 313/498) were *bla*_{CTX-M-9}; the next most common was *bla*_{TEM} (36.9%; 184/498). This distribution is comparable to patterns observed in a small sample of healthy adults residing in HCMC, where the *bla*_{CTX-M} class of genes was the most dominant among ESBL producing *E. coli* isolates (100%; 10/10), followed by *bla*_{TEM} (50%; 5/10)²⁵². A relatively consistent prevalence of *qnr* genes were found as previously reported in this setting¹⁸². Further, *qnrB* and *qnrS* were the most prevalent PMQR genes (67%; 335/498 and 61%; 306/498, respectively). This prevalence was higher in comparison to data from China and some settings in Latin American²⁵³. However, this disparity may again be associated with a difference in the population sampled. In a recent study, the prevalence of *qnrB* was 94% (16/17) but this result was recovered from *E. coli* isolated only, while my study revealed the prevalence of a whole population of bacteria in the faecal samples of healthy children. However, while *qnrS* was not commonly detected in other locations²⁵³, my results shows that *qnrS* is among the most common AMR genes in children within HCMC.

A significant association between children carrying *qnr* genes and a higher weight-for-height z-score were found in this study. An explanation for this association could be that overweight and obese children are more likely to be treated with antimicrobials. In this setting, children that are overweight are more commonly from families with a higher socio-economic status, which in turn may be more likely to seek healthcare, and therefore receive antimicrobials. Besides, childhood obesity is known to trigger chronic low-grade inflammation²⁶², which may lead to their vulnerability to gastrointestinal diseases and

diarrhoea²⁶³. This in turn results in a higher likelihood of antimicrobial consumption prior to hospitalization in cases of a diarrhoeal episode²⁶⁴. Furthermore, quantitative microbiological examination has reported a significant expansion of *E. coli*, coupled with the contraction of *Bifidobacterium*, in obese children in China when compared to age-matched controls²⁶⁵. As I screened for PMQR genes, which chiefly circulate within the Enterobacteriaceae, I speculate a link between shifts in the gut microbiome during obesity and the selection of genotypic resistance. Fluoroquinolones are amongst the most commonly used antimicrobials worldwide, and their use is particularly common in Viet Nam^{211,231}. The association of the carriage of *bla*_{CTX-M} genes and younger children may be induced by reducing antimicrobial usage with age. It is difficult to assess the nature of the selective pressure for specific AMR genes during therapeutic use, but we predict that selection would reduce as children mature.

This study has limitations. First, the point prevalence of phenotypic and genotypic resistance could identify non-causal associations with the variable assessed in the children. Second, I investigated associations between the carriage of various AMR genes and a wide range of heterogeneous epidemiological variables among a healthy population, which may explain the limited number of significant epidemiological associations. Furthermore, by screening for the presence of PMQR and β -lactamase genes directly from rectal swabs using conventional PCRs I was unable to assess the direct association between resistant phenotypes and associated genotypes. Notwithstanding these limitations, these results from this first of its type study in Viet Nam will provide an important step in understanding the role of the microbiome in maintaining AMR genes in the community.

5.4 Conclusion

I conclude that there is a high prevalence of commensal *E. coli* in children aged under five years in an urban setting within Viet Nam exhibiting resistance against fluoroquinolones and third generation cephalosporins. In addition, I have determined that the prevalence of PMQR and β -lactamase encoding genes is extremely high among healthy children. I highlight high weight-to-height z-scores as a major risk for carrying *qnr* genes and that younger children were more likely to carry CTX-M genes in combination with *qnr*. I advocate that future longitudinal studies in Asia assess the maturing of AMR component of the human microbiome. These data are imperative for understating the ecology of AMR gene carriage and transmission within humans. Results from this work emphasize the role of the commensal bacteria, especially among children, as being keys for the transmission and maintenance of AMR organisms/genes.

6. High rates of Caesarean section and mother/child separation prevents optimal breastfeeding practices in a population of urban and semi-rural Vietnamese mothers

Initiating early breastfeeding, which is a good proxy for continued breastfeeding¹⁵⁷ and is beneficial for infant health, is recommended by most international health organizations. However, there is a paucity of investigating risk factors associated with suboptimal breastfeeding practices in urbanized area of low-middle income countries (LMICs). Using data from the large prospective birth cohort (Method section 2.1.1 Birth cohort) among 6,706 infants in HCMC and Dong Thap, Viet Nam between 2009 and 2013, in this chapter I investigate the practice of breastfeeding in an urban (HCMC) and semi-rural (Dong Thap) population including the exclusive breastfeeding at birth during the hospital stay and aim to identify factors associated with the suboptimal breastfeeding practice. Additionally, I explore potential associations between breastfeeding practices and diarrhoeal disease, including time to the first event of diarrhoeal disease and the number of diarrhoea episodes in the first year of life among children less than twelve months of age. Findings from these analyses would propose suggestions for breastfeeding promotion strategies in prioritizing common risk factors in hospital and others location specific factors associated with socioeconomics.

6.1 Background

There is a large body of evidence demonstrating that exclusive breastfeeding has a positive effect on reducing infant mortality and morbidity from diarrhoea^{76,144–146}; breast milk is nutritionally beneficial and provides some gastrointestinal immunity against enteric

pathogens^{76,147}. It has been estimated that >823,000 deaths among children under five years of age could be prevented annually by improving breastfeeding practices¹⁶³. Despite the general benefits of breastfeeding, breastfeeding rates, particularly exclusive breastfeeding (without supplemental milk products), which is recommended by the WHO in the first six months of life, are low in many LMICs²⁶⁶. The global rate of exclusive breastfeeding of children aged <6 months was estimated to be approximately 43% in 2015²⁶⁷. Viet Nam and other countries in East Asia and the Pacific area share similar trends of slowly improving breastfeeding rates. The most updated estimate in Viet Nam in 2014 suggested that around 24% of Vietnamese mothers practiced exclusive breastfeeding in the first 6 months²⁶⁷.

Previous studies have shown that good breastfeeding practices in the first few months of life are associated with early correct infant feeding after birth in the first few days before hospital discharge¹⁵⁷. However, there is a paucity of data on following up early optimal breastfeeding practices, which means exclusive breastfeeding in the first few days in the hospital. Recent studies in Viet Nam have measured the initiation of breastfeeding within one hour after birth, these were: 47.5% in an urban southern area in 2000²⁶⁸, 73.6% in a rural northern area in 2002²⁶⁹, and 90.6% in an urban central area in 2017²⁷⁰. Notably, there were locations where children born in less affluent households are subjected to early breastfeeding more commonly than more affluent children²⁶⁷. In contrast, a recent study from northern Viet Nam showed that initiating breastfeeding within one hour after delivery was slightly higher in urban areas than in rural areas²⁷¹. However, there are little accurate current data regarding breastfeeding practices in LMICs undergoing economic development and urbanization, such as Viet Nam²⁶⁷. In the effort of promoting health for

infants and young children, there is a need to investigate factors associated with breastfeeding practices in different settings in such locations.

Here, using data from the birth cohort established in 2009, which is described in the method section (2.1.1 Birth cohort), I aim to measure the rates of breastfeeding practices, including early breastfeeding before hospital discharge and any breastfeeding activity, and to explore the patterns of infant feeding during the first year of life. In addition, I aim to identify risk factors associated with inadequate breastfeeding practices at birth in these urban and semi-rural areas. Understanding these factors may assist in providing supporting information for those promoting health strategies for young children. A final aim is to investigate whether breastfeeding practice at birth had any potential association with time until the first diarrhoeal episode and numbers of diarrhoeal episodes in the first year of life.

6.2 Results

6.2.1 Baseline characteristics

From 2009 to 2013 there were 6,706 infants enrolled in the birth cohort. Figure 2.2 shows the study location of HCMC (urban area) (4,248) and Dong Thap province (semi-rural area) (2,458). 7,274 mothers were invited to participate this cohort study; 6,743 (93%) completed the baseline enrolment interview. Ultimately, 6,706 infants from 6,679 mothers were enrolled in the birth cohort. Except for one mother, who was interviewed nine days after delivery, the breastfeeding practices of all mothers/infants were assessed within the first seven days after birth and 6,413/6,706 (96%) were assessed within 2 days of delivery. The follow-up rate was high, with 5,307/6,706 (79%) infants attending all follow-up visits; 1,202/6,706 (18%) infants missed several follow-up visits and 197/6,706 (3%) of infants

did not attend any follow-up visits. Common causes of attrition were living too far from the study location and a reluctance of having blood draws. In comparison to those attending all follow-up visits, infants who missed follow-up visits were more likely to live in the semi-rural area, be from a family with a low household income, not be the first child in the household, have parents with lower level education, have mothers without complications during pregnancy, and be borne by vaginal delivery (Table 6.1). The majority of infants (5,778/6,706, 86%) attended the 12-month follow-up visit.

Table 6.1 Epidemiological characteristics of participants who completed all, missed several or missed all follow-up visits.

Characteristics	Completed all FU visits (n=5,307)		Missed several FU visits (n=1,202)		Missed all FU visits (n=197)		p- value
	n	Frequency (%)	n	Frequency (%)	n	Frequency (%)	
Study site: Semi-rural	5,307	1,723 (32)	1,202	594 (49)	197	141 (72)	<0.001
Household wealth	5,305		1,201		196		<0.001
1 st quintile		932 (18)		311 (26)		95 (48)	
2 nd quintile		1,018 (19)		342 (28)		58 (30)	
3 rd quintile		1,420 (27)		268 (22)		25 (13)	
4 th quintile		801 (15)		114 (9)		6 (3)	
5 th quintile		1,134 (21)		166 (14)		12 (6)	
Mother							
Primiparous	5,305	3,102 (58)	1,201	668 (56)	196	93 (47)	0.003
Age ^a (years)	5,304	27 (23, 31)	1,201	26 (22, 30)	197	25 (21, 30)	<0.001 ^b
Ethnic	5,305		1,200		196		0.078 ^c
Kinh		5,048 (95)		1,159 (97)		190 (97)	
Chinese		212 (4)		30 (2)		4 (2)	
Other		45 (1)		11 (1)		2 (1)	
High education	5,305	1,885 (36)	1,201	343 (29)	196	25 (13)	<0.001
Currently married	5,305	5,236 (99)	1,201	1,186 (99)	196	189 (96)	0.045 ^c
In-paid employment mother	5,305	3,558 (67)	1,201	745 (62)	196	122 (62)	0.002
Living with others	5,302	5,178 (98)	1,201	1,164 (97)	196	187 (95)	0.056 ^c
Complication during pregnancy	5,307	1,055 (20)	1,202	181 (15)	197	23 (12)	<0.001
HIV and/or Hepatitis B infection	5,307	262 (5)	1,202	43 (4)	197	8 (4)	0.120
Father							
Ethnic	5,299		1,200		196		0.203 ^c
Kinh		4,922 (93)		1,120 (93)		190 (97)	
Chinese		329 (6)		67 (6)		5 (3)	
Other		48 (1)		13 (1)		1 (1)	
High education	5,300	2,172 (41)	1,200	428 (36)	196	29 (15)	<0.001
In-paid employment father	5,300	5,273 (99)	1,200	1,192 (99)	196	194 (99)	0.334 ^c
Infant							
Premature at birth	5,307	150 (3)	1,202	43 (4)	197	6 (3)	0.382
Male	5,307	2,764 (52)	1,202	636 (53)	197	103 (52)	0.873
Caesarean section	5,307	1,603 (30)	1,202	249 (21)	197	25 (13)	<0.001
Low birth weight	5,307	232 (4)	1,202	65 (5)	197	12 (6)	0.182
Neonatal complication at birth	5,307	210 (4)	1,202	53 (4)	197	12 (6)	0.279

^a described in median (interquartiles); all p-values based on Chi-squared test, except for: ^b based on Kruskal-Wallis test, ^c based on Fisher's exact test

High education: completed lower secondary school (>9 years of education), Premature at birth: gestational age at birth <37 weeks, Low birth weight: birth weight <2500g, FU = follow-up

Sixty three percent (4,248/6,706) resided in the urban area (HCMC) and 37% (2,458/6,706) resided in the semi-rural area (Dong Thap) (Table 6.2). Mothers in the urban area were generally of a higher social economic status (92%; 3,880/4,247 in the three highest quintiles) in comparison to mothers in the semi-rural area (98%; 2,399/2,455 in the two lowest quintiles). The majority of mothers were bearing a child for the first time (58%; 3,863/6,702) and had a median age of 27 years. Almost all mothers were of Kinh ethnicity, with only 5% belonging to other minorities; most (4%; 246/6,701) were Chinese in the urban area. The level of parents' education was higher in the urban area (40% of mothers and 46% of fathers had >9 years of education in the urban area compared to only 23% and 28%, respectively, in semi-rural area). In addition, Caesarean sections were more common in the urban area than the rural area (40% vs. 7%, respectively). A comparable trend (urban vs. rural) was observed for maternal complications (22% vs. 13%), maternal infections (7% vs. 1%), births delivered before week 37th (4% vs. 2%), and infant complications at birth (5% vs. 2%).

Table 6.2 The epidemiological characteristics of the study participants.

Characteristic	Total (n = 6,706)		Urban (n = 4,248)		Semi-rural (n = 2,458)	
	n	Frequency (%)	n	Frequency (%)	n	Frequency (%)
Household income	6,702		4,247		2,455	
1 st quintile		1,338 (20)		47 (1)		1,291 (53)
2 nd quintile		1,418 (21)		310 (7)		1,108 (45)
3 rd quintile		1,713 (26)		1,657 (39)		56 (2)
4 th quintile		921 (14)		921 (22)		0 (0)
5 th quintile		1,312 (20)		1,312 (31)		0 (0)
Mother						
Primiparous	6,702	3863 (58)	4,247	2,371 (56)	2,455	1,492 (61)
Age ^a (years)	6,702	27 (23, 31)	4,245	28 (24, 32)	2,457	25 (21, 29)
Ethnic	6,701		4,247		2,454	
Kinh		6,397 (95)		3,948 (93)		2,449 (99)
Chinese		246 (4)		246 (6)		0 (0)
Other		58 (1)		53 (1)		5 (<1)
Education: >9 years	6,702	2,253 (34)	4,247	1,692 (40)	2,455	561 (23)
Currently married	6,702	6,611 (99)	4,247	4,171 (98)	2,455	2,440 (99)
In-paid employment	6,702	4,425 (66)	4,247	2,737 (64)	2,455	1,688 (69)
mother						
Living with others	6,699	6,529 (97)	4,247	4,095 (96)	2,452	2,434 (99)
Complication during pregnancy	6,706	1,259 (19)	4,248	950 (22)	2,458	309 (13)
HIV and/or Hepatitis B infection	6,706	313 (5)	4,248	290 (7)	2,458	23 (1)
Father						
Ethnic	6,695		4,242		2,453	
Kinh		6,232 (93)		3,784 (89)		2,448 (99)
Chinese		401 (6)		401 (9)		0 (0)
Other		62 (1)		57 (1)		5 (<1)
High level of education	6,696	2,629 (39)	4,242	1,931 (46)	2,454	698 (28)
In-paid employment father	6,696	6,659 (99)	4,242	4,210 (99)	2,454	2,449 (100)
Infant						
Premature at birth	6,706	199 (3)	4,248	149 (4)	2,458	50 (2)
Male	6,706	3,503 (52)	4,248	2,248 (53)	2,458	1,255 (51)
Caesarean section	6,706	1,877 (28)	4,248	1,715 (40)	2,458	162 (7)
Low birth weight	6,706	309 (5)	4,248	199 (5)	2,458	110 (4)
Neonatal complication at birth	6,706	275 (4)	4,248	218 (5)	2,458	57 (2)

^a described in median (interquartiles)

High education: completed lower secondary school (>9 years of education), Premature at birth: gestational age at birth <37 weeks, Low birth weight: birth weight <2500g

6.2.2 Breastfeeding practices

The majority of mothers (91%; 6,106/6,706) fed their infants with breast milk on at least one occasion during the time they were in the hospital for delivery; however, only a third (2,187/6,706) exclusively breastfed at birth (Table 6.3). Consistently, only 15% (959/6,210) of infants were exclusively breastfed for up to four months and <1% (56/6,093) were exclusively breastfed up to six months (Table 6.3). Amongst the infants who were exclusively breastfed at birth and were followed up to 4 and 6 months, the frequency of exclusive breastfeeding for 4 and 6 months was 49% (959/1,949) and 3% (56/1,902), respectively. Figures 6.1 and 6.2 provide more information on breastfeeding rates over months during the first year of life of the children.

In both areas, the frequency of mothers reporting any breastfeeding and exclusive breastfeeding at each of the follow-up visits decreased considerably at months 4 and 6 with the introduction of solid food (Figure 6.1 and Figure 6.2). In addition, most mothers reported breastfeeding their infant on at least one occasion within the first year (96%, 6,222/6,509 in total; 94%, 3,942/4,192 in urban area; 98%, 2,280/2,317 in semi-rural area).

The prevalence of exclusive breastfeeding at birth was significantly lower in the urban population (9%; 364/4,248) than the semi-rural population (74%; 1,823/2,458) ($p < 0.0001$, Fisher's exact test). Similarly, all other indicators of breastfeeding practices (any breastfeeding at birth, exclusive breastfeeding for 4 and 6 months) were significantly lower in the urban area than the semi-rural area (Table 6.2). Furthermore, at the final follow-up visit (12 months after delivery), 79% (1,522/1,928) of mothers in the semi-rural area reported some breastfeeding within the first year, while 68% (2,620/3850) of mothers in the

urban area reported no breastfeeding within the first year (Figure 6.1 and Figure 6.2). This association corresponded with the use of infant formula, which was more commonly used in urban area, with a frequency of 58%–93%; the comparable frequency in the semi-rural area was 26%–48% (Figure 6.1).

Table 6.3 Breastfeeding practices in the urban and semi-rural areas in Viet Nam.

Breastfeeding practice	Total (n = 6,706)		Urban (n = 4,248)		Semi-rural (n = 2,458)		p value
	n	Frequency (%)	n	Frequency (%)	n	Frequency (%)	
Any breastfeeding at birth	6,706	6,106 (91)	4,248	3,663 (86)	2,458	2,443 (99)	<0.001
Exclusive breastfeeding at birth	6,706	2,187 (33)	4,248	364 (9)	2,458	1,823 (74)	<0.001
Exclusive breastfeeding for 4 months	6,210 ^a	959 (15)	4,048 ^a	70 (2)	2,162 ^a	889 (41)	<0.001
Exclusive breastfeeding for 6 months	6,093 ^b	56 (<1)	3,983 ^b	5 (<1)	2,110 ^b	51 (2)	<0.001

^a Number of infants were followed up to 4 months.

^b Number of infants were followed up to 6 months.

All *p*-values based on Fisher's exact test.

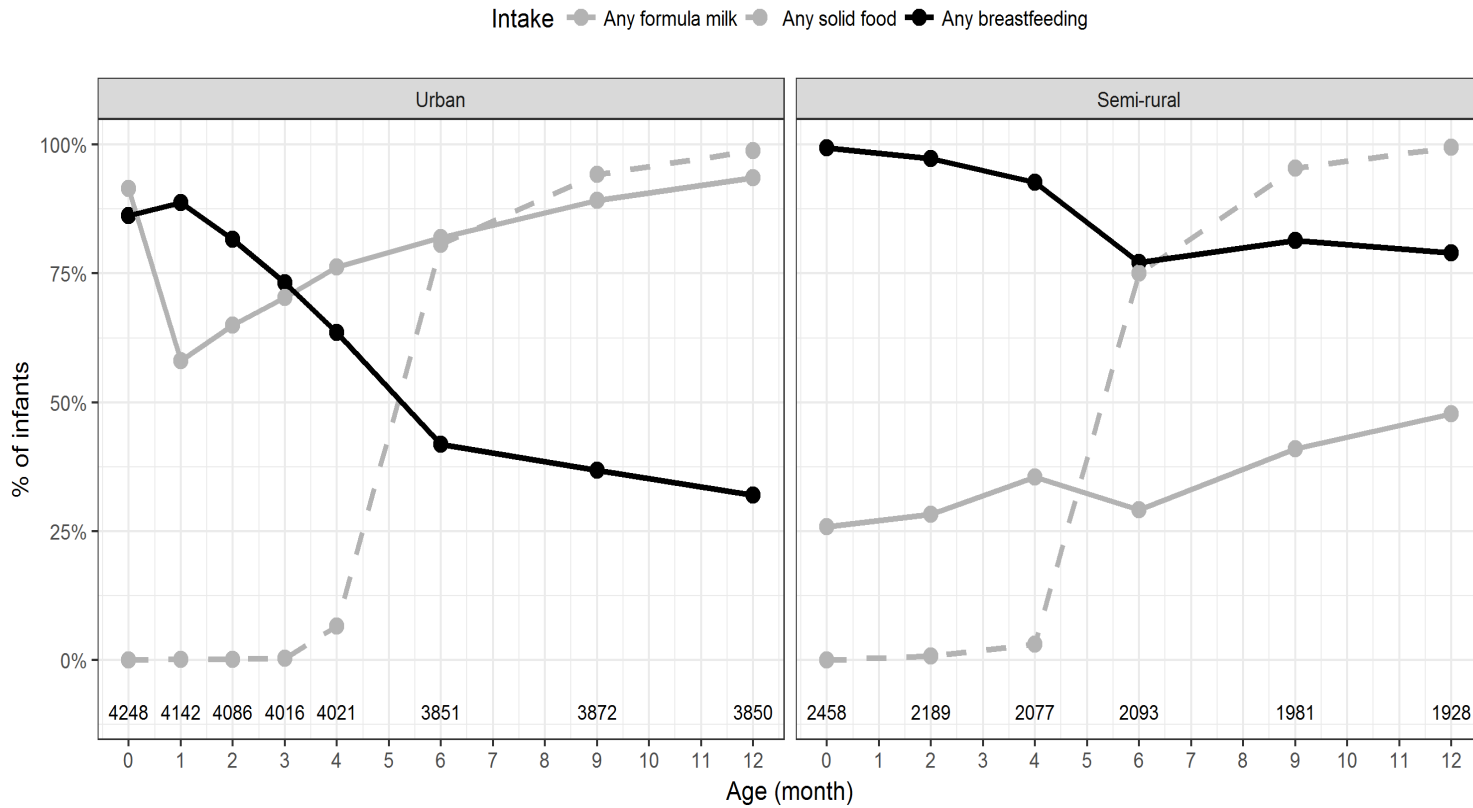


Figure 6.1 Cohort members’ dietary intake at routine follow-up visits during in first year of life
Plots showing the dietary intake of children in the cohort during the first year of life in the urban (left panel) and semi-rural (right panel). The dots and lines indicate the proportion for each type of intake that mothers fed their infants at each routine follow-up visit; formula milk, any solid food, and any breastfeeding (see key). Numbers at base of the x axis are numbers of responses received at each visit.

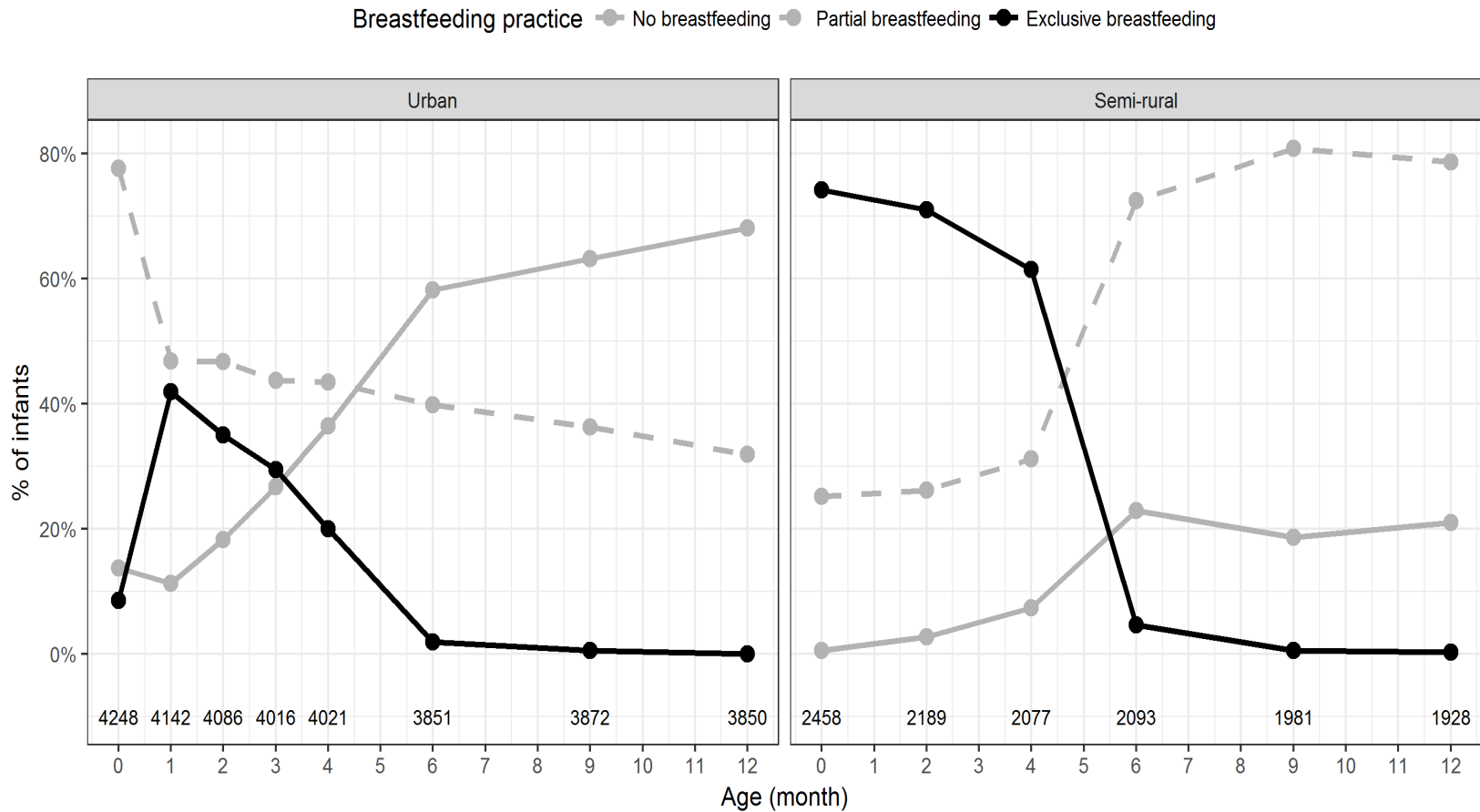


Figure 6.2 Cohort member's breastfeeding practices at routine follow-up visits during the first year of life

Plots showing the breastfeeding practices in the cohort during the first year of life in the urban (left panel) and semi-rural (right panel). The dots and lines indicate the proportion for each type of breastfeeding activity in the previous month prior to each routine follow-up visit; no breastfeeding, partial breastfeeding, and exclusive breastfeeding (see key). Numbers at base of the x axis are numbers of responses received at each visit.

6.2.3 Risk factors of suboptimal breastfeeding in urban and rural areas in Viet Nam

To explore factors associated with exclusive breastfeeding at birth in the urban and semi-rural areas, I stratified the cohort members by location (i.e. urban; n=4,248 and semi-rural; n=2,458) and conducted discrete analyses in these populations. Table 6.4 and Table 6.5 show the results of a univariable and multivariate analysis regarding the explanatory factors associated with the behaviour of exclusive breastfeeding at birth (before hospital discharge) in the urban and semi-rural areas, respectively. In the urban population mothers of higher social economic status were found to be more likely to not initiate exclusive breastfeeding at birth in comparison to those in the lowest socioeconomic quintile (ORs and 95%CI for the fourth and the fifth quintiles compared to the lowest quintile: 0.38, 95%CI:0.18–0.88 and 0.29, 95%CI:0.14–0.68, respectively, $p<0.0001$). Additionally, not initiating exclusive breastfeeding at birth was associated with mothers who were currently married (OR:0.23, 95%CI:0.11–0.50, $p<0.001$), reporting complications during pregnancy (OR: 0.53, 95%CI:0.38–0.74, $p<0.001$), mothers with infection during pregnancy (OR:0.53, 95%CI:0.30–0.87, $p=0.011$), mothers delivering before week 37th of gestation (OR:0.37, 95%CI:0.15–0.81, $p=0.011$), mothers delivering by Caesarean section (OR: 0.07, 95%CI:0.04–0.11, $p=0.001$), and those with neonatal complications at birth (OR=0.2, 95%CI:0.07–0.47, $p<0.001$). Conversely, older mothers were found to be more likely to early breastfeed their infants in hospital (OR of exclusive breastfeeding at birth for each year of age increase: 1.04, 95%CI:1.01–1.06, $p=0.003$). Additionally, mothers giving birth to infants with a birth weight <2,500g were more likely to initiate early breastfeeding (OR: 2.02, 95%CI:1.18–3.33, $p<0.001$). In the semi-rural area, two factors were identified to have a negative effect on early breastfeeding in hospital. In a comparable manner to the

urban population, these included mothers delivering by Caesarean section (OR: 0.05, 95%CI:0.03–0.08, $p<0.001$) and the infant having neonatal complications at birth (OR:0.25, 95%CI: 0.14–0.46, $p<0.001$) (Table 6.5).

Table 6.4 Factors associated with exclusive breastfeeding at birth in the urban population.

Characteristics	n	Yes (n=364) Frequency (%)	n	No (n=3,884) Frequency (%)	OR	Unadjusted (95% CI)	p	OR	Adjusted (95% CI)	p
Household wealth (Ref: 1 st quintile)	364		3,883				<0.001			<0.001
2 nd quintile		41 (11)		269 (7)	0.50	(0.24, 1.10)		0.71	(0.32, 1.65)	
3 rd quintile		194 (53)		1,463 (38)	0.43	(0.22, 0.91)		0.68	(0.33, 1.50)	
4 th quintile		58 (16)		863 (22)	0.22	(0.11, 0.47)		0.38	(0.18, 0.88)	
5 th quintile		60 (16)		1,252 (32)	0.16	(0.08, 0.34)		0.29	(0.14, 0.68)	
Mother										
Primiparous	364	176 (48)	3,883	2,195 (57)	0.72	(0.58, 0.89)	0.003	0.86	(0.66, 1.12)	0.258
Age ^a (years)	364	28 (24, 32)	3,881	28 (24, 32)	1.00	(0.98, 1.02)	0.875	1.04	(1.01, 1.06)	0.003
Ethnic (Ref: Kinh)	364		3,883				0.036			0.109
Chinese		11 (3)		235 (6)	0.48	(0.25, 0.85)		0.53	(0.26, 0.98)	
Other		5 (1)		48 (1)	1.08	(0.37, 2.48)		0.74	(0.24, 1.88)	
High education	364	114 (31)	3,883	1,578 (41)	0.67	(0.53, 0.84)	<0.001	1.09	(0.81, 1.46)	0.574
Currently married	364	344 (95)	3,883	3,827 (99)	0.25	(0.15, 0.43)	<0.001	0.23	(0.11, 0.50)	<0.001
In-paid employment mother	364	208 (57)	3,883	2,529 (65)	0.71	(0.57, 0.89)	0.003	0.81	(0.64, 1.04)	0.096
Living with others	364	344 (95)	3,883	3,751 (97)	0.61	(0.38, 1.01)	0.054	1.35	(0.70, 2.84)	0.386
Complication during pregnancy	364	49 (13)	3,884	901 (23)	0.52	(0.37, 0.70)	<0.001	0.53	(0.38, 0.74)	<0.001
HIV and/or Hepatitis B infection	364	16 (4)	3,884	274 (7)	0.61	(0.35, 0.98)	0.042	0.53	(0.30, 0.87)	0.011
Father										
Ethnic (Ref: Kinh)	363		3,879				0.006			0.095
Chinese		27 (7)		374 (10)	0.77	(0.50, 1.14)		0.94	(0.60, 1.44)	
Other		12 (3)		45 (1)	2.85	(1.43, 5.26)		2.36	(1.08, 4.84)	
High education	363	145 (40)	3,879	1,786 (46)	0.78	(0.63, 0.97)	0.025	1.25	(0.94, 1.64)	0.120
In-paid employment father	363	359 (99)	3,879	3,851 (99)	0.65	(0.25, 2.21)	0.451	0.86	(0.29, 3.17)	0.796
Infant										
Premature at birth	364	7 (2)	3,884	142 (4)	0.52	(0.22, 1.03)	0.063	0.37	(0.15, 0.81)	0.011
Male	364	187 (51)	3,884	2,061 (53)	0.93	(0.75, 1.16)	0.537	0.93	(0.74, 1.16)	0.510
Caesarean section	364	19 (5)	3,884	1,696 (44)	0.07	(0.04, 0.11)	<0.001	0.07	(0.04, 0.11)	<0.001
Low birth weight	364	23 (6)	3,884	176 (5)	1.42	(0.89, 2.18)	0.140	2.02	(1.18, 3.33)	0.011
Neonatal complication at birth	364	5 (1)	3,884	213 (5)	0.24	(0.09, 0.53)	<0.001	0.20	(0.07, 0.47)	<0.001

^a described in median (interquartiles) OR: odds ratio; 95% CI: 95% confidence interval. OR, 95% CI and p values were estimated using univariable (unadjusted) and multivariable (adjusted) logistic regression models. High education: completed lower secondary school (>9 years of education), Premature at birth: gestational age at birth <37 weeks, Low birth weight: birthweight <2500g

Table 6.5 Factors associated with exclusive breastfeeding at birth in the semi-rural population.

Characteristics	Yes (n=1823)		No (n=635)		Unadjusted			Adjusted		
	n	Frequency (%)	n	Frequency (%)	OR	(95% CI)	p	OR	(95% CI)	p
Household wealth (Ref: 1 st quintile)	1,821		634				0.217			0.444
2 nd quintile		803 (44)		305 (48)	0.85	(0.71, 1.02)		0.94	(0.76, 1.16)	
3 rd quintile		42 (2)		14 (2)	0.97	(0.53, 1.86)		1.43	(0.71, 3.06)	
Mother										
Primiparous	1,821	1,085 (60)	634	407 (64)	0.82	(0.68, 0.99)	0.040	0.79	(0.62, 1.02)	0.071
Age ^a (years)	1,822	24 (21, 29)	635	25 (22, 29)	0.98	(0.96, 1.00)	0.024	0.99	(0.96, 1.01)	0.234
Ethnic: Other (Ref: Kinh)	1,820	4 (<1)	634	1 (<1)	1.39	(0.21, 27.30)	0.759	1.10	(0.16, 21.69)	0.929
High education	1,821	394 (22)	634	167 (26)	0.77	(0.63, 0.95)	0.016	0.91	(0.69, 1.22)	0.531
Currently married	1,821	1,811 (99)	634	629 (99)	1.44	(0.45, 4.07)	0.517	1.19	(0.05, 43.13)	0.920
In-paid employment mother	1,821	1,238 (68)	634	450 (71)	0.87	(0.71, 1.06)	0.159	0.87	(0.69, 1.08)	0.203
Living with others	1,819	1,806 (99)	633	628 (99)	1.11	(0.35, 2.95)	0.850	0.25	(0.01, 3.56)	0.359
Complication during pregnancy	1,823	212 (12)	635	97 (15)	0.73	(0.56, 0.95)	0.019	0.86	(0.65, 1.16)	0.325
HIV and/or Hepatitis B infection	1,823	14 (1)	635	9 (1)	0.54	(0.23, 1.30)	0.161	0.70	(0.28, 1.93)	0.475
Father										
Ethnic: Other (Ref: Kinh)	1,820	3 (<1)	633	2 (<1)	0.52	(0.09, 3.96)	0.488	0.57	(0.09, 4.75)	0.570
High education	1,821	495 (27)	633	203 (32)	0.79	(0.65, 0.96)	0.020	0.96	(0.74, 1.26)	0.778
In-paid employment father	1,821	1,818 (100)	633	631 (100)	1.92	(0.25, 11.62)	0.488	2.24	(0.29, 13.85)	0.402
Infant										
Premature at birth	1,823	32 (2)	635	18 (3)	0.61	(0.35, 1.12)	0.109	0.72	(0.38, 1.44)	0.346
Male	1,823	925 (51)	635	330 (52)	0.95	(0.79, 1.14)	0.594	1.03	(0.85, 1.26)	0.744
Caesarean section	1,823	25 (1)	635	137 (22)	0.05	(0.03, 0.08)	<0.001	0.05	(0.03, 0.08)	<0.001
Low birth weight	1,823	75 (4)	635	35 (6)	0.74	(0.49, 1.12)	0.151	0.77	(0.50, 1.23)	0.276
Neonatal complication at birth	1,823	21 (1)	635	36 (6)	0.19	(0.11, 0.33)	<0.001	0.25	(0.14, 0.46)	<0.001

^a described in median (interquartiles)

OR: odds ratio; 95% CI: 95% confidence interval. OR, 95% CI and p values were estimated using univariable (unadjusted) and multivariable (adjusted) logistic regression models.

High education: completed lower secondary school (>9 years of education), Premature at birth: gestational age at birth <37 weeks, Low birth weight: birthweight <2500g

6.2.4 Association between breastfeeding practices and diarrhoeal disease

Lastly, I investigated the effect of breastfeeding practices (exclusive breastfeeding during hospital stay and any breastfeeding at birth) on time to the first episode of diarrhoeal disease (Table 6.6), and the total number of reported diarrhoeal disease episodes in the first year of life (Table 6.7). The number of diarrhoeal episodes was derived from self-reporting by the participant's mother or caregiver at each routine health check (follow-up) and from passive surveillance of diarrhoeal episodes when participants attended a study hospital to seek healthcare for diarrhoea. After adjusting for maternal age, maternal education, infant sex, low birth weight, and mode of delivery, I found no statistically significant evidence of the effect of any breastfeeding or early breastfeeding at birth on the time from birth to the first episode of diarrhoea or the number of diarrhoea.

Table 6.6 Breastfeeding practices at birth and time to the first diarrhoeal episode.

Breastfeeding practice	Urban (n=4,248)					Semi-rural (n=2,458)				
	n	Event (%)	HR	(95% CI)	p	n	Event (%)	HR	(95% CI)	p
Self-reported ^a										
Any breastfeeding at birth										
No	585	130 (22)	1.00			15	3 (20)	1.00		
Yes	3,658	705 (19)	0.84	(0.69, 1.02)	0.071	2,439	508 (21)	0.93	(0.30, 2.91)	0.898
Exclusive breastfeeding at birth										
No	3,879	757 (19)	1.00			634	120 (19)	1.00		
Yes	364	78 (21)	1.16	(0.92, 1.48)	0.214	1,820	391 (21)	1.16	(0.93, 1.45)	0.185
Passive surveillance ^b										
Any breastfeeding at birth										
No	585	31 (5)	1.00			15	5 (33)	1.00		
Yes	3,659	181 (5)	0.82	(0.55, 1.22)	0.331	2,439	737 (30)	0.74	(0.31, 1.80)	0.508
Exclusive breastfeeding at birth										
No	3,880	193 (5)	1.00			634	178 (28)	1.00		
Yes	364	19 (5)	0.97	(0.60, 1.58)	0.916	1,820	564 (31)	1.10	(0.92, 1.32)	0.300

^aDiarrhoeal episodes recorded by self-reporting from participants at follow up health check; ^bDiarrhoeal episodes recorded from passive surveillance when participants visited study sites;

Event: having any diarrhoea episode within the first year of life; HR: hazard ratio; 95% CI: 95% confidence interval. HR, 95% CI and p values were estimated using Cox proportional hazards models with adjustment for maternal age, maternal education, gender, low birth weight and delivery method.

Table 6.7 Breastfeeding practices at birth and numbers of diarrhoeal episodes.

Breastfeeding practice	Urban (n = 4,248)					Semi-rural (n = 2,458)				
	n	CI	IRR	(95% CI)	p value	n	CI	IRR	(95% CI)	p value
Self-reported ^a										
Any breastfeeding at birth										
No	585	278.5	1.00			15	251.3	1.00		
Yes	3,658	234.6	0.83	(0.69, 1.00)	0.053	2,439	320.3	1.18	(0.38, 5.16)	0.792
Exclusive breastfeeding at birth										
No	3,879	238.3	1.00			634	291.6	1.00		
Yes	364	266.6	1.13	(0.90, 1.42)	0.289	1,820	329.8	1.16	(0.93, 1.44)	0.184
Passive surveillance ^b										
Any breastfeeding at birth										
No	585	61.9	1.00			15	502.5	1.00		
Yes	3,659	60.8	0.89	(0.60, 1.37)	0.591	2,439	563.0	1.08	(0.42, 3.10)	0.884
Exclusive breastfeeding at birth										
No	3,880	61.1	1.00			634	548.1	1.00		
Yes	364	59.9	0.90	(0.52, 1.48)	0.692	1,820	567.7	1.05	(0.88, 1.25)	0.615

^aDiarrhoeal episodes recorded by self-reporting from participants at follow up health check; ^bDiarrhoeal episodes recorded from passive surveillance when participants visited study sites;

CI: cumulative incidence of diarrhoea per 1,000 infant years; IRR: incidence rate ratio; 95% CI: 95% confidence interval. IRR, 95%CI and p values were estimated using negative binomial regression models with adjustment for maternal age, maternal education, gender, low birth weight and delivery method.

6.3 Discussion

This was a large longitudinal prospective study conducted in urban and semi-rural areas in a transitional economic LMIC. I used data from this cohort to assess breastfeeding practices during the first year of life. My results show a low prevalence of exclusive breastfeeding in hospitals immediately after birth, identifying differences in breastfeeding practices between regions with a different socioeconomic structure^{271–273}. Similar to previous cross-sectional breastfeeding studies conducted in Viet Nam, this study confirmed that Vietnamese mothers generally consider breast milk as an important component of infant nutrition, with 94% and 98% of them breastfeeding their infant on at least one occasion in the first year after birth in urban area and semi-rural area, respectively²⁷⁴. However, the prevalence of optimal breastfeeding practices in our study was low. The rate of optimal breastfeeding practices was especially low in the urban area, with only 9% and <1% practicing exclusive breastfeeding at birth and when the child was six months of age, respectively. This prevalence of exclusive breastfeeding was similar to that described in a cohort study conducted in Hong Kong in 2010²⁷⁵, but was only half the prevalence measured in Taiwan and the USA²⁷⁶. These findings suggest specific breastfeeding promotions need to be tailored to the local population.

The key finding from this study was that the factors associated with exclusively breastfeeding at birth were different between urban and semi-rural areas. A greater number of factors were associated with exclusive breastfeeding at birth in the urban population than the semi-rural population. In both areas, delivering by Caesarean sections and having neonatal complications were strongly associated with not introducing exclusive breastfeeding at birth. An explanation for these trends is the fact that current practice in obstetric hospitals in Viet Nam is to separate mothers from their

children after Caesarean sections and when the child has an infection, despite the benefits of early skin to skin contact¹⁵⁰.

I found a significantly lower rate of early breastfeeding in hospital in urban area of southern Viet Nam (9%, 364/4,248) in comparison to the semi-rural area (74%, 1,823/2,458). This figure was concordant with data from previous cross-sectional study conducted in HVH in HCMC in 2014²⁷⁷, and is comparable with data arising from China concerning exclusive breastfeeding on hospital discharge²⁷⁸. However, in contrast to my result, a previous study from northern Viet Nam found that mothers residing in urban areas were more likely to initiate breastfeeding than women in rural areas²⁷¹. This discrepancy may be associated with a difference in methods for measuring “early exclusive breastfeeding”. In this study mothers were interviewed at discharge (three to seven days after birth), whereas Huong *et al.* measured the initiation of breastfeeding within one hour, or one day after birth²⁷¹. Mothers may correctly initiate breastfeeding within one hour after birth but introduce infant formula later, as suggested by our results that the most common feeding behaviour of mothers at birth in the urban area was to combine breast milk and infant formula. In the urban area, women of a higher socio-economic status were less likely to exclusively breastfeed their infants. This difference may be linked to the capability of being able afford an alternative to breast milk²⁶⁹.

Participants in this study exhibited a similar pattern of breastfeeding behaviour to a population in northern Viet Nam in which urban mothers in Hanoi with a higher socio-economic status, undergoing Caesarean section, were less likely to practice breast feeding²⁷¹. Here, the rate of delivery by Caesarean section was high (28%, 1,877/6,706), which was amplified in the urban setting (40; 1,715/4,248). This number is considerably higher than the range of 5–15% recommended by the WHO²⁷⁹. However, this elevated

figure is consistent with other available data regarding the most common birthing practices in this location^{270,271,277,278}, and in the majority of industrialized countries globally²⁸⁰. Potential explanations for not initiating breastfeeding in these cases are separation of mothers and infants, a large work load for hospital staff, other priorities of healthcare professionals, and a lack of lactation consultation in large obstetric hospitals such as HVH, which has 40,000 deliveries annually²⁸¹. Furthermore, a stressful delivery affects the initiation of breastfeeding among mothers²⁸², and it is perceived that antimicrobials and other drugs for the post-partum infectious have a negative impact on the benefits of breast milk²⁶⁹.

Although breastfeeding is known to provide some protection against gastrointestinal infections, a previously published analysis using the same data did not find any association between breastfeeding practice at birth and the incidence of diarrhoea¹⁸⁴. Here, in this detailed analysis, there was no association between exclusive breastfeeding or any breastfeeding during the first few days in hospital and the first diarrhoeal episode reported, or the number of the disease episodes in the first year of life. A potential reason for this lack of association is that we may have missed a large proportion of disease episodes during the first year of life as diarrhoeal events were not recorded through active surveillance in the cohort. A further explanation is that the association between breastfeeding and diarrhoea may be underestimated as infant diarrhoea is likely to be associated with food or fluid given in recent days²⁸³. In addition, I did not stratify for potential confounders, which may include socioeconomic statuses such as maternal and paternal education, household wealth, source of drinking water, or child's age and gender. This finding should be interpreted with caution and further research with a primary focus on the impact of breastfeeding practices on health outcomes in translational economic LMICs like Viet Nam are required.

This study contains limitations. First, as this cohort was restricted to areas with high burden of infectious diseases, this cohort may not be a population-representative sample and therefore the generalisability of our prevalence of breastfeeding practices is limited¹⁷⁰. Second, the estimated prevalence of exclusive breastfeeding for 4 and 6 months in the semi-rural area may be less precise than in the urban area due to differences in the number of participants recruited and in the schedules of follow-up visits. Third, the study areas were geographically large and access to healthcare facilities is broad, therefore participants may seek medical assistance from a variety of sources including pharmacies, private clinics, private hospitals, or other hospitals. Therefore, different healthcare practices may underestimate the true burden of diarrhoeal disease. Fourth, breastfeeding practices and the occurrence of diarrhoeal episodes were assessed at birth and follow-up visits with at least one-month gap in between via interview only; therefore, these data are subject to recall bias. Lastly, investigating breastfeeding was not the primary aim of this cohort; therefore, the study criteria of breastfeeding were more simplistic than those suggested by the WHO. For example, we did not collect data on the time of early breastfeeding within one hour after birth. Nevertheless, this cohort, which used the same approach for accessing breastfeeding on the unique population over time²⁸⁴, offsets the point-of-time data derived from population survey in depicting the exact measurement of breastfeeding practice in a population. My results show that exclusive breastfeeding drops dramatically after two months after birth, identifying a specific time for the initiation of breastfeeding promotion strategies.

6.4 Conclusion

This is one of the largest studies of its type to investigate factors associated with exclusive breastfeeding in hospital at birth in Viet Nam. I conclude that the practice of breastfeeding in Viet Nam is a national health issue, with a low prevalence of exclusive breastfeeding (33% at birth and <1% up to six months), especially in an urban area. Here, I have highlighted common risk factors associated with not initiating exclusive breastfeeding at birth in urban and semi-rural areas, which included Caesarean section and neonatal complications. These factors are strongly associated with the suboptimal practice in hospital; hence, future studies are recommended for further investigating implementation strategies in order to improve breastfeeding among these groups, which have not, as of yet, been conducted in Viet Nam. Of note, early initiation of breastfeeding after birth by Caesarean section was incorporated into formal Vietnamese health policy, as part of the implementation of the Baby-friendly Hospital Initiative (BFHI), in 2016²⁸⁵. Even though BFHI was launched in Viet Nam in 1994 and has been integrating into national hospital criteria since 2013, there has been no evaluation of this implementation on breastfeeding rates in Viet Nam²⁸⁵.

7. General discussion

Paediatric diarrhoea is still a problem within the healthcare system in Viet Nam; antimicrobials are commonly used for treating these infections, and consequently AMR in the bacteria carried by Vietnamese children is highly prevalent. The work presented in this thesis has identified the frequent presence of AMR in commensal *E. coli*, and additionally highlighted resistance genes associated with cephalosporins and fluoroquinolones among healthy children. For the first time, and on a large scale, I additionally measured the incidence of diarrhoeal disease in the urban community, both in terms of true disease burden and age and pathogens. My work contributes to the understanding of the bigger picture of AMR in urban communities and how diarrhoeal disease management among children contributes to this problem. The AMR data from *E. coli* and the pharmacy access data may be further used as a resource for improved antimicrobial stewardship. In addition, data regarding breastfeeding suggest further implementation research to improve breastfeeding practice in hospitals and propose further clinical studies to investigate breastfeeding as a cost-effective intervention for antimicrobial resistant infections.

In chapter 3 I estimated the true burden of diarrhoeal disease in the community in HCMC. The use of a novel diagnostic approach (Luminex xTAG GPP) allowed for a comprehensive understanding of the incidence of a range of pathogens and their clinical significance in mild and moderate diarrhoeal diseases. Norovirus and *Salmonella* were the leading pathogens detected in stool samples from mild and moderate diarrhoea in the community; these data are indicative of an epidemiological transition in Viet Nam. Further studies should be conducted to explore the association between specific infectious agents and diarrhoea and whether the quantity of specific pathogens in faecal material is associated with diarrhoea.

My findings demonstrated a high rate of antimicrobial usage for diarrhoeal disease among young children in both community and hospital settings. My results found a low proportion of antimicrobial vending in community pharmacies, representing a trend of decreasing antimicrobial usage for diarrhoeal disease. However, I found that antimicrobials were prescribed more commonly in hospitals than in the community. In combination with data on the low level of knowledge regarding antimicrobials of parents and caregivers, my work affirms a high burden of inappropriate antimicrobial usage in Viet Nam, which is consistent with other areas²⁸⁶. Limitations of this approach include an underestimation of actual antimicrobial use. However, I speculate that the AMR in this setting is likely attributable to inappropriate antimicrobial usage. For the first time in Viet Nam, I provided estimations on antimicrobial usage for paediatric diarrhoea. Further research aiming to reduce antimicrobial resistance in LMICs should focus on implementation research to improve knowledge, attitude, and practice of antimicrobials and antimicrobial resistance in the community in Viet Nam.

I found that genotypic and phenotypic resistance against fluoroquinolones and third generation cephalosporins was common among healthy children in the community (Chapter 5). As exposure of susceptible bacteria to antimicrobial drugs can result in the emergence of resistant mutants²⁸⁶, my findings emphasize the emerging issue of antimicrobial resistance in this setting. I acknowledge that data from this analysis did not take into account any potential antimicrobial treatment that children may have received prior to the routine visit and the rectal swabbing. However, my findings fill a knowledge gap regarding AROs and the associated ARGs in commensal *E. coli* in urban Vietnamese children. Given that fluoroquinolones are dispensed widely in urban pharmacies, and are prescribed for paediatric diarrhoea, I speculate on the sustained

genotypic selection in overweight and obese children. Future research should examine AMR in terms of the progression of the carriage from birth and structure of the microbiome in healthy children in whom commensal bacteria have been shown to be highly resistant to common antimicrobials.

Strong evidence in the literature suggests that exclusive breastfeeding is beneficial for gastrointestinal health^{76,147}. Exclusive breastfeeding is triggered by optimal early exclusive breastfeeding in hospital. However, I found a low prevalence of early exclusive breastfeeding in hospital and found an association between this practice and socioeconomic status. These findings reflect a disparity in development between rural and urban populations^{287,288}. I identified that having a Caesarean birth and having infants with complications at birth were common risk factors for suboptimal breastfeeding in both the urban and semi-rural area. My findings are informative for further implementation research aiming to improve breastfeeding in hospital. In the scope of this analysis, I assumed that exclusive breastfeeding was a pragmatic solution to reduce diarrhoeal disease and consequently reduce the needs for using antimicrobials. However, I found no statistically significant association between breastfeeding practice at birth and the incidence of diarrhoea in the first year of life¹⁸⁴, or the time to the first diarrhoeal episode and number of diarrhoeal episodes in the first year of life.

Finally, findings from this thesis demonstrate that diarrhoeal disease is likely decreasing in Viet Nam but is still a common problem in children aged one to five years in urban HCMC. These infections are dominated by viruses and bacteria, among which there is a large diversity of pathogens. Importantly, antimicrobials are widely used in both hospital and community settings, which may be, at least in part, an explanation for a

high prevalence of antimicrobial resistance among commensal *E. coli* and resistance against fluoroquinolones and cephalosporins.

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9. Appendices

9.1 Informed consent forms (ICF) for the diarrhoea cohort

9.1.1 ICF for the potential participants coming from the birth cohort extension (English version)

Information sheet for the “Diarrhoeal Cohort Study”

For children who are enrolled in the original birth cohort extension

OXTREC – 1058-13

Information about this program

We are inviting mothers and their children who have been enrolled in our “Healthy Children” program at HVH to be involved in “**Diarrhoeal Cohort Study**”. This form will give you the information you will need to help you decide whether or not to participate. Please read the form carefully. You may ask questions about this program, the possible risks and benefits, your rights as a volunteer, and anything related or this form that is not clear. When all of your questions have been answered, you can decide if you and your child will participate. This process is called ‘informed’ consent.

The aims of this research program are to identify how often young Vietnamese children get sick with diarrhoea, what causes the illness and why children get sick. Additionally, we are interested in understanding how your child’s blood responds to diarrhoeal diseases. We hope to learn more about diarrhoeal disease in HCMC so that we can make a vaccine to prevent disease in children in the future.

This program will be part of the current program you are already enrolled in. The specific goal of this new part will be to study a bacterium called *Shigella*, which often causes severe diarrhoea in young children. To learn more about *Shigella* and other diarrhoeal diseases in young Vietnamese children, we would like to collect information on where and how children live through a series of surveys. We will do this by following the health of your child for two years. During this time we will monitor your child’s blood for evidence of infection, and will ask you about any illness that your child and your family members have had, including any admission to hospital. The information we get from

these surveys will help us to make a vaccine to prevent diarrhoeal disease due to *Shigella* in the future.

Part of this program will mean we will collect your child's blood to perform genetic tests. These tests will help us to know why some people get sick with this disease, while others do not. If you agree, the genetic code in your child's blood will be tested. The results of these tests will be made available to other researchers but you and your child will not be identified. Some samples will be sent out of the country for testing, which may be considered to be an invasion of privacy. Providing blood for these tests is optional. If you do not agree for these genetic tests to be conducted, you (or your child) may still take part in the study.

Who is doing this program ?

The partners in the study are HVH and HTD.

If I consent on behalf of my child, what will happen to my child and me in this program and what are the risks?

At enrolment (today):

When you attend the HVH-Well Baby Clinic for your routine follow up appointment as part of the Healthy Baby Cohort program in early 2014, our nurse will invite you to participate in the diarrhoeal birth cohort. If you agree for you and your child to participate in this new program our nurse will review previously collected information about where you live, your family structure, and the child's health status. For the existing "Healthy Children" program, the nurse will collect a 2ml blood sample from your child. A maximum of 2ml of blood will be collected from your child today. The nurse will also collect a stool sample, or an rectal swab if stool is unavailable, from your child. Finally, the nurse will collect a respiratory swab from your child's nose.

At each follow-up visit (every 6 months for 2 years):

In order to monitor your child's health and development, we need to see you and your child one time every six months. Your child will be aged between 18-36 months at the time of enrolment, meaning this study will finish when your child is aged between 42-60 months.

Each visit will take approximately half an hour, and will take place here at the same clinic where you are enrolled. The nurse will give you a card to remind you of when you should visit for follow-up visits. At each follow-up visit the baby will receive a health check from a hospital doctor. We will record some simple information about your child's development and also ask you some simple questions about your child's health. When your child is 18-35 months of age, we would like to collect 2ml of blood (about 20 drops) from your child. When your child is 36 to 60 months of age, we would like to collect 3 ml of blood (about 30 drops) from your child. Only one blood sample will be drawn from your child at each visit. A highly experienced nurse will collect the blood using a small needle from your child's hand. This will hurt for a moment, and might leave a small bruise. At each follow-up visit the nurse will also collect a stool sample or rectal swab and a respiratory swab from your child. These procedures are quick and painless.

When you are home with your child and you think your child is sick with diarrhoea;

We will send you a short SMS message routinely to ask if your child has experienced an episode of diarrhoea. If you respond with "yes", our nurses will call you to arrange a convenient time to visit your house to collect a stool sample, ask a set of simple questions and provide medical advice to help your child. If you feel that you need to seek hospitalization, we ask that you consider bringing your child to the HTD. If you attend the HTD when your child has diarrhoea, we request that you call our nurse so she can support you and your child when he/she is evaluated by a doctor from HTD. You can also bring your child to other clinics or hospitals that are convenient for you.

Risks and benefits to being in the program:

By being in the program, you will have access to experienced doctors in the Well Baby Clinic at HVH who will examine your child for no cost at scheduled visits that will monitor your child's health and development. The amount of blood we collect from your infant at each visit is small (20-30 drops). Your child will probably cry out when the sample is collected. You will receive money to compensate you for travel costs in bringing your child to the Well Baby clinic, HVH for follow-up visits in addition to free diagnostic services and supported travel costs for diarrhoeal examinations at HTD. If you bring your child to a private clinic or an emergency department of the hospital other than HTD when your child is sick with diarrhoea, compensation will not be given.

What we will do with the blood and other samples we collect from your child

The blood and stool samples we collect from your child will be used for research. The specimens will be stored in a freezer. We will do various tests on the blood, stool and respiratory samples we collect from your child to understand if your child has ever had *Shigella* or other diarrhoeal and respiratory diseases that are common in Viet Nam. The tests we do on the blood and respiratory samples we collect from your child will not have a direct benefit to you, but might help other children in the future. Samples we collect from your child may be used in future studies.

Confidentiality

All of the information we get from you is strictly private. Your child's name will not be on any samples we collect or test results—we will use a number instead of a name. Your name, or your child's name, will not be mentioned in any output from this program. We will ask for information on the district and ward in which you live, but we will not use this information for any purpose outside this program or give it to anyone else.

Costs

There will be no cost to you for participating in this program. The costs of examination by a paediatrician at scheduled program visits will be paid for by the program. You will be compensated for the travel costs you incur by bringing your child to the clinic for scheduled program visits.

Voluntary participation

If you do not want to be part of this program, it will not affect the care your child will receive in any way. If you do agree to become a program participant, you can withdraw from the program at any time (verbally). The care your child receives will not be affected. However, information collected on your child up until you withdraw will still be used. If you choose not to participate in this program, your child will not be monitored for diarrhoeal illness. However, you will still be able to participate in the current Healthy Baby Cohort Extension that your child is already enrolled in.

End of the program

We will stop seeing your child at HVH after two years of follow up. No information on your child health and no samples will be collected after this time.

Obtaining additional information

You are encouraged to ask any questions related to this program during the time of participation. If you have any questions about this program, its procedures, risks and benefits, or alternatives please call Dr. Nguyễn Trọng Hiếu at 0903 159 285. If you have any questions about your child's rights as a subject in this program, you may want to talk to Dr. Lu Lan Vi, or if you want to speak to someone outside of the program you may contact the Ethics Committee at the HTD at 083 855 8532.

Consent form for the “Diarrhoeal Cohort Study”

OXTREC -1058-13

Consent from: MOTHER/FATHER/GUARDIAN

- I have been fully informed of the possible risks and benefits of taking part in this diarrhoeal cohort program and agree that I and my child will take part.
- I know who to contact if I need more information. I understand that confidentiality will be preserved. I understand that I am free to withdraw from the program at any time without affecting the care I or my child will receive.
- I understand there will be limited direct benefit to me or my child.
- I agree to allow nurses to come to my home when I report a diarrhoeal disease episode in my child
- I agree that the geographical location of my house may be recorded in order to understand any relationship between location and disease.
- ☐ **I AGREE OR** ☐ **I DO NOT AGREE** that the samples taken can be stored for other research studies about infectious diseases, including genetic testing and these tests may be done outside of Viet Nam.

Participant Number: BCB- ____ - ____ ____ ____

Participant’s name: _____

Signature of person giving consent _____

Relationship to participant _____

Print name _____

Date of signature _____

Investigator’s statement

I, the undersigned, have fully explained the relevant information of this research to the participant named above and will provide her/him with a copy of this signed and dated informed consent form.

Investigator / designee signature _____

Print name _____

Date of signature _____

If the person giving consent cannot read the form, a witness must be present and sign here:

I was present throughout the entire informed consent process with the participant. All questions from the participant were answered and the participant has agreed to take part in the research.

Witness signature _____

Print name _____

Date of signature _____

Consent for genetic testing OXTREC – 1058-13
(to be signed by the participant's parent or guardian)

By signing/marketing my name here, I confirm my willingness for my child's blood to be used for genetic tests.

Participant Number: BCB- ____ - ____ ____ ____

Participant's name: _____

Signature of person giving consent _____

Relationship to participant _____

Print name _____

Date of signature _____

I, the undersigned, have fully explained the relevant information of this research to the participant named above and will provide her/him with a copy of this signed and dated ICF.

Investigator / designee signature _____

Print name _____

Date of signature _____

If the person giving consent cannot read the form her/himself, a witness must be present and sign here:

I was present throughout the entire informed consent process with the participant. All questions from the participant were answered and the participant has agreed to take part in the research.

Witness signature _____

Print name _____

Date of signature _____

9.1.2 ICF for the potential participants coming from the birth cohort extension (Vietnamese version)

Phiếu thông tin cho “Nghiên cứu Đoàn hệ Bệnh Tiêu chảy”

Cho trẻ đã tham gia vào nghiên cứu mở rộng của "Sức khỏe trẻ em"

OXTREC-1058-13

Thông tin về chương trình

Chúng tôi mời những bà mẹ và em bé đã tham gia trong chương trình "Sức Khỏe Trẻ Em" của chúng tôi tại Bệnh viện Hùng Vương cùng tham gia vào **"Nghiên Cứu Đoàn Hệ Bệnh Tiêu Chảy"**. Phiếu này sẽ cung cấp cho bạn những thông tin cần thiết để bạn quyết định có tham gia hay không. Vui lòng đọc kỹ tờ thông tin này. Bạn có thể hỏi về chương trình này, những lợi ích và nguy cơ có thể xảy ra, quyền của người tham gia nghiên cứu, và bất kỳ vấn đề nào có liên quan hay thông tin trên phiếu này mà bạn thấy chưa rõ. Khi mọi thắc mắc đã được giải đáp, bạn có thể quyết định cho bạn và con bạn có tham gia hay không. Quá trình này được gọi là sự lấy chấp thuận tham gia nghiên cứu.

Mục tiêu của chương trình này là để xác định tần suất bị bệnh tiêu chảy ở trẻ em Việt Nam, nguyên nhân gây tiêu chảy và tại sao trẻ em lại bị tiêu chảy. Ngoài ra chúng tôi cũng muốn tìm hiểu về việc máu của con bạn phản ứng với bệnh tiêu chảy như thế nào. Chúng tôi hy vọng có thể hiểu hơn về bệnh tiêu chảy ở thành phố Hồ Chí Minh để có thể tạo vắc xin giúp các trẻ em ngừa bệnh sau này.

Đây là một phần của chương trình “Sức khỏe trẻ em” mà bạn đang tham gia. Mục tiêu cụ thể của phần mới này là để tìm hiểu một chủng vi khuẩn có tên gọi *Shigella*, thường gây bệnh tiêu chảy nặng ở trẻ nhỏ. Chúng tôi cần khảo sát thu thập thông tin về nơi ở và điều kiện sống của trẻ em để hiểu hơn về bệnh do vi khuẩn *Shigella* và các nguyên nhân gây bệnh tiêu chảy khác ở trẻ em Việt Nam. Để thực hiện được điều này, chúng tôi sẽ theo dõi sức khỏe của con bạn trong 2 năm. Trong suốt thời gian đó chúng tôi sẽ theo dõi máu của bé để tìm chứng cứ của nhiễm khuẩn, và sẽ hỏi bạn về tất cả những bệnh mà bé và các thành viên gia đình bạn mắc phải, kể cả những lần phải nhập viện. Những thông tin có được từ các khảo sát này có thể giúp chúng tôi trong việc tạo ra vắc xin ngăn ngừa bệnh tiêu chảy do nhiễm khuẩn *Shigella* trong tương lai.

Chương trình này cũng sẽ có một phần thực hiện các xét nghiệm di truyền trên mẫu máu của con bạn. Xét nghiệm di truyền này giúp chúng ta hiểu vì sao một số người bị bệnh

này trong khi một số khác thì không bị. Nếu bạn đồng ý, thông tin di truyền trong máu của con bạn sẽ được xét nghiệm. Kết quả xét nghiệm này sẽ được sử dụng để tiến hành các nghiên cứu khác, tuy nhiên danh tính của bạn và con bạn sẽ được ẩn để không ai biết thông tin di truyền đó là của ai. Một số mẫu sẽ được gửi ra nước ngoài để xét nghiệm, có thể điều này được cho là xâm phạm đến sự riêng tư. Việc đồng ý cho máu để xét nghiệm di truyền là tùy ở bạn. Bạn và con bạn vẫn có thể tham gia vào chương trình này dù bạn không đồng ý để mẫu máu được dùng cho xét nghiệm di truyền.

Các đơn vị tham gia chương trình ?

Các đơn vị cộng tác tham gia nghiên cứu bao gồm Bệnh viện Hùng Vương (TPHCM) và Bệnh viện Bệnh Nhiệt Đới (TPHCM).

Nếu như tôi thay mặt con tôi chấp thuận tham gia, điều gì sẽ xảy ra trong chương trình và có những nguy cơ thế nào?

Khi tham gia (hôm nay):

Khi bạn đưa bé đến hẹn khám theo dõi cho nghiên cứu Sức Khỏe Trẻ Em ở Phòng khám nhi - Bệnh viện Hùng Vương vào đầu năm 2014, bạn và con bạn sẽ được mời tham gia vào "Nghiên cứu Đoàn hệ Bệnh Tiêu Chảy". Nếu bạn và con bạn đồng ý tham gia, một điều dưỡng sẽ cập nhật những thông tin về bạn như nơi bạn ở, cấu trúc gia đình, và tình trạng sức khỏe của bé. Điều dưỡng cũng sẽ lấy 2ml mẫu máu của bé cho chương trình "Sức khỏe trẻ em". Chỉ lấy tối đa 2ml máu từ bé trong lần khám này. Điều dưỡng cũng sẽ lấy một mẫu phân, hoặc một mẫu phết hậu môn nếu không có phân, và mẫu phết mũi họng của con bạn.

Các lần tái khám (mỗi 6 tháng một lần trong 2 năm):

Để theo dõi tình trạng sức khỏe và sự phát triển của con bạn, chúng tôi cần gặp bạn và bé vào mỗi 6 tháng một lần. Quá trình theo dõi như sau: nếu con bạn tham gia chương trình này lúc 18 tháng tuổi thì lần khám theo dõi cuối cùng là khi bé được 42 tháng tuổi; nếu tham gia lúc 24 tháng tuổi thì lần khám cuối là khi 48 tháng tuổi; nếu tham gia lúc 30 tháng tuổi thì lần khám cuối là khi 54 tháng tuổi; nếu tham gia lúc 36 tháng tuổi thì lần khám cuối là khi 60 tháng tuổi.

Mỗi lần tái khám mất khoảng nửa tiếng tại ngay phòng khám nơi bạn được nhận vào nghiên cứu. Điều dưỡng sẽ phát cho bạn một thẻ ghi lịch tái khám của chương trình. Tại mỗi lần tái khám, con của bạn sẽ được bác sĩ kiểm tra sức khỏe. Chúng tôi sẽ ghi lại

những thông tin về sự phát triển của con bạn và hỏi thêm một số câu hỏi đơn giản về sức khỏe của bé. Khi con bạn được 18 – 35 tháng tuổi, chúng tôi sẽ lấy khoảng 2ml máu (khoảng 20 giọt) của con bạn cho nghiên cứu. Khi con bạn được 36 – 60 tháng tuổi chúng tôi sẽ lấy 3ml máu (khoảng 30 giọt). Mỗi lần khám chỉ lấy 1 mẫu máu của bé. Một điều dưỡng nhiều kinh nghiệm sẽ dùng cây kim nhỏ để lấy máu trên tay của bé. Việc lấy máu sẽ gây đau một tí và có thể lại một vết bầm nhỏ. Tại mỗi lần tái khám, điều dưỡng cũng sẽ lấy một mẫu phân hoặc phết hậu môn và một mẫu phết mũi họng của bé. Việc thu mẫu này rất nhanh và không gây đau.

Khi bạn ở nhà với bé và bạn nghĩ rằng bé bị bệnh tiêu chảy;

Chúng tôi sẽ giữ liên lạc với bạn bằng tin nhắn SMS định kỳ để hỏi thăm bé có bị tiêu chảy không. Nếu bạn trả lời "Có", một điều dưỡng của chúng tôi sẽ gọi điện cho bạn để sắp xếp thời gian thuận tiện để đến thăm nhà bạn để lấy một mẫu phân, hỏi một số câu hỏi đơn giản và tư vấn y tế để giúp con bạn. Nếu bạn cảm thấy rằng con bạn cần nhập viện, chúng tôi mong bạn xem xét đưa bé đến Bệnh viện Bệnh Nhiệt Đới. Nếu bạn muốn đưa bé đến Bệnh viện Bệnh Nhiệt Đới khi bé bị tiêu chảy, hãy gọi cho điều dưỡng của chúng tôi để được hỗ trợ và bé sẽ được khám bởi một bác sĩ chuyên khoa của bệnh viện Bệnh Nhiệt đới. Chúng tôi sẽ thu thập một mẫu phân của bé khi bạn cho bé đến khám tại đây. Bạn cũng có thể đưa bé đến các phòng khám hoặc bệnh viện khác nếu điều đó thuận tiện cho bạn.

Các nguy cơ và lợi ích khi tham gia:

Khi tham gia vào chương trình, con bạn sẽ được những bác sĩ có kinh nghiệm tại phòng khám Nhi bệnh viện Hùng Vương khám và tư vấn miễn phí vào những lần tái khám định kỳ để theo dõi tình trạng sức khỏe và sự phát triển của bé. Lượng máu chúng tôi lấy từ con bạn tại mỗi lần tái khám là rất nhỏ (khoảng 20-30 giọt). Bé có thể khóc khi bị lấy máu. Bạn sẽ nhận được một khoản tiền hỗ trợ cho chi phí đi lại cho các lần tái khám tại phòng khám Nhi, Bệnh viện Hùng Vương, các lần đi khám bệnh vì tiêu chảy tại bệnh viện bệnh Nhiệt đới cũng như miễn phí xét nghiệm khi đến khám tại bệnh viện này vì tiêu chảy. Nếu bạn cho con đến khám ở một phòng khám tư hoặc đến khoa cấp cứu tại một bệnh viện khác, không phải là Bệnh viện Bệnh Nhiệt đới khi bé bị tiêu chảy, chúng tôi sẽ không hỗ trợ các chi phí này.

Chúng tôi sẽ làm gì với máu và các mẫu khác thu từ con bạn

Mẫu máu và phân mà chúng tôi thu được từ con của bạn sẽ được dùng vào nghiên cứu. Các mẫu đó sẽ được lưu trữ trong tủ đông lạnh. Chúng tôi sẽ làm một số xét nghiệm trên mẫu máu, phân và mẫu phết mũi họng của con bạn để xem con bạn có từng bị nhiễm *Shigella* hoặc một số bệnh tiêu chảy và hô hấp phổ biến khác ở Việt Nam hay không. Những xét nghiệm mà chúng tôi tiến hành trên các mẫu máu và mũi họng thu được từ con bạn có thể không mang lại lợi ích trực tiếp cho bạn, nhưng điều này có thể giúp những trẻ em khác trong tương lai. Các mẫu chúng tôi thu được từ con bạn cũng có thể được sử dụng cho các nghiên cứu về sau.

Tính bảo mật

Tất cả những thông tin chúng tôi có được từ bạn sẽ được bảo mật nghiêm ngặt. Tên của con bạn sẽ không hiện diện trên bất kỳ một mẫu xét nghiệm nào cũng như trên kết quả của chúng tôi - chúng tôi sẽ dùng mã số thay cho tên. Tên của bạn, tên của con bạn sẽ không được đề cập đến trên kết quả của chương trình. Chúng tôi sẽ hỏi thông tin về quận và phường nơi bạn ở nhưng sẽ không sử dụng thông tin này vào bất kỳ mục đích nào khác ngoài chương trình này và cũng không đưa cho ai khác.

Chi phí

Bạn không tốn bất kỳ chi phí nào khi tham gia vào chương trình này. Việc khám bệnh theo lịch sẽ được chương trình chi trả. Bạn sẽ được hỗ trợ chi phí đi lại khi mang bé đến phòng khám để tái khám theo lịch đã hẹn. Bạn cũng được miễn phí chi phí xét nghiệm chẩn đoán bệnh và được hỗ trợ chi phí đi lại khi cho bé đến khám tại bệnh viện Bệnh Nhiệt Đới vì tiêu chảy.

Sự tự nguyện tham gia chương trình

Dù bạn chọn không tham gia vào chương trình thì việc đó cũng không ảnh hưởng đến việc chăm sóc sức khỏe cho con bạn. Ngay cả khi bạn đã đồng ý tham gia chương trình, bạn cũng có thể xin rút khỏi chương trình bất kỳ lúc nào (bằng lời). Việc chăm sóc sức khỏe cho con bạn sẽ không bị ảnh hưởng. Tuy nhiên những thông tin đã được thu thập về con bạn cho đến thời điểm đó vẫn sẽ được dùng cho nghiên cứu. Nếu bạn chọn không tham gia vào nghiên cứu này, con bạn sẽ không được theo dõi bệnh tiêu chảy. Tuy nhiên, con bạn vẫn có thể tiếp tục tham gia nghiên cứu Sức khỏe Trẻ em mở rộng.

Kết thúc chương trình

Chúng tôi sẽ ngừng khám theo dõi con bạn sau 2 năm. Sau đó, chúng tôi sẽ không thu thập thêm mẫu và thông tin từ con bạn nữa.

Các thông tin thêm

Chúng tôi khuyến khích bạn hỏi thêm bất kỳ câu hỏi gì liên quan đến chương trình này trong suốt thời gian tham gia. Nếu bạn có thắc mắc về chương trình, các quy trình, nguy cơ và lợi ích, hay các câu hỏi khác, vui lòng gọi BS. Nguyễn Trọng Hiếu tại số 0903 159 285.

Nếu bạn có bất kỳ thắc mắc gì về các quyền của con bạn khi tham gia chương trình này, bạn có thể liên hệ BS. Lư Lan Vi, hoặc nếu bạn muốn nói chuyện với ai khác ngoài nhóm nghiên cứu, bạn có thể liên hệ với Hội Đồng Đạo Đức tại Bệnh Nhiệt đới ở số 083 855 8532.

Phiếu chấp thuận tham gia "Nghiên cứu Đoàn hệ Bệnh Tiêu chảy"**OXTREC – 1058-13****Chấp thuận từ: BỐ/MẸ/ NGƯỜI GIÁM HỘ**

- Tôi đã được thông tin đầy đủ về các nguy cơ và lợi ích có thể có của việc cho con tôi tham gia vào chương trình nghiên cứu đoàn hệ bệnh tiêu chảy này và đồng ý rằng tôi và con của tôi sẽ tham gia.
- Tôi biết sẽ liên lạc với ai khi cần hỏi thêm thông tin. Tôi hiểu rằng các thông tin về tôi hay con tôi sẽ được bảo mật. Tôi hiểu được tôi có quyền rút khỏi chương trình vào bất kỳ lúc nào mà không ảnh hưởng đến việc chăm sóc sức khỏe mà tôi hay con tôi sẽ được nhận.
- Tôi hiểu là việc nghiên cứu này sẽ không mang đến nhiều lợi ích trực tiếp cho tôi và con tôi
- Tôi đồng ý để điều dưỡng của chương trình đến nhà tôi khi tôi báo con tôi bị nhiễm bệnh tiêu chảy
- Tôi đồng ý việc định vị nhà ở của tôi có thể được thực hiện để tìm hiểu sự liên quan giữa nơi ở và bệnh tật.

☐ **Tôi đồng ý** hoặc ☐ **Tôi không đồng ý** cho phép mẫu của con tôi được lưu trữ cho các nghiên cứu về sau trên các bệnh truyền nhiễm, bao gồm các xét nghiệm di truyền và các xét nghiệm này có thể được thực hiện bên ngoài Việt Nam.

Mã số tham gia: 14EN- ____ - ____ ____ ____

Tên người tham gia: _____

Chữ ký của người chấp thuận _____

Quan hệ với người tham gia _____

Họ Tên (chữ in) _____

Ngày ký _____

Xác nhận của nghiên cứu viên

Tôi, người ký tên bên dưới, đã giải thích đầy đủ các thông tin liên quan đến chương trình nghiên cứu này cho người tham gia có tên bên trên và sẽ cung cấp cho cô/anh ấy một bản sao của phiếu chấp thuận đã được ký và ghi ngày tháng này.

Chữ ký của nghiên cứu viên**hoặc người được chỉ định****Họ Tên (chữ in)****Ngày ký**

Nếu người cho chấp thuận không thể tự đọc phiếu này, một nhân chứng phải có mặt và ký tên dưới đây:

Tôi đã có mặt cùng với người tham gia trong suốt quá trình lấy chấp thuận. Tất cả các câu hỏi của người tham gia đã được trả lời và người tham gia đã đồng ý tham gia vào chương trình nghiên cứu.

Chữ ký nhân chứng**Họ Tên (chữ in)****Ngày ký**

Phiếu chấp thuận cho xét nghiệm di truyền**OXTREC - 1058-13****(được ký tên bởi bố mẹ hoặc người giám hộ của trẻ tham gia nghiên cứu)**

Bằng việc ký tên/đánh dấu ở đây, tôi xác nhận đồng ý cho phép mẫu máu của con tôi được dùng vào các xét nghiệm di truyền.

Mã số tham gia: 14EN- ____ - ____ ____ ____

Tên người tham gia: _____

Chữ ký của người chấp thuận _____

Quan hệ với người tham gia _____

Họ Tên (chữ in) _____

Ngày ký _____

Tôi, người ký tên bên dưới, đã giải thích đầy đủ các thông tin liên quan đến nghiên cứu này cho người tham gia có tên bên trên và sẽ cung cấp cho cô/anh ấy một bản sao của phiếu chấp thuận đã được ký và ghi ngày tháng này.

Chữ ký của nghiên cứu viên

hoặc người được chỉ định _____

Họ Tên (chữ in) _____

Ngày ký _____

Nếu người cho chấp thuận không thể tự đọc phiếu này, một nhân chứng phải có mặt và ký tên dưới đây:

Tôi đã có mặt cùng với người tham gia trong suốt quá trình lấy chấp thuận. Tất cả các câu hỏi của người tham gia đã được trả lời và người tham gia đã đồng ý tham gia vào chương trình nghiên cứu.

Chữ ký nhân chứng _____

Họ Tên (chữ in) _____

Ngày ký _____

9.1.3 ICF for the potential participants coming from the original cohort (English version)

Information sheet for the “Diarrhoeal Cohort Study”

For children who are enrolled in the original birth cohort

OXTREC – 1058-13

Information about this program

We are inviting mothers and their children who have been enrolled in our “Healthy Children” program at HVH to be involved in “**Diarrhoeal Cohort Study**”. This form will give you the information you will need to help you decide whether or not to participate. Please read the form carefully. You may ask questions about this program, the possible risks and benefits, your rights as a volunteer, and anything related or this form that is not clear. When all of your questions have been answered, you can decide if you and your child will participate. This process is called ‘informed’ consent.

The aims of this program are to identify how often young Vietnamese children get sick with diarrhoea, what causes the illness and why children get sick. Additionally, we are interested in understanding how your child’s blood responds to diarrhoeal diseases. We hope to learn more about diarrhoeal disease in HCMC so that we can make a vaccine to prevent disease in children in the future.

This will be part of the current program you are already enrolled in. The specific goal of this new part of the program will be to study a bacterium called *Shigella*, which often causes severe diarrhoea in young children. To learn more about *Shigella* and other diarrhoeal diseases in young Vietnamese children, we would like to collect information on where and how children live through a series of surveys. We will do this by following the health of your child for two years. During this time we will monitor your child’s blood for evidence of infection, and will ask you about any illness that your child and your family members have had, including any admission to hospital. The information we get from these surveys will help us to make a vaccine to prevent diarrhoeal disease due to *Shigella* in the future.

Part of this program will mean we will collect your child’s blood to perform genetic tests. These tests will help us to know why some people get sick with this disease, while others do not. If you agree, the genetic code in your child’s blood will be tested. The

results of these tests will be made available to other researchers but you and your child will not be identified. Some samples will be sent out of the country for testing, which may be considered to be an invasion of privacy. Providing blood for these tests is optional. If you do not agree for these genetic tests to be conducted, you (or your child) may still take part in the study.

Who is doing this program?

The partners in the study are HVH and HTD.

If I consent on behalf of my child, what will happen to my child and me in this program and what are the risks?**At enrolment (today):**

When you attend the HVH-WBC for your final routine follow up appointment as part of the Healthy Baby Cohort program at 12 months of age, our nurse will invite you to participate in the diarrhoeal birth cohort. If you agree for you and your child to participate in this new program, a nurse will review previously collected information about where you live, your family structure, and the child's health status. For the existing "Healthy Children" program, the nurse will collect a 2ml blood sample (about 20 drops) from your child, instead of the 1ml sample (about 10 drops) that would otherwise have been collected. A maximum of 2ml of blood will be collected from your child today. The nurse will also collect a stool sample, or an rectal swab if stool is unavailable, from your child. Finally, the nurse will collect a respiratory swab from your child's nose.

At each follow-up visit (every 6 months for 2 years):

In order to monitor your child's health and development, we need to see you and your child one time every six months, at 18, 24, 30 and 36 months of age.

Each visit will take approximately half an hour, and will take place here at the same clinic where you are enrolled. The nurse will give you a card to remind you of when you should visit for follow-up visits. At each follow-up visit the baby will receive a health check from a hospital doctor. We will record some simple information about your child's development and also ask you some simple questions about your child's health. When your child is 18, 24, and 30 months of age, we would like to collect 2ml of blood (about 20 drops) from your child. When your child is 36 months of age, we would like to collect 3 ml of blood (about 30 drops) from your child. Only one blood sample will be drawn

from your child during this visit. A highly experienced nurse will collect the blood using a small needle from your child's hand. This will hurt for a moment, and might leave a small bruise. At each follow-up visit the nurse will also collect a stool sample or rectal swab and a respiratory swab from your child. These procedures are quick and painless.

When you are home with your child and you think your child is sick with diarrhoea;

We will send you a short SMS message routinely to ask if your child has experienced an episode of diarrhoea. If you respond with "yes", our nurses will call you to arrange a convenient time to visit your house to collect a stool sample, ask a set of simple questions and provide medical advice to help your child. If you feel that you need to seek hospitalization, we ask that you consider bringing your child to the HTD. If you attend the HTD when your child has diarrhoea, we request that you call our nurse so she can support you and your child when he/she is evaluated by a doctor from HTD. You can also bring your child to other clinics or hospitals that are convenient for you.

Risks and benefits to being in the program:

By being in the program, you will have access to experienced doctors in the Well Baby Clinic at HVH who will examine your child for no cost at scheduled visits that will monitor your child's health and development. The amount of blood we collect from your infant at each visit is small (20-30 drops). Your child will probably cry out when the sample is collected. You will receive money to compensate you for travel costs in bringing your child to the Well Baby Clinic, HVH for follow-up visits in addition to free diagnostic services and supported travel costs for diarrhoeal examinations at HTD. If you bring your child to a private clinic or an emergency department of the hospital other than HTD when your child is sick with diarrhoea, compensation will not be given.

What we will do with the blood and other samples we collect from your child

The blood and stool samples we collect from your child will be used for research. The specimens will be stored in a freezer. We will do various tests on the blood, stool and respiratory samples we collect from your child to understand if your child has ever had *Shigella* or other diarrhoeal and respiratory diseases that are common in Viet Nam. The tests we do on the blood and respiratory samples we collect from your child will not have a direct benefit to you, but might help other children in the future. Samples we collect from your child may be used in future studies.

Confidentiality

All of the information we get from you is strictly private. Your child's name will not be on any samples we collect or test results—we will use a number instead of a name. Your name, or your child's name, will not be mentioned in any output from this program. We will ask for information on the district and ward in which you live, but we will not use this information for any purpose outside this program or give it to anyone else.

Costs

There will be no cost to you for participating in this program. The costs of examination by a paediatrician at scheduled visits will be paid for by the program. You will be compensated for the travel costs you incur by bringing your child to the clinic for scheduled visits.

Voluntary participation

If you do not want to be part of this program, it will not affect the care your child will receive in any way. If you do agree to become a program participant, you can withdraw from the program at any time (verbally). The care your child receives will not be affected. However, information collected on your child up until you withdraw will still be used.

End of the program

We will stop seeing your child at HVH after four follow up visits during two years after today. . No information on your child health and no samples will be collected after this time.

Obtaining additional information

You are encouraged to ask any questions related to this program during the time of participation. If you have any questions about this program, its procedures, risks and benefits, or alternatives please call Dr. Nguyễn Trọng Hiếu at 0903 159 285.

If you have any questions about your child's rights as a subject in this program, you may want to talk to Dr. Lu Lan Vi, or if you want to speak to someone outside of the program you may contact the Ethics Committee at the HTD at 083 855 8532.

Consent form for the “Diarrhoeal Cohort Study”

OXTREC – 1058-13

Consent from: MOTHER/FATHER/GUARDIAN

- I have been fully informed of the possible risks and benefits of taking part in this diarrhoeal cohort program and agree that I and my child will take part.
- I know who to contact if I need more information. I understand that confidentiality will be preserved. I understand that I am free to withdraw from the program at any time without affecting the care I or my child will receive.
- I understand there will be limited direct benefit to me or my child.
- I agree to allow nurses to come to my home when I report a diarrhoeal disease episode in my child
- I agree that the geographical location of my house may be recorded in order to understand any relationship between location and disease.
- ☐ **I AGREE OR** ☐ **I DO NOT AGREE** that the samples taken can be stored for other research studies about infectious diseases, including genetic testing and these tests may be done outside of Viet Nam.

Participant Number: BCB- ____ - ____ ____ ____

Participant’s name: _____

Signature of person giving consent _____

Relationship to participant _____

Print name _____

Date of signature _____

Investigator’s statement

I, the undersigned, have fully explained the relevant information of this research to the participant named above and will provide her/him with a copy of this signed and dated ICF.

Investigator / designee signature _____

Print name _____

Date of signature _____

If the person giving consent cannot read the form, a witness must be present and sign here:

I was present throughout the entire informed consent process with the participant. All questions from the participant were answered and the participant has agreed to take part in the research.

Witness signature _____

Print name _____

Date of signature _____

Consent for genetic testing

OXTREC – 1058-13

(to be signed by the participant's parent or guardian)

By signing/marketing my name here, I confirm my willingness for my child's blood to be used for genetic tests.

Participant Number: BCB- ____ - ____ ____ ____

Participant's name: _____

Signature of person giving consent _____

Relationship to participant _____

Print name _____

Date of signature _____

I, the undersigned, have fully explained the relevant information of this research to the participant named above and will provide her/him with a copy of this signed and dated ICF.

Investigator / designee signature _____

Print name _____

Date of signature _____

If the person giving consent cannot read the form her/himself, a witness must be present and sign here:

I was present throughout the entire informed consent process with the participant. All questions from the participant were answered and the participant has agreed to take part in the research.

Witness signature _____

Print name _____

Date of signature _____

9.1.4 ICF for the potential participants coming from the original cohort (Vietnamese version)

Phiếu thông tin cho “Nghiên cứu Đoàn hệ Bệnh Tiêu chảy”

Cho trẻ đã tham gia vào chương trình "Sức khỏe trẻ em"

OXTREC - 1058-13

Thông tin về chương trình

Chúng tôi mời những bà mẹ và em bé đã tham gia trong chương trình "Sức Khỏe Trẻ Em" của chúng tôi tại Bệnh viện Hùng Vương cùng tham gia vào **"Nghiên Cứu Đoàn Hệ Bệnh Tiêu Chảy"**. Phiếu này sẽ cung cấp cho bạn những thông tin cần thiết để bạn quyết định có tham gia hay không. Vui lòng đọc kỹ tờ thông tin này. Bạn có thể hỏi về chương trình này, những lợi ích và nguy cơ có thể xảy ra, quyền của người tham gia, và bất kỳ vấn đề nào có liên quan hay thông tin trên phiếu này mà bạn thấy chưa rõ. Khi mọi thắc mắc đã được giải đáp, bạn có thể quyết định cho bạn và con bạn có tham gia hay không. Quá trình này được gọi là sự lấy đồng thuận tham gia nghiên cứu.

Mục tiêu của chương trình này là để xác định tần suất bị bệnh tiêu chảy ở trẻ em Việt Nam, nguyên nhân gây tiêu chảy và tại sao trẻ em lại bị tiêu chảy. Ngoài ra chúng tôi cũng muốn tìm hiểu về việc máu của con bạn phản ứng với bệnh tiêu chảy như thế nào. Chúng tôi hy vọng có thể hiểu hơn về bệnh tiêu chảy ở thành phố Hồ Chí Minh để có thể tạo vắc xin giúp các trẻ em ngừa bệnh sau này.

Đây là một phần của chương trình “Sức khỏe trẻ em” mà bạn đang tham gia. Mục tiêu cụ thể của phần mới này là để tìm hiểu một chủng vi khuẩn có tên gọi *Shigella*, thường gây bệnh tiêu chảy nặng ở trẻ nhỏ. Chúng tôi cần khảo sát thu thập thông tin về nơi ở và điều kiện sống của trẻ em để hiểu hơn về bệnh do vi khuẩn *Shigella* và các nguyên nhân gây bệnh tiêu chảy khác ở trẻ em Việt Nam. Để thực hiện được điều này, chúng tôi sẽ theo dõi sức khỏe của con bạn trong 2 năm. Trong suốt thời gian đó chúng tôi sẽ theo dõi máu của bé để tìm chứng cứ của nhiễm khuẩn, và sẽ hỏi bạn về tất cả những bệnh mà bé và các thành viên gia đình bạn mắc phải, kể cả những lần phải nhập viện. Những thông tin có được từ các khảo sát này có thể giúp chúng tôi trong việc tạo ra vắc xin ngăn ngừa bệnh tiêu chảy do nhiễm khuẩn *Shigella* trong tương lai.

Chương trình này cũng sẽ có một phần thực hiện xét nghiệm di truyền trên mẫu máu của con bạn. Xét nghiệm di truyền này giúp chúng tôi hiểu vì sao một số người bị bệnh này

trong khi một số khác thì không bị. Nếu bạn đồng ý, thông tin di truyền trong máu của con bạn sẽ được xét nghiệm. Kết quả xét nghiệm này sẽ được sử dụng để tiến hành các nghiên cứu, tuy nhiên danh tính của bạn và con bạn sẽ được ẩn để không ai biết thông tin di truyền đó là của ai. Một số mẫu sẽ được gửi ra nước ngoài để xét nghiệm, có thể điều này được cho là xâm phạm đến sự riêng tư. Việc đồng ý cho máu để xét nghiệm di truyền là tùy ở bạn. Bạn và con bạn vẫn có thể tham gia vào chương trình này dù bạn không đồng ý để mẫu máu được dùng cho xét nghiệm di truyền.

Các đơn vị tham gia chương trình?

Các đơn vị cộng tác tham gia nghiên cứu bao gồm Bệnh viện Hùng Vương (TPHCM) và Bệnh viện Bệnh Nhiệt Đới (TPHCM).

Nếu như tôi thay mặt con tôi chấp thuận tham gia, điều gì sẽ xảy ra trong chương trình và có những nguy cơ thế nào?

Khi tham gia (hôm nay):

Khi bé được 12 tháng tuổi và bạn đưa bé đến hẹn khám theo dõi lần cuối cùng cho chương trình Sức Khỏe Trẻ Em ở Phòng khám nhi - Bệnh viện Hùng Vương, bạn và con bạn sẽ được mời tham gia vào "Nghiên cứu Đoàn hệ Bệnh Tiêu Chảy". Nếu bạn và con bạn đồng ý tham gia, một điều dưỡng sẽ cập nhật những thông tin về bạn như nơi bạn ở, cấu trúc gia đình, và tình trạng sức khỏe của bé. Điều dưỡng cũng sẽ lấy 2ml mẫu máu (khoảng 20 giọt) của bé, cho chương trình "Sức khỏe trẻ em" thay vì 1ml (khoảng 10 giọt) như trước đây. Chỉ lấy tối đa 2ml máu từ bé trong lần khám này. Điều dưỡng cũng sẽ lấy một mẫu phân, hoặc một mẫu phết hậu môn nếu không có phân, và mẫu phết mũi họng của con bạn.

Các lần tái khám (mỗi 6 tháng một lần trong 2 năm):

Để theo dõi sức khỏe và sự phát triển của con bạn, chúng tôi cần gặp bạn và bé vào mỗi 6 tháng một lần, vào các thời điểm bé được 18, 24, 30 và 36 tháng tuổi.

Mỗi lần tái khám mất khoảng nửa tiếng tại ngay phòng khám nơi bạn được nhận vào nghiên cứu. Điều dưỡng sẽ phát cho bạn một thẻ ghi lịch tái khám của chương trình. Tại mỗi lần tái khám, con của bạn sẽ được bác sĩ kiểm tra sức khỏe. Chúng tôi sẽ ghi lại những thông tin về sự phát triển của con bạn và hỏi thêm một số câu hỏi đơn giản về sức khỏe của bé. Khi con bạn được 18, 24 và 30 tháng, chúng tôi sẽ lấy khoảng 2ml máu (khoảng 20 giọt) của con bạn cho nghiên cứu. Khi con bạn được 36 tháng, chúng tôi sẽ

lấy 3ml máu (khoảng 30 giọt). Mỗi lần khám chỉ lấy 1 mẫu máu của bé. Một điều dưỡng nhiều kinh nghiệm sẽ dùng cây kim nhỏ để lấy máu trên tay của bé. Việc lấy máu sẽ gây đau một tí và có thể lại một vết bầm nhỏ. Tại mỗi lần tái khám, điều dưỡng cũng sẽ lấy một mẫu phân hoặc phết hậu môn và một mẫu phết mũi họng của bé. Việc thu mẫu này rất nhanh và không gây đau.

Khi bạn ở nhà với bé và bạn nghĩ rằng bé bị bệnh tiêu chảy;

Chúng tôi sẽ giữ liên lạc với bạn bằng tin nhắn SMS định kỳ để hỏi thăm bé có bị tiêu chảy không. Nếu bạn trả lời "Có", một điều dưỡng của chúng tôi sẽ gọi điện cho bạn để sắp xếp thời gian thuận tiện để đến thăm nhà bạn để lấy một mẫu phân, hỏi một số câu hỏi đơn giản và tư vấn y tế để giúp con bạn. Nếu bạn cảm thấy rằng con bạn cần nhập viện, chúng tôi mong bạn xem xét đưa bé đến Bệnh viện Bệnh Nhiệt Đới. Nếu bạn muốn đưa bé đến Bệnh viện Bệnh Nhiệt Đới khi bé bị tiêu chảy, hãy gọi cho điều dưỡng của chúng tôi để được hỗ trợ và bé sẽ được khám bởi một bác sĩ chuyên khoa của bệnh viện Bệnh Nhiệt Đới. Chúng tôi sẽ thu thập một mẫu phân của bé khi bạn cho bé đến khám tại đây. Bạn cũng có thể đưa bé đến các phòng khám hoặc bệnh viện khác nếu điều đó thuận tiện cho bạn.

Các nguy cơ và lợi ích khi tham gia:

Khi tham gia chương trình, con bạn sẽ được những bác sĩ có kinh nghiệm tại phòng khám Nhi, bệnh viện Hùng Vương khám và tư vấn miễn phí vào những lần tái khám định kỳ để theo dõi tình trạng sức khỏe và sự phát triển của bé. Lượng máu chúng tôi lấy từ con bạn tại mỗi lần tái khám là rất nhỏ (khoảng 20-30 giọt). Bé có thể khóc khi bị lấy máu. Bạn sẽ nhận được một khoản tiền hỗ trợ cho chi phí đi lại cho các lần tái khám tại phòng khám Nhi, Bệnh viện Hùng Vương, các lần đi khám bệnh vì tiêu chảy tại bệnh viện bệnh Nhiệt đới cũng như miễn phí xét nghiệm khi đến khám tại bệnh viện này vì tiêu chảy. Nếu bạn cho con đến khám ở một phòng khám tư hoặc đến khoa cấp cứu tại một bệnh viện khác, không phải là Bệnh viện Bệnh Nhiệt đới khi bé bị tiêu chảy, chúng tôi sẽ không hỗ trợ các chi phí này.

Chúng tôi sẽ làm gì với máu và các mẫu khác thu từ con bạn

Mẫu máu và phân mà chúng tôi thu được từ con của bạn sẽ được dùng vào nghiên cứu. Các mẫu đó sẽ được lưu trữ trong tủ đông lạnh. Chúng tôi sẽ làm một số xét nghiệm trên mẫu máu, phân và mẫu phết mũi họng của con bạn để xem con bạn có từng bị nhiễm

Shigella hoặc một số bệnh tiêu chảy và hô hấp phổ biến khác ở Việt Nam hay không. Những xét nghiệm mà chúng tôi tiến hành trên các mẫu máu và mũi họng thu được từ con bạn có thể không mang lại lợi ích trực tiếp cho bạn, nhưng điều này có thể giúp những trẻ em khác trong tương lai. Các mẫu chúng tôi thu được từ con bạn cũng có thể được sử dụng cho các nghiên cứu về sau.

Tính bảo mật

Tất cả những thông tin chúng tôi có được từ bạn sẽ được bảo mật nghiêm ngặt. Tên của con bạn sẽ không hiện diện trên bất kỳ một mẫu xét nghiệm nào cũng như trên kết quả của chúng tôi - chúng tôi sẽ dùng mã số thay cho tên. Tên của bạn, tên của con bạn sẽ không được đề cập đến trên kết quả của chương trình. Chúng tôi sẽ hỏi thông tin về quận và phường nơi bạn ở nhưng sẽ không sử dụng thông tin này vào bất kỳ mục đích nào khác ngoài chương trình này và cũng không đưa cho ai khác.

Chi phí

Bạn không tốn bất kỳ chi phí nào khi tham gia vào chương trình này. Việc khám bệnh theo lịch sẽ được chương trình chi trả. Bạn sẽ được hỗ trợ chi phí đi lại khi mang bé đến phòng khám để tái khám theo lịch đã hẹn. Bạn cũng được miễn phí chi phí xét nghiệm chẩn đoán bệnh và được hỗ trợ chi phí đi lại khi cho bé đến khám tại bệnh viện Bệnh Nhiệt Đới vì tiêu chảy.

Sự tự nguyện tham gia chương trình

Dù bạn chọn không tham gia vào chương trình thì việc đó cũng không ảnh hưởng đến việc chăm sóc sức khỏe cho con bạn. Ngay cả khi bạn đã đồng ý tham gia chương trình, bạn cũng có thể xin rút khỏi chương trình bất kỳ lúc nào (bằng lời). Việc chăm sóc sức khỏe cho con bạn sẽ không bị ảnh hưởng. Tuy nhiên những thông tin đã được thu thập về con bạn cho đến thời điểm đó vẫn sẽ được dùng cho nghiên cứu.

Kết thúc chương trình

Chúng tôi sẽ ngừng khám theo dõi con bạn sau bốn lần theo dõi trong vòng 2 năm tại bệnh viện Hùng Vương kể từ ngày hôm nay. Sau đó, chúng tôi sẽ không thu thập thêm mẫu và thông tin từ con bạn nữa.

Các thông tin thêm

Chúng tôi khuyến khích bạn hỏi thêm bất kỳ câu hỏi gì liên quan đến chương trình này trong suốt thời gian tham gia. Nếu bạn có thắc mắc về chương trình, các quy trình, nguy cơ và lợi ích, hay các câu hỏi khác, vui lòng gọi BS. Nguyễn Trọng Hiếu tại số 0903 159 285

Nếu bạn có bất kỳ thắc mắc gì về các quyền của con bạn khi tham gia chương trình này, bạn có thể liên hệ BS. Lư Lan Vi hoặc nếu bạn muốn nói chuyện với ai khác ngoài nhóm nghiên cứu, bạn có thể liên hệ với Hội Đồng Đạo Đức tại Bệnh viện Bệnh Nhiệt đới ở số 083 855 8532.

Phiếu chấp thuận tham gia "Nghiên cứu Đoàn hệ Bệnh Tiêu chảy"**OXTREC – 1058-13****Chấp thuận từ: BỐ/MẸ/NGƯỜI GIÁM HỘ**

- Tôi đã được thông tin đầy đủ về các nguy cơ và lợi ích có thể có của việc cho con tôi tham gia vào chương trình nghiên cứu đoàn hệ bệnh tiêu chảy này và đồng ý rằng tôi và con của tôi sẽ tham gia.
- Tôi biết sẽ liên lạc với ai khi cần hỏi thêm thông tin. Tôi hiểu rằng các thông tin về tôi hay con tôi sẽ được bảo mật. Tôi hiểu được tôi có quyền rút khỏi chương trình vào bất kỳ lúc nào mà không ảnh hưởng đến việc chăm sóc sức khỏe mà tôi hay con tôi sẽ được nhận.
- Tôi hiểu là việc nghiên cứu này sẽ không mang đến nhiều lợi ích trực tiếp cho tôi và con tôi
- Tôi đồng ý để điều dưỡng của chương trình đến nhà tôi khi tôi báo con tôi bị nhiễm bệnh tiêu chảy
- Tôi đồng ý việc định vị nhà ở của tôi có thể được thực hiện để tìm hiểu sự liên quan giữa nơi ở và bệnh tật.

☐ **Tôi đồng ý** hoặc ☐ **Tôi không đồng ý** cho phép mẫu của con tôi được lưu trữ cho các nghiên cứu về sau về các bệnh truyền nhiễm, bao gồm các xét nghiệm di truyền và các xét nghiệm này có thể được thực hiện bên ngoài Việt Nam.

Mã số tham gia: 14EN- ____ - ____ ____ ____

Tên người tham gia: _____

Chữ ký của người chấp thuận _____

Quan hệ với người tham gia _____

Họ Tên (chữ in) _____

Ngày ký _____

Xác nhận của nghiên cứu viên

Tôi, người ký tên bên dưới, đã giải thích đầy đủ các thông tin liên quan đến chương trình nghiên cứu này cho người tham gia có tên bên trên và sẽ cung cấp cho cô/anh ấy một bản sao của phiếu chấp thuận đã được ký và ghi ngày tháng này.

Chữ ký của nghiên cứu viên**hoặc người được chỉ định****Họ Tên (chữ in)****Ngày ký**

Nếu người chấp thuận không thể tự đọc phiếu này, một nhân chứng phải có mặt và ký tên dưới đây:

Tôi đã có mặt cùng với người tham gia trong suốt quá trình lấy chấp thuận. Tất cả các câu hỏi của người tham gia đã được trả lời và người tham gia đã đồng ý tham gia vào chương trình nghiên cứu.

Chữ ký nhân chứng**Họ Tên (chữ in)****Ngày ký**

Phiếu chấp thuận cho xét nghiệm di truyền**OXTREC - 1058-13****(được ký tên bởi bố mẹ hoặc người giám hộ của trẻ tham gia nghiên cứu)**

Bằng việc ký tên/đánh dấu ở đây, tôi xác nhận đồng ý cho phép mẫu máu của con tôi được dùng vào các xét nghiệm di truyền.

Mã số tham gia: 14EN- ____ - ____ ____ ____

Tên người tham gia: _____

Chữ ký của người chấp thuận _____

Quan hệ với người tham gia _____

Họ Tên (chữ in) _____

Ngày ký _____

Tôi, người ký tên bên dưới, đã giải thích đầy đủ các thông tin liên quan đến chương trình nghiên cứu này cho người tham gia có tên bên trên và sẽ cung cấp cho cô/anh ấy một bản sao của phiếu chấp thuận đã được ký và ghi ngày tháng này.

Chữ ký của nghiên cứu viên

hoặc người được chỉ định _____

Họ Tên (chữ in) _____

Ngày ký _____

Nếu người cho chấp thuận không thể tự đọc phiếu này, một nhân chứng phải có mặt và ký tên dưới đây:

Tôi đã có mặt cùng với người tham gia trong suốt quá trình lấy chấp thuận. Tất cả các câu hỏi của người tham gia đã được trả lời và người tham gia đã đồng ý tham gia vào chương trình nghiên cứu.

Chữ ký nhân chứng _____

Họ Tên (chữ in) _____

Ngày ký _____

9.2 ICF for the community survey

9.2.1 ICF for the community survey (English version)

PARTICIPANT INFORMATION SHEET AND ICF

Diarrhoea Management among children under 5 in the community in HCMC, Viet Nam

Introduction and summary

You are invited to take part in this research study to help us explore behavior for the treatment of diarrhoeal disease among children less than five years of age in the community. We want to know which options for outside-the-home healthcare you would seek if your child had an episode of diarrhoeal disease. We also want to know about your attitude on the use of antimicrobials and antimicrobial resistance issues.

You will have a copy of this sheet to keep for your records.

What do I do if I participate in the study?

We will ask you to sign in the informed consent form and we will ask you a few questions regarding your demographics, your child's health status during the last month, what you would do if your child has diarrhoea and where would you choose to seek healthcare outside of the home. We also ask you some questions on your attitude regarding antimicrobial usage and antimicrobial resistance. The interview will take approximately 15 minutes.

You are free to not answer any questions that you do not want to answer.

What happens to the information I give?

The interview will be private between yourself and the interviewer. The questionnaire is anonymous so no one will know the answers are yours. Only the project team members have access to your answers but your information will be anonymous.

What are the risks and the benefits if I participate in the study?

There are no disadvantages if you decide to participate, except that you need to spend time for the interview. Your answers will contribute to the understanding of management of diarrhoea in the community in Viet Nam, and help inform future public

health promotion for diarrhoeal diseases in Vietnamese children. There are also no disadvantages if you choose not to participate.

You will receive a compensation for the time you spend taking part in this study (50,000VND).

Is my participation is compulsory or voluntary?

Your choice to participate is completely voluntary. You are free to decide whether you want to join in this project. If you agree you can then withdraw from the project at any time without any consequences. You can also withdraw any given information before the analysis process.

When I need some more information, who should I contact?

We encourage you to discuss any problems with the study team. If you need any more information about the participation, you can contact Ms Le Thi Quynh Nhi at 0946309385

INFORMED CONSENT FORM
Diarrhoea Management In HCMC, Viet Nam

- I have read the information given to me and freely agree to be in this study. I will be given a copy of this form to keep.

I have been told about the risks and benefits.

I understand that I may stop participating in the study at any time.

(Please check one)

- ☐ I AGREE OR ☐ I DO NOT AGREE to take part in the interview.

By signing/marking my name here, I confirm what is written above

Signature of Participant: _____	Full Name: _____	Date of Signature: ____/____/____
--	-------------------------	--

I, the undersigned, have fully explained the relevant information of this study to the person named above and will provide her/him with a copy of this signed and dated ICF.

_____ Investigator/Designee Signature	_____ Print Name	____/____/____ Date of Signature
---	---------------------	-------------------------------------

If the person giving consent cannot read the form themselves, a witness must be present and sign here: I was present throughout the entire informed consent process with the participant. This form was read accurately to the volunteer, all questions from the volunteer were answered and the volunteer has agreed to take part in the research.

_____ Witness Signature	_____ Full Name	____/____/____ Date of Signature
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9.2.2 ICF for community survey (Vietnamese version)

BẢNG THÔNG TIN VÀ PHIẾU CHẤP THUẬN THAM GIA KHẢO SÁT

Khảo sát về thực trạng xử trí tiêu chảy cho trẻ em dưới 5 tuổi trong cộng đồng tại thành phố Hồ Chí Minh, Việt Nam

Giới thiệu và Tóm tắt

Chúng tôi mời bạn tham gia vào khảo sát thực hành của ba mẹ hoặc người chăm sóc trẻ dưới 5 tuổi khi trẻ bị tiêu chảy trong cộng đồng. Chúng tôi muốn biết anh/chị sẽ lựa chọn những dịch vụ y tế nào khi có tình huống bé bị tiêu chảy. Chúng tôi cũng muốn tìm hiểu thái độ của anh chị trong việc sử dụng thuốc kháng sinh và vấn đề kháng kháng sinh.

Chúng tôi có 02 bản giống hệt nhau và sẽ gửi cho anh chị 01 bản để giữ.

Tôi sẽ làm gì khi tham gia khảo sát này?

Nếu anh/ chị đồng ý tham gia, anh chị sẽ ký tên vào “Phiếu chấp thuận tham gia” và chúng tôi sẽ hỏi một vài câu hỏi về các đặc điểm liên quan đến dân số của anh/chị, vài câu về tình trạng sức khỏe của trẻ trong vòng 01 tháng qua, chúng tôi sẽ hỏi về lựa chọn anh chị sẽ thực hiện nếu trẻ bị tiêu chảy. Sẽ mất khoảng 15 phút cho việc phỏng vấn. Anh chị được quyền trả lời hoặc từ chối trả lời bất cứ câu hỏi nào anh chị không muốn.

Những thông tin tôi cung cấp sẽ được dùng làm gì?

Nghiên cứu này sử dụng bộ câu hỏi ẩn danh nên không ai được biết anh/ chị đã trả lời phiếu câu hỏi nào. Chỉ có nhóm nghiên cứu mới được tiếp cận các thông tin anh/ chị đã trả lời nhưng trên đó không ghi rõ họ và tên anh/ chị.

Các nguy cơ và bất lợi nếu tôi tham gia?

Đây là khảo sát thông qua phỏng vấn hỏi – trả lời nên nếu anh chị đồng ý tham gia, sẽ không có bất lợi gì về sức khỏe hoặc bất cứ bất lợi gì khác, ngoại trừ mất một chút thời gian trả lời câu hỏi.

Các câu trả lời của anh/ chị sẽ giúp chúng tôi hiểu được cách xử trí của người dân trong cộng đồng nói chung khi trẻ bị tiêu chảy, từ đó sẽ giúp cho việc thông tin đến các chiến lược truyền thông nhằm cải thiện tình hình tiêu chảy ở Việt Nam.

Nếu anh/ chị không tham gia thì cũng không có bất lợi nào cho mình.

Anh/ chị sẽ được gửi một món quà nhỏ (trị giá 50.000VND) để cảm ơn thời gian anh/ chị đã dành cho trả lời câu hỏi.

Tham gia tự nguyện hay bắt buộc?

Việc tham gia hoàn toàn tự nguyện. Anh/ chị hoàn toàn được tự ý quyết định tham gia hoặc không tham gia. Anh/ chị có thể rút lui ở bất kỳ thời điểm nào mà không bị ảnh hưởng gì cả. Nếu anh/ chị muốn rút khỏi khảo sát, bất cứ lúc nào cũng được, trước giai đoạn các dữ liệu được phân tích.

Tôi cần biết thêm thông tin, tôi liên hệ với ai?

Chúng tôi khuyến khích anh/ chị tham gia thảo luận bất cứ vấn đề gì liên quan với nhóm nghiên cứu. Khi cần anh/ chị có thể liên hệ: ThS. Lê Thị Quỳnh Nhi, 0946309385.

PHIẾU CHẤP THUẬN THAM GIA

Khảo sát về thực trạng xử trí tiêu chảy cho trẻ em dưới 5 tuổi trong cộng đồng tại thành phố Hồ Chí Minh, Việt Nam

- Tôi đã đọc và hiểu thông tin trên đây, đã có cơ hội xem xét và đặt câu hỏi về thông tin liên quan đến nội dung trong nghiên cứu này.
- Tôi đã nói chuyện trực tiếp về nguy cơ và lợi ích
- Tôi sẽ nhận một bản sao của Bản Thông tin và phiếu chấp thuận tham gia.
- Tôi hiểu rằng mình có thể dừng tham gia khảo sát bất cứ lúc nào.

Tôi chọn

☐ **TÔI ĐỒNG Ý THAM GIA**

☐ **TÔI KHÔNG ĐỒNG Ý THAM GIA**

Chữ ký của người tham gia:

Chữ ký _____	Họ tên người tham gia _____	Ngày tháng năm ____/____/____
-----------------	--------------------------------	----------------------------------

Chữ ký của Nghiên cứu viên/người lấy chấp thuận:

Tôi, người ký tên dưới đây, xác nhận rằng bệnh nhân/người tình nguyện tham gia nghiên cứu ký bản chấp thuận đã đọc toàn bộ bản thông tin trên đây, các thông tin này đã được giải thích cặn kẽ cho Anh/ Chị và Anh/ Chị đã hiểu rõ bản chất, các nguy cơ và lợi ích của việc tham gia vào khảo sát này.

_____	_____	____/____/____
Chữ ký	Họ tên nghiên cứu viên	Ngày tháng năm

Chữ ký của người làm chứng hoặc của người đại diện hợp pháp (nếu áp dụng):

_____	_____	____/____/____
Chữ ký	Họ tên người làm chứng	Ngày tháng năm

9.3 CRFs for the community survey

9.3.1 English version

**Questionnaire: A descriptive study on the management of diarrhoeal disease in
the community in HCMC, Viet Nam**

ICF ID: _ _ _ _

Investigator: _____

Date of interview: _ _ / _ _ / _ _

Ward : _____ District 8 HCMC

Instruction: Please fill in the box that match the interviewees' answers

Part A. Demographic information

A1. Gender of interviewee

1. Male ☐2. Female ☐

A2. What is your role with the kid

1. Parents ☐2. Care-giver ☐

A3. What year of birth you were born

_____(year)

A4. Did you have any health training

1. Yes ☐2. No ☐

A5. How many kids do you have?

1. 01 child ☐2. From 2 + ☐

A6. How many kids under 5 do you have?

1. 01 child ☐2. From 2 + ☐

A7. DOB of the first kid under 5 _ _ / _ _ / _ _

A8. DOB of the second kid under 5 _ _ / _ _ / _ _

A9. DOB of the third kid under 5 _ _ / _ _ / _ _

A10. What do you do for living?

1. Worker, shop assistant ☐2. Officer, teacher (long term paid) ☐3. Health staff ☐4. Business (shop owner, company) ☐5. Housewife ☐6. Other ☐ _____

A11. Have you finished?

1. Primary ☐
2. Secondary ☐
3. Highschool ☐
4. Vocational training, college, undergraduate ☐
5. Postgraduate ☐

A12. How much is your monthly income?

1. < 900.000VND/ month ☐
2. 900.000 - 1.300.000 /month ☐
3. 1.300.000VND – less than 9.000.000/ month ☐
4. > 9.000.000/ month ☐
5. No answer ☐

Part B. Information about the child under 5 during the last one month. Please tell us during the last month if your child,

B13. Has the child had any infections (eg: diarrhoea, pneumonia, ...)?

1. Yes ☐
2. No ☐
3. Don't known ☐

B14. Has the child taken any medication?

1. Yes ☐ If yes, what disease _____
2. No ☐
3. Don't known ☐

B15. Has the child taken any antibiotics?

1. Yes ☐
2. No ☐
3. Don't known ☐

Part C. In this part we are going to propose a scenario that there is one child under 5 in your house has diarrhoea (meaning that the kid has abnormal loose stool such as loose stool, watery stool, mucus, bloody, more than 3 times during the previous 24 hours. The kid may have small amount of abnormal stool, many times, watery, etc. Please tell us

C16. Given the kid has diarrhoea, what would you do (multiple options)

1. Stay at home ☐
2. Buy drugs from pharmacy ☐

3. Visit doctors ☐

4. Visit hospital ☐

C17. First scenario: the kid has loose stool, 4-5 times/ day, please tell us 03 options of your choice (noted that in this scenario the kid has no fever and no vomit)

1. Stay at home
2. Buy drugs from pharmacy
3. Visit district hospital
4. Visit private and/ or international hospitals
5. Visit Health center at the ward
6. Visit private doctors, clinics
7. Visit pediatrics hospital or HTD
8. Buy drugs from traditional medical shops (thuốc bắc, thuốc nam)

C18. In the 1st scenario, that the kid has loose stool and no fever, no vomit, if you do not stay at home, on which day of the disease would you go to seek medical care (such as buy drugs or visit doctors)

1. Day 1 ☐
2. Day 2 ☐
3. Day 3 ☐
4. Other _____

C19. Second scenario: the kid has mucus stool, 4-5 times/ day please tell us 03 options of your choice (noted that in this scenario the kid has no vomit, but mild fever)

1. Stay at home
2. Buy drugs from pharmacy
3. Visit district hospital
4. Visit private and/ or international hospitals
5. Visit Health center at the ward
6. Visit private doctors, clinics
7. Visit pediatrics hospital or HTD
8. Buy drugs from traditional medical shops (thuốc bắc, thuốc nam)

C20. In the scenario that the kid has mucus and mild fever, no vomit, if you do not stay at home, on which day of the disease would you go to seek medical care (such as buy drugs or visit doctors)

1. Day 1 ☐

2. Day 2 ☐
3. Day 3 ☐
4. Other _____

C21. During the disease onset, would you provide more water to the kid?

1. Yes ☐
2. No ☐
3. Don't known ☐

C22. During the disease onset, would you provide any more fluid or water?

1. Yes ☐
2. No ☐
3. If yes, please clarify _____

C23. Would you supplement zinc (a kind of nutritional mineral)?

1. Yes ☐
2. No ☐
3. Don't known ☐

C24. Would you provide any probiotics?

1. Yes ☐
2. No ☐
3. Don't known ☐
4. Other _____

C25. Would you feed the kid yoghurt?

1. Yes ☐
2. No ☐
3. Don't known ☐
4. Other _____

Part D. We'd like to ask about your behavior in real life (these can be applied for you and/ or you children)

D26. Have you ever bought drugs from pharmacy

1. Yes ☐
2. No ☐

D27. Have you ever bought antibiotics from pharmacy?

1. Yes ☐
2. No ☐

D28. When have you bought antibiotics?

1. In the last month ☐
2. In the last 6 month ☐
3. In the last year ☐
4. More than 01 year ☐
5. Don't remember ☐

D29. According to you, antibiotics are used for (multiple options)

1. Headache ☐
2. Diarrhoea ☐
3. Teething ☐
4. Fever ☐
5. Cold ☐
6. Coughing ☐
7. Others _____

D30. According to you, how could we buy antibiotics (multiple options)

1. Doctors' prescription ☐
2. Require the doctor to prescribe ☐
3. Require drug seller to sell antibiotics ☐
4. Don't know ☐
5. Other ☐

D31. Have you ever bought antibiotics without doctors' prescription?

1. Yes ☐
- 2.No ☐

D32. Have you ever stored antibiotics at home for further uses?

1. Yes ☐
- 2.No ☐

D33. Have you ever stopped antibiotics if you get better?

1. Yes ☐
- 2.No ☐

D34. Have you ever reduced doses if you get better?

1. Yes ☐
- 2.No ☐

D35. Have you ever exchange prescriptions with others?

1. Yes ☐
- 2.No ☐

Part F. Please let us know your idea bout these statements

F36. Some infections can be hard to treat if there is antibiotic resistance.

1. Agree ☐
2. Don't agree ☐

3. No idea/ don't know ☐

4. Other_____

F37. Some bacteria can be resistant if you do not take enough doses

1. Agree ☐

2. Don't agree ☐

3. No idea/ don't know ☐

4. Other_____

9.3.2 Vietnamese version

Bộ câu hỏi

Nghiên cứu mô tả về thực trạng xử trí tiêu chảy cho trẻ em dưới 5 tuổi trong cộng đồng tại Tp.HCM

Mã số BCH: _____

Ngày thực hiện: __/__/__

Điều tra viên: _____

Phường: _____ Quận 8 Tp.HCM

Hướng dẫn hoàn tất bộ câu hỏi: Đánh dấu vào ô phù hợp với phần trả lời của người được phỏng vấn

Phần A. Thông tin nền

A1. Giới tính người trả lời 1.Nam ☐ 2.Nữ ☐

A2. Anh/ Chị là gì đối với trẻ 1.Ba/ mẹ ☐ 2.Người chăm sóc ☐

A3. Anh/ Chị sinh năm nào? _____ (năm)

A4. Anh/ Chị có được đào tạo về y tế không? Có ☐ Không ☐

A5. Anh/ Chị có mấy người con? 01 con ☐ Từ 2 trở lên ☐

A6. Anh/ Chị có mấy con dưới 5 tuổi? 01 con ☐ Từ 2 trở lên ☐

A7. Ngày sinh của trẻ dưới 5 tuổi thứ nhất __/__/__

A8. Ngày sinh của trẻ dưới 5 tuổi thứ hai __/__/__

A9. Ngày sinh của trẻ dưới 5 tuổi thứ ba __/__/__

A10. Anh/ Chị làm nghề gì kiếm sống?

1.Công nhân, bán hàng ☐

2.Văn phòng, giáo viên (hưởng lương dài hạn) ☐

3.Nhân viên y tế ☐

4.Buôn bán, kinh doanh (làm chủ cửa hàng, DN) ☐

5.Nội trợ ☐

6.Khác ☐ (ghi rõ) _____

A11. Anh/ Chị đã học xong cấp nào?

1.Cấp 1 ☐

2.Cấp 2 ☐

3.Cấp 3 ☐

4.Trung học nghề, cao đẳng, đại học ☐

5.Sau đại học ☐

A12. Thu nhập hàng tháng của anh/ chị là bao nhiêu?

1.< 900.000VND/ tháng ☐

- | | |
|--|--------------------------|
| 2.900.000 - 1.300.000 tháng | ☐ |
| 3.1.300.000VND - dưới 9.000.000/ tháng | ☐ |
| 4.> 9.000.000/ tháng | ☐ |
| 5.Không trả lời | ☐ |

Phần B. Thông tin về trẻ trong tháng qua. Hãy cho chúng tôi biết trong một tháng qua bé (<5 tuổi) của anh/chị

B13. Bé có bị bệnh nhiễm trùng nào không (ví dụ: tiêu chảy, viêm phổi, ...)

- | | |
|--------------|--------------------------|
| 1.Có | ☐ |
| 2.Không | ☐ |
| 3.Không biết | ☐ |

B14. Bé có uống thuốc trị bệnh gì không?

- | | |
|---------------------------|--------------------------|
| 1.Có | ☐ |
| 2.Không | ☐ |
| 3.Không biết | ☐ |
| 4.Nếu có, ghi rõ bệnh gì? | _____ |

B15. Bé có uống thuốc kháng sinh không?

- | | |
|--------------|--------------------------|
| 1.Có | ☐ |
| 2.Không | ☐ |
| 3.Không biết | ☐ |

Phần C. Sau đây chúng tôi sẽ đặt giả sử có tình huống một em bé dưới 5 tuổi trong nhà bị tiêu chảy, (nghĩa là bé đi cầu phân lỏng bất thường như: phân lỏng, phân toé nước, phân có nhầy, máu, mủ ,... từ 3 lần trở lên trong vòng 24 giờ). Bé có thể đi xoẹt xoẹt, đi phân nhiều nước, hoặc có thể bé đi nhiều lần, mỗi lần một chút phân lỏng. Hãy cho chúng tôi biết

C16. Giả sử khi bé bị bệnh tiêu chảy, Anh/ chị sẽ làm gì (đánh dấu nhiều lựa chọn)

- | | |
|-------------------------|--------------------------|
| 1.Ở nhà theo dõi | ☐ |
| 2.Mua thuốc ở nhà thuốc | ☐ |
| 3.Đi khám bác sĩ | ☐ |
| 4.Đến bệnh viện | ☐ |

C17. Tình huống 1 là em bé đi phân lỏng khoảng 4 đến 5 lần, Anh/chị hãy cho biết thứ tự ưu tiên 03 lựa chọn đầu tiên của Anh/Chị (ghi chú thêm: trường hợp này em bé không sốt, không ói)

- | | |
|---|-----|
| 1.Ở nhà theo dõi | _ _ |
| 2.Mua thuốc ở nhà thuốc | _ _ |
| 3.Cho trẻ đi khám ở bệnh viện Quận | _ _ |
| 4.Cho trẻ đi khám ở bệnh viện tư, bệnh viện quốc tế | _ _ |
| 5.Cho trẻ đi khám ở Trạm y tế | _ _ |
| 6.Cho trẻ đi khám ở phòng mạch bác sĩ, phòng khám đa khoa | _ _ |
| 7.Cho trẻ đi khám ở bệnh viện nhi đồng hoặc Bệnh viện nhiệt đới | _ _ |
| 8.Mua thuốc từ nhà thuốc Y học cổ truyền (thuốc bắc, thuốc nam) | _ _ |

C18. Trong tình huống 1, trường hợp em bé chỉ đi phân lỏng và không sốt, không ói, nếu không cho ở nhà, thì vào ngày thứ mấy của bệnh Anh/ chị cho bé đi tìm dịch vụ y tế (như mua thuốc hoặc khám bệnh)

- | | |
|----------|--|
| 1.Ngày 1 | ☐ |
| 2.Ngày 2 | ☐ |
| 3.Ngày 3 | ☐ |
| 4.Khác | ☐ (ghi rõ)_____ |

C19. Tình huống 2 là em bé đi phân có nhầy, cũng 4-5 lần một ngày, Anh/chị hãy cho biết thứ tự ưu tiên 03 lựa chọn đầu tiên của Anh/Chị (ghi chú thêm: trường hợp này em bé không ói nhưng có sốt nhẹ)

- | | |
|---|-----|
| 1.Ở nhà theo dõi | _ _ |
| 2.Mua thuốc ở nhà thuốc | _ _ |
| 3.Cho trẻ đi khám ở bệnh viện Quận | _ _ |
| 4.Cho trẻ đi khám ở bệnh viện tư, bệnh viện quốc tế | _ _ |
| 5.Cho trẻ đi khám ở Trạm y tế | _ _ |
| 6.Cho trẻ đi khám ở phòng mạch bác sĩ, phòng khám đa khoa | _ _ |
| 7.Cho trẻ đi khám ở bệnh viện nhi đồng hoặc Bệnh viện nhiệt đới | _ _ |
| 8.Mua thuốc từ nhà thuốc Y học cổ truyền (thuốc bắc, thuốc nam) | _ _ |

C20. Trong tình huống 2, em bé đi cầu phân có nhầy, có sốt nhẹ, nếu không cho ở nhà, thì vào ngày thứ mấy của bệnh Anh/ chị cho bé đi tìm dịch vụ y tế (như mua thuốc hoặc khám bệnh)

- 1. Ngày 1 ☐
- 2. Ngày 2 ☐
- 3. Ngày 3 ☐
- 4. Khác ☐ (ghi rõ) _____

C21. Trong những ngày bé bị tiêu chảy, Anh/ Chị có cho bé uống thêm nước không?

- 1. Có ☐
- 2. Không ☐
- 3. Không biết ☐

C22. Trong những ngày bé bị tiêu chảy, Anh/ chị có cho bé uống thêm dịch/nước uống khác không?

- 1. Có ☐ Nếu có, ghi rõ loại dịch, nước _____
- 2. Không ☐

C23. Anh/ chị có cho bé bổ sung kẽm (vi chất dinh dưỡng) không?

- 1. Có ☐
- 2. Không ☐
- 3. Không biết ☐

C24. Anh/ chị có cho bé uống men vi sinh không?

- 1. Có ☐
- 2. Không ☐
- 3. Không biết ☐
- 4. Khác ☐ (ghi rõ) _____

C25. Anh/ chị có cho bé ăn sữa chua không?

- 1. Có ☐
- 2. Không ☐
- 3. Không biết ☐
- 4. Khác ☐ (ghi rõ) _____

Phần D. Chúng tôi muốn hỏi về thực hành của anh/ chị trong thực tế (cho bản thân anh/ chị và/ hoặc cho bé)

D26. Anh/ chị có từng mua thuốc từ nhà thuốc không?

1.Có ☐

2.Không ☐

3.Không biết ☐

D27. Anh/ chị có từng mua thuốc kháng sinh từ nhà thuốc không?

1.Có ☐

2.Không ☐

3.Không biết ☐

D28. Anh/ chị từng mua thuốc kháng sinh khi nào?

1.Trong tháng qua ☐

2.Trong 6 tháng qua ☐

3.Trong năm qua ☐

4.Hơn 01 năm ☐

5.Không nhớ ☐

D29. Theo Anh/ chị, thuốc kháng sinh được dùng để điều trị (nhiều lựa chọn)

1.Đau đầu ☐

2.Tiêu chảy ☐

3.Mọc răng ☐

4.Sốt ☐

5.Cảm lạnh ☐

6.Ho ☐

7.Khác ☐ (ghi rõ)_____

D30. Theo Anh/chị, có thể mua được thuốc kháng sinh bằng cách nào (nhiều lựa chọn)

1.Từ bác sĩ kê toa theo bệnh ☐

2.Đề nghị bác sĩ kê toa ☐

3.Đề nghị người bán thuốc bán cho mình ☐

4.Không biết ☐

5.Khác ☐ (ghi rõ)_____

1 Có

2

3

Không

Không biết

D31. Anh/ chị có từng mua thuốc kháng sinh theo toa

BS chỉ định không?

1 ☐

2 ☐

3 ☐

D32. Anh/ chị có từng giữ kháng sinh ở nhà để sau này dùng không? 1 ☐ 2 ☐ 3 ☐

D33. Anh/ chị có từng dùng thuốc kháng sinh nếu thấy đỡ hơn không? 1 ☐ 2 ☐ 3 ☐

D34. Anh/ chị có từng giảm liều nếu thấy bệnh đỡ hơn không? 1 ☐ 2 ☐ 3 ☐

D35. Anh/ chị có từng đổi toa thuốc với người khác hoặc các ba mẹ khác không; hoặc xin toa thuốc đã trị dứt bệnh của người khác cho con mình không? 1 ☐ 2 ☐ 3 ☐

Phần F. Hãy cho chúng tôi biết ý kiến của Anh/ chị về các câu nói sau đây

F36. Một số bệnh nhiễm trùng có thể khó trị hơn nếu có sự đề kháng kháng sinh

- 1. Đồng ý ☐
- 2. Không đồng ý ☐
- 3. Không ý kiến/ không biết ☐
- 4. Khác ☐ (ghi rõ) _____

F37. Một số vi khuẩn có thể trở nên kháng thuốc nếu uống thuốc không đủ liều

- 1. Đồng ý ☐
- 2. Không đồng ý ☐
- 3. Không ý kiến/ không biết ☐
- 4. Khác ☐ (ghi rõ) _____

9.4 The diarrhoea cohort study protocol

Title: A community cohort study to define the age-specific incidence and risk factors of *Shigella* diarrhoeal infections in Vietnamese children

Study code: 14EN

OXTREC number: 1058-13

Short title: *Shigella* cohort study

Version 1.3

Introduction

Diarrhoea remains a major cause of childhood morbidity and mortality globally ^{2,3}, with the vast majority of the 2 billion annual infections and the 2.5 million resulting deaths occurring in low and middle-income countries ^{13,30}. The aetiologies of diarrhoea are broad and can induce a range of symptoms. The four species of the Gram-negative bacterial genus *Shigella* (*boydii*, *dysenteriae*, *flexneri* and *sonnei*)³⁷, are amongst the most common causes of dysenteric diarrhoea, with >164 million infections annually ³⁸. However, the burden of *Shigella* is disproportionately distributed between the species. *S. flexneri* has historically been responsible for the majority of disease in developing countries ^{39,40}, whilst *S. sonnei* has dominated in developed countries ^{41–43}. This pattern, however, is changing with *S. sonnei* replacing *S. flexneri* as the primary agent of dysentery in many countries ^{40,44,45}.

There is a necessity to control *Shigella* infections and investigate the dramatic emergence of *S. sonnei* in industrialising countries. Yet, there are extensive knowledge gaps regarding disease distribution, incidence and exposure. Longitudinal, population-based rates of age-related morbidity and complications are an important gap in *Shigella* knowledge ²⁸⁹. Age-specific incidence is essential for assessing disease burden and implementing and assessing the potential benefits of future vaccines. There is also a need, for *Shigella* as well as other diarrhoeal pathogens, to identify individual risk effects of malnutrition, low education levels, poor sanitation and lack of vitamins and micronutrients in acquisition of diarrhoea in children in the developing world ²⁹⁰.

Shigella in Vietnam

Factors determining the distribution of the species are not well understood but the *S. flexneri*/*S. sonnei* ratio is related to the economic status of the population ²⁸⁹. The collaborative work between Oxford University Clinical Research Unit (OUCRU), Children's Hospital 1 (CH1), Children's Hospital 2 (CH2) and the Hospital for Tropical Disease (HTD) in Ho Chi Minh City (HCMC), Vietnam, has reaffirmed this association, demonstrating a transition from *S. flexneri* to *S. sonnei* over the past 15 years ⁴⁵. Now, >95% of *Shigella* infections in HCMC are *S. sonnei*, making it the principal bacterial agent of hospitalised diarrhoea in HCMC. These *Shigella* infections can be aggressive, frequently necessitating antimicrobial treatment ^{291,292}. Yet, the increasing global dominance of *S. sonnei* is associated with resistance to “last resort” antimicrobials such as fluoroquinolones and 3rd generation cephalosporins ^{179,293}, and there is no vaccine ²⁹⁴.

The burden of shigellosis in Vietnam remains unacceptably high. According to data from government surveillance records, shigellosis in all ages was reported to have an annual incidence rate of 58.8/100,000 in 2001¹⁹⁷; this was estimated to be 490/100,000/year in children under 5 in a population-based study conducted in the early 2000s in the coastal province of Khanh Hoa⁴⁰. Due to this substantial burden of disease, if a vaccine for *Shigella* becomes available as part of a routine schedule for childhood immunisation, this could have a substantial impact on the incidence of diarrhoea and improvement of child health in Vietnam²⁹⁵. However, in the face of growing antimicrobial resistance and the continued lack of a vaccine^{296,297}, diarrhoeal admissions due to *Shigella* are predicted to become increasingly more common and more difficult to treat.

Although various studies have identified *Shigella* as a pathogen in children in this setting, very few have focused exclusively on *Shigella* and its associated risk factors in Vietnam. However, previous work has shown as in other developing nations, *Shigella* infections tend to present most often in the summer months during the monsoon season (which occurs April-November in southern Vietnam) and is most common in children 2-3 years of age^{40,45,187,197}. Additionally, from a study evaluating *Shigella* isolates over a period of 15 years in HCMC, district 8 has been shown to contribute proportionally more shigellosis patients than other districts in the city. District 8 is an area associated with high population density and poor sanitation (see Figure 2)⁴⁵.

Relationship between the original 07DX Birth Cohort and the *Shigella* cohort

The original Children's Health Birth Cohort (07DX: *A birth cohort study to identify immunological, genetic, environmental, and viral risk factors for dengue and viral infections of the respiratory and gastrointestinal tract in Vietnamese infants and children*) (Oxtrec: 0209) was established in 2009 as a collaboration between Hung Vuong Hospital, Dong Thap Provincial Hospital, District 8 Hospital, the Hospital for Tropical Diseases and the Oxford University Clinical Research Unit. The 07DX study was designed to address major questions in childhood infectious diseases transmission in southern Vietnam. Specifically, 07DX aimed to investigate immunity and pathogenesis in young children exposed to dengue fever and to describe infectious causes of respiratory and diarrhoeal disease in the first 12 months of life.

As of December 2013, the 07DX Birth Cohort has enrolled over 6,500 children. Children enrolled in 07DX are currently followed up through 12 months of age with 5 scheduled study follow-up visits, usually overlapped with standard care immunisation visits. Passive surveillance of common childhood illnesses is also performed when parents bring their children into the study clinic during periods of illness. Enrolment into 07DX has been

completed. A smaller cohort of 1,000 children enrolled to the 07DX Birth Cohort have been enrolled into an extended follow-up period until 6 years of age as per the protocol amendment version 6.0 of the 07DX study (07DXA Birth Cohort Extension) (Oxtrec: 0209), known as study 07DXA. .

This newly proposed *Shigella cohort* aims to address additional scientific questions related to diarrhoeal disease in children in the first 5 years of life. The proposed start date for recruitment into the *Shigella cohort* is within the first half of 2014. The *Shigella cohort* will recruit participants aged 12–36 months from the existing 07DX Birth Cohort and 07DXA Extension studies, with follow-up for 2 years to 36-60 months of age (depending on the age of recruitment). The *Shigella cohort* will utilise the existing participants, staff and infrastructure of 07DX. Limited changes to the routine follow-up procedures of the existing 07DXA extension and extensive coordination amongst study organisers will reduce confusion and work burden amongst study staff to achieve a smooth transition between studies. The databases for the 07DX, 07DXA and *Shigella cohort* studies will be linked to facilitate combined data analysis.

As *Shigella* is most often reported in children between 2-3 years of age, the *Shigella cohort* will expand on the original infrastructure and aims of the 07DX study to answer questions specifically on this pathogen, as well as other enteric pathogens, in children in this age range. Serological samples collected in this study will also be used to explore dynamics of other infectious diseases in young Vietnamese children including hand foot and mouth disease, dengue virus and common respiratory pathogens, as per the objectives of the 07DXA Birth Cohort Extension.

Together, the 07DX, 07DXA and *Shigella cohort* studies create a strategic and comprehensive platform to address major scientific questions in communicable diseases in southern Vietnam. Longitudinal information and samples collected from birth will allow for robust evaluation of a variety of epidemiological, immunological and genetic aspects of infection and disease. Such data is crucial to develop appropriate diagnostics, treatment regimens and disease control interventions among many other activities to improve the health of Vietnamese children.

Shigella cohort objectives

Primary objective

To define the age-specific incidence and exposure of *Shigella* infection in children ≤ 60 months of age in the community in HCMC

Secondary objectives

To determine the age-specific incidence of other agents of diarrhoeal infection including *Salmonella*, *Campylobacter*, pathogenic *E. coli* variants, rotavirus and norovirus in children ≤ 60 months of age in the community in HCMC

To describe risk factors for both *Shigella* as well as other diarrhoeal aetiologies in the community

Evaluate levels of seroconversion to diarrhoeal pathogens in children at 6 month intervals among children ≤ 60 months of age

Explore the relationship between levels of cord blood IgG titres at birth and rates of seroconversion to *Shigella* infection

A collection of DNA from all enrollees that will be used to investigate host genetic susceptibilities for diarrhoeal disease. These data will be compared to data from cross-sectional human genetics data that is already available for the Vietnamese population.

Longitudinal anal and nasopharyngeal swabs collected during the period of study will be used to investigate the development of the gastrointestinal tract and the respiratory tract microbiota.

Deliverables

This project will aim to describe the epidemiology of *Shigella* infections in Ho Chi Minh City and to inform the development and use of a *Shigella* vaccine. Specifically, we aim to determine age-specific incidence and risk factors for *Shigella* infections and other agents of diarrhoea across a spectrum of disease severity. Additionally, serum collected through this

project will provide an estimation of the true burden of *S. flexneri* and *S. sonnei* exposure and seroincidence rates in this population through longitudinal serological analyses. We will also investigate the longitudinal immune response to a range of *Shigella* antigens in infected/exposed individuals in an endemic setting with a view of identifying vaccine and diagnostic candidates and their serological relationship to O-antigen. It is hoped that local and international partners will use data collected through this project as a resource to not only influence vaccine development but also local deployment. The host DNA samples will be stored and used for genotyping to investigate potential genetic associations with diarrhoeal disease and the development of the gastrointestinal and respiratory tract flora will be measured by performing metagenomic analysis from the anal and nasopharyngeal swabs.

Enrolment

There will be two different sets of children invited for enrolment into the *Shigella cohort*: (1) children enrolled in 07DX who were born in August 2011 – December 2012 and subsequently enrolled in the 07DXA Extension, and who attend a scheduled follow-up visit (at 18m, 24m, 30m or 36m old) for 07DXA during 2014 and (2) children enrolled in 07DX at birth during 2013, who complete follow-up for that study at 12 months of age during 2014. A schematic of these sets of children is shown in Figure 1. Enrolment procedures will be slightly different in each set of children (see sections below). However, in all cases, parents will be provided with both verbal and written information about the study objectives and procedures and will be able to consider and discuss participation with other family members before making a decision on study participation. Written informed consent must be provided for enrolment.

Children born during August 2011 – December 2012

Babies born during August 2011 – December 2012 will have aged out of the original 07DX birth cohort and will have been invited to participate in the 07DXA Extension at their 12 month follow up appointment. When children come in for their first routine 07DXA Extension appointment of 2014 – born between August 2011- December 2012 - the family will be invited to consider participation in the *Shigella cohort*. All children currently enrolled in the 07DXA Extension will be eligible for participation for 2 years of follow up from enrolment.

Existing 07DXA Extension participants who consent to enrol in the *Shigella cohort* will be enrolled in both 07DXA and the *Shigella cohort* studies. Existing 07DXA Extension participants who decline to enrol in the *Shigella cohort* will continue their follow-up under the 07DXA Extension protocol only.

Children born during 2013

Mothers and babies enrolled in the original 07DX Birth Cohort study in 2013 will be invited to participate in the *Shigella cohort* study at their scheduled 12 month follow up visit during the original 07DX study. For babies attending their 12 month follow up visit in 2014 prior to the start of the *Shigella cohort*, study nurses will request permission to contact the family once the study begins to invite them for participation. All infants who were enrolled in 07DX will be eligible to participate in the *Shigella cohort*.

Activities on enrolment for all children

Once enrolled in the *Shigella cohort*, a study nurse will administer a questionnaire to review information on contact details as well as demographic and socioeconomic status data in an existing database and correct if necessary. An additional questionnaire regarding specific risk factors for diarrhoea such as water usage, sanitation and hygiene habits as well as concurrent familial illness will be administered to all children. The study nurses will also register the mother for the next follow up appointment.

At enrolment, an anal swab will be collected at HVH-WBC as described in section 7.1, a blood sample as in section 7.3 and a nasopharyngeal swab as in section 7.4. If there is stool available, stool sample will be collected as described in section 7.2 instead of anal swab,

Routine follow up appointments

All routine follow up appointments will take place at HVH-WBC. Parents will be requested to attend appointments every 6 months, as detailed in section 5.1. At these appointments, the study nurse will provide general health advice to the families as needed or requested in addition to *Shigella cohort* activities detailed in section 5.2. Families will be compensated for travel costs associated with attending the routine follow up appointments. Routine follow up appointments will last no longer than 30 minutes.

Scheduled appointments

Children born in August 2011 - December 2012 will have routine follow up appointments every 6 months for 2 years from enrolment. Children born in 2013 will also have scheduled routine follow up appointments every 6 months for 2 years, and will be ≥ 12 months at enrolment. To encourage completeness in attendance, study nurses will call the mother in advance of the scheduled appointment to remind her and reschedule if needed.

Activities at routine follow up appointments

Questionnaire Administration

At each scheduled routine follow up visit, a brief questionnaire will be administered to collect information on the child's weight and height, any changes in risks associated with

diarrhoea in the household and any disease history of the child or other children in the household in the preceding period since the last appointment.

Sample Collection

At each scheduled routine follow up visit, an anal swab will be collected at HVH-WBC as described in section 7.1, a blood sample as in section 7.3 and a nasopharyngeal swab as in section 7.4. If there is stool available, stool sample will be collected as described in section 7.2 instead of anal swab,

Diarrhoeal Events

A diarrhoeal event is defined as the occurrence of ≥ 3 non-formed stools (or ≥ 1 , if bloody) in a 24 hour period¹⁹. Additionally, a diarrhoeal event can be defined as an increase in frequency, reduction in consistency or notable change in quality of the stools, compared to what the family considers to be normal for the child²⁹⁸.

Active surveillance of diarrhoeal events

Contact with study nurses & weekly messaging

In order to perform active surveillance for diarrhoeal events in cohort children, parents will be requested to alert a member of study staff as soon as possible when the child experiences an episode of diarrhoea through calling or texting a study nurse or doctor. Additionally, a designated family member will be contacted weekly by phone (SMS or call) enquiring if the enrolled child has experienced an episode of diarrhoea in the preceding 7 days. If the family member responds positively, a study nurse will be made aware and will follow procedures for household visits as outlined in section 6.1.2. Costs associated with text messaging and phone calls from study nurses will be billed to OUCRU.

Household visits

Once a study nurse is alerted to a diarrhoeal episode in a cohort member, they will call the family to arrange a mutually convenient time for a household visit. Upon arrival, a short disease history questionnaire will be administered including details of illness onset, symptoms, travel history, and illness of close contacts. Weight and height of the child, dehydration status and other clinical symptoms will be recorded in a short eCRF. All information will be recorded on a handheld tablet.

The study nurse will clinically evaluate the child and advise as to hospital attendance. Oral rehydration solution and zinc supplements will be provided to the family. Study nurses will be trained carefully to ensure that patients seek treatment when necessary. The nurse will

provide clinical advice only and will recommend whether the parent seeks standard of care at a local healthcare facility. This visit will take no more than one hour.

Household sample collection and transport

At the household visit, the study nurse will collect a stool sample from the child if a specimen is available at the time of visit. If no sample is available at the visit, the study nurse will give the family a sterile container and will arrange for collection and transport within 24 hours.

Outpatient visits & Hospitalisation

If a child experiences an episode of diarrhoeal disease that the parent feels is severe enough to seek medical care, we will request that the parents (1) alert one of our study nurses and (2) consider attending HTD for consultation. If the family attends HTD for diarrhoeal illness, they will be requested to call a study nurse or doctor prior to their visit to alert staff that they are coming. If the parent fails to call our study nurse the compensation fees may not be paid. If the family chooses to attend this hospital, outpatient department registration fees will be provided to the patient in addition to a travel reimbursement as described in section 13.3.3. Alternatively, families can choose to attend a private clinic or an emergency department of the hospital of their choice in which case compensation will not be given.

If a child in the *Shigella cohort* attends HTD with diarrhoeal illness, they will be directed to Paediatric Ward B for evaluation. Both outpatients and inpatients will be evaluated by a dedicated study doctor. If the child is admitted to the PICU at HTD for diarrhoeal illness, stool samples will be taken in PICU.

CRFs will be completed for all patients from the *Shigella cohort* attending HTD. Additionally, a stool sample will be collected from children as described in section 7.2. Bacterial culture and viral PCR results from the OUCRU microbiology laboratory will be reported back to the treating clinician promptly. Patients will not be responsible to pay for any laboratory analyses.

Sample collection & transport

Throughout the study, all specimens will be labelled with the child's study ID and not with the participant's name to ensure confidentiality. All samples will be collected and transported to the OUCRU Microbiology Laboratory for analysis on the same day.

Anal swab

In order to obtain faecal matter from a child with no available stool sample at a routine study visit, an anal swab will be collected by an experienced study nurse and placed in a

sterile container for transport to the Microbiology Laboratory at OUCRU within 24 hours of collection.

Stool samples

Stool samples will be collected from a diaper and placed in a sterile container by a study nurse. Samples must be transported within 24 hours of collection to the Microbiology Laboratory at OUCRU.

Blood samples

Venous blood samples from the antecubital fossa area of the arm will be collected by an experienced nurse in an EDTA tube and stored at room temperature. A total of 2ml will be collected from children aged 12-35 months, and 3ml for children ≥ 36 months of age. The difference in volume of blood collected by age is due to precedents set by other research studies conducted through OUCRU. We appreciate that collecting blood samples from very young children is stressful for both the child and the parents. Therefore, collecting less blood from smaller children is an attempt to reduce any potential discomfort for those involved. Blood must be kept at room temperature and transported to the Microbiology Laboratory at OUCRU within 24 hours of collection.

The maximum volume of blood drawn at each timepoint will be 2ml for children aged 12-35 months and 3ml for children ≥ 36 months. If a child is enrolled in 07DXA and the *Shigella* cohort simultaneously, only one blood sample will be requested during routine follow up.

Nasopharyngeal swabs

A nasopharyngeal swab will be collected by a study nurse and placed in bacterial transport medium and stored at 4°C. Two nasopharyngeal swabs will be collected for each patient (one from each nostril). Samples must be transported within 24 hours of collection to the Microbiology Laboratory at OUCRU.

A maximum of two nasopharyngeal swabs will be collected at the routine follow up appointments, even if the child is enrolled in both 07DXA and the *Shigella* cohort.

Data management and completeness

Data will be collected electronically on laptops at HVH-WBC and on tablets provided to the study nurses during home visits. Laptops or paper CRFs will be used during diarrhoeal illness presentations at the HTD study clinic. Electronic data entry devices will be password protected and accessible only by authorised users. A central database will be developed in collaboration with IT services at OUCRU to ensure secure and confidential data management. Any information collected on the tablets will be sent immediately to the

OUCRU server through a 3G internet connection. No information will be retained on the tablet once it is sent to the server.

Laboratory data will be recorded on the standard microbiology forms and subsequently entered by a dedicated member of the OUCRU Microbiology staff into the database. This information will be integrated into the appropriate patient record through the use of a unique identifying code per patient.

In order to minimise bias due to loss of follow up, study nurses will contact mothers who fail to attend their routinely scheduled appointments by telephone to reschedule and enquire reasons for non-attendance. We will aim to record reasons for loss to follow up in all cases.

Study duration and number of participants

This study is projected to last for approximately 3 years beginning in the first half of 2014. We expect to enrol approximately 250 - 300 children who were born between August 2011 – December 2012 and 400 children born in 2013 with follow up at approximately 18-36-42-60 months and 12-36 months through January 2017 for each group, respectively.

Clinical and laboratory research investigations

Microscopy, stool culturing and bacterial identification

Parasite screening

For parasitic examination, a fresh smear of faecal specimen will be prepared in phosphate buffered saline; 10µl of this solution will be examined at 400 times magnification to identify *Giardia lamblia* and *Entamoeba histolytica* at the OUCRU Microbiology Laboratory. *Cryptosporidium* cysts will be detected by ZN stain.

Blood cells screening

A fresh faecal smear of each sample will be screened under microscope at the OUCRU microbiology laboratory (HPF ($\times 400$)) for the presence of white blood cells and/or red blood cells and will be scored on scale from zero to four, scale 0 = 0 cells/HPF, scale 1 = 1 to 10 cells/HPF, scale 2 = 11 to 20 cells/HPF, scale 3 = 21 to 30 cells/HPF and scale 4 = >30 cells/HPF.

Bacterial culture

Culturing and bacterial identification will be performed at OUCRU Microbiology Laboratory. Stool samples and anal swabs will be cultured on MacConkey Agar (MC), Xylose-Lysine-Deoxycholate Agar (XLD), Selenite broth, and Campylobacter media. The purpose of these plates is to identify and isolate *Shigella spp*, *Salmonella spp*, *Campylobacter spp*, and *Escherichia coli*. The MC, XLD, and Selenite broth will be

incubated at 37°C overnight and the *Campylobacter* plates will be incubated micro-aerophically at 42°C. Identification of potential pathogens will be performed using conventional biochemical (API E20 tests) and serological testing, as per the standard operating procedure of the microbiology department of OUCRU. All isolated pathogens will be stored for future testing and the isolated organism will be recorded in the study database.

To identify pathogenic *E. coli* variants (ETEC, EPEC, EHEC and EIEC) sweeps of Gram-negative colonies indicative of *E. coli* will be collected from MacConkey Agar plates and stored in 20% glycerol at -80°C at OUCRU. Samples will be subjected to PCR to identify pathogenic *E. coli* variants after DNA extraction.

Antimicrobial resistance profiling

Antimicrobial susceptibility testing of the pathogens isolated will be performed by the disk diffusion method using guidelines established by the Clinical and Laboratory Standards Institute (CLSI) and, when required, by minimum inhibitory concentrations (MICs) by Etest. Antimicrobial susceptibilities tests will be performed against Nalidixic acid, Ciprofloxacin, Ceftriaxone, Ceftazidime, Augmentin, Ampicillin, Trimethoprim-sulfamethoxazole, Azithromycin, Chloramphenicol, Imepenem and Amikacin.

The production of extended-spectrum β -lactamases (ESBL) will be investigated using the double-disc synergy test by comparing zone sizes between ceftazidime discs against ceftazidime-clavulanic acid discs and cefotaxime discs against cefotaxime-clavulanic acid discs. Isolates with an increase in diameter of inhibitory zone of equal to or more than 5 mm by the synergy of clavulanate will be considered ESBL positive.

Isolation of Nucleic Acid & PCR

Extraction

Total nucleic (RNA and DNA) will be extracted from 10% (in phosphate-buffered saline [PBS]) faecal specimens using the QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations or using the Roche MagNA pure 96 automated nucleic acid extraction machine (Roche) at OUCRU. RNA will be converted to complementary DNA (cDNA) by reverse transcription (RT), and an aliquot of RNA of each sample will be stored at -80°C until required. For RT, extracted RNA will be 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 will be stored at -80°C.

Bacterial PCR

To potentially increase the diagnosis rate of *Shigella*, *Salmonella* and *Campylobacter* a multiplex realtime PCR will be performed on extracted nucleic acid to detect these pathogens. These methods will be performed following an OUCRU SOP.

Viral PCR

Extracted RNA (cDNA) will be used as template in a multiplex realtime PCR to detect rotavirus and norovirus, these methods will be performed following an OUCRU SOP ²⁹⁹.

Blood separation and sample storage

Plasma for serology

Blood from the EDTA tubes will be separated into blood cells and plasma by centrifugation for 10 minutes at 1,500 RCF. The plasma will be removed and aliquoted carefully (so not to disturb the red cells or the buffy coat) into a screw top cryovial tubes for storage at -80°C or -20°C until required for serology.

Cells for future host genetics studies

The cells (red cells and buffy coat) will be aspirated into screw top cryovial tubes for storage at -80°C until required for host genetics or other analyses.

Stool samples, anal swabs and nasopharyngeal swabs

Aliquots of stool specimens (2 per subject) will be placed in 2ml cryovials and stored at -80°C at OUCRU until required for further analysis. All anal and nasopharyngeal swabs will be also stored at -80°C at OUCRU until further analysis (i.e. at the end of the follow-up period of the investigation).

Bacterial isolates

All isolates will be stored at -80°C (and freeze dried) at OUCRU for future studies.

Nucleic acid from stool samples (-80°C)

Nucleic acid extracted from all stool samples and bacterial isolates will be stored at -80°C OUCRU for future studies.

Sample size

The median age of hospitalised *S. sonnei* infections in HCMC is 24 months ⁴⁵. Additionally, the annual incidence of diarrhoea in Vietnam in children under 5 years of age is 1.5 events/child/year ⁴⁰. With our estimated 650-700 enrollees from the previous 07DX cohort, this would result in an estimated 2,025 diarrhoeal episodes over 2 years of follow up. From preliminary investigations on stool samples acquired through passive diarrhoeal

reporting in this cohort in infants ≤ 12 months, 7.5% (92/1,229) were PCR-positive for *Shigella*. This estimate is consistent with previously collected data¹⁹⁰. Therefore, we conservatively estimate that we will collect approximately 152 *S. sonnei* episodes over the study period. We believe this estimate to be conservative due to the fact that *Shigella* infections do not always present with dysentery and therefore are likely to go undetected in previous estimates of incidence which screened dysenteric cases only³⁰⁰. Secondly, this estimate is based on passively collected prevalence estimates from isolates in infants ≤ 12 months. As the average age of hospitalised *Shigella* infection is in children 2-3 years of age, we believe we will in fact collect more than the predicted 152 infections as children in the *Shigella* cohort will be ≥ 12 months⁴⁰.

Known risk factors for *Shigella* infection due to direct transmission include the presence of multiple children in a household as well as attendance at daycare centres^{301,302}. Identifying common routes of transmission is an important aspect of this study. From baseline data of the existing 07DX Birth Cohort, we know that 70% of children have siblings and 10% regularly attend daycare. As such, this study will have 80% power to detect a relative risk ≥ 2.5 for *Shigella*-associated diarrhoeal disease in children with siblings compared to children without and in children attending daycare compared to those who do not (type 1 error probability = 5%).

Data analysis

Diarrhoea and pathogen-specific incidence rates will be calculated by dividing the number of relevant episodes by the number of child-months at risk. This will be stratified by year of age.

Descriptive analyses will be performed to explore the characteristics of children with *Shigella* infection as well as infection due to other diarrhoeal pathogens. Variation in demographic, socioeconomic and exposure variables between *Shigella* cases and non-cases will be evaluated through parametric and non-parametric tests including the chi-square or Fisher's exact test for categorical variables and through Student's t-test or Kruskal-Wallis tests for continuous data, respectively. Simple Spearman or Pearson correlations will be generated to assess associations between continuous variables such as rainfall and proportion of reported diarrhoea due to *Shigella* by month.

Multivariate logistic regression methods will be performed to assess which variables of interest are independently associated with the outcome of *Shigella* infection. Covariates included in the multivariate model will include, *a priori*, age, sex, household income, breastfeeding and household crowding status as well as any covariates deemed to be strongly associated with the outcome of *Shigella* infection in the univariate analyses.

Spatiotemporal analysis will use the address information of each family's household. Clustering of infections in time and space will be investigated through the spatial scan

statistic and the local Moran's I statistic using SaTScan and GeoDa software, respectively. For patient confidentiality purposes, resultant analyses will report spatial data at an aggregated scale (ie infections per ward). If point (latitude/longitude) data are reported, exact household locations will be jittered through visualisation software so as to not precisely locate each house on a map.

Serology

There is a correlation between pre-existing IgG to O-antigen and natural immunity against *S. sonnei* infections. Furthermore, a rise in circulatory IgA to O-antigen has been shown to indicate recent *Shigella* infection and elevated IgG and low IgA to O-antigen is a marker of past exposure. These methods are robust, exhibit limited cross-reactivity and are able to identify recent and distant infections/exposures. Therefore, ELISAs will be used to measure IgA, IgM and IgG subtypes to *S. sonnei* O-antigen in all plasma samples using defined cut-offs from culture positive *Shigella* cases. Furthermore, these plasma samples will be used to screen across a panel of novel *Shigella* antigens to identify immunogenic proteins that may be used in future vaccine candidates (future work). To interrogate the diversity, specificity and longevity of the humoral immune response to pan-*Shigella* antigens, an antigen-array will be constructed in collaboration with the Wellcome Trust Sanger Institute. This method will be similar to those used to study the immune response, define diagnostic antigens and identify potential vaccine candidates for several bacterial pathogens.

Host genetic studies

Genetic Association Studies

The relationship between genetic variation and disease susceptibility can be determined using genetic association studies. The most commonly used method is a population based case/control association study. Association of a gene with disease is when the disease causative allele, or genotype, occurs at a significantly different frequency in affected individuals than in unaffected individuals. Identification of *Shigella* susceptibility genes can be approached either by selecting candidate genes or by scanning the whole genome. Proposed genetic studies will be genetic association studies.

Candidate gene studies

Candidate gene or gene regions are selected based on prior knowledge. There must be sufficient experimental evidence to support the investigation of a particular gene in a given disease. The selection is therefore reliant on prior knowledge of the biology and pathogenesis of the disease, or related diseases, in the human or animal host. Therefore the

list of potential candidate genes can include both general i.e. immune response genes, and disease specific genes i.e. a specific receptor molecule.

Once the candidate genes have been selected, then variants (polymorphisms) are identified within and near to the candidate gene. Our genetic association studies focus on genotyping single nucleotide polymorphism (SNPs) which are abundant throughout the human genome and amenable to high throughput and large scale genotyping. A SNP is defined as a position in the genome where two alternative bases (eg. A to T) occur in the human population at a frequency $>1\%$. Candidate gene studies may require genotyping a small number (10-100) of SNPs in a moderately sized case and control population (500-1000 cases and equal numbers of controls), based on the allele frequency of the SNP, the size of the effect (odds ratio) and the level of significance (p value).

Genome wide association studies

With recent technological advances we are now able to determine whether genetic variation across the complete genome affects an individual's ability to fight infection. The whole genome approach does not specifically target particular genes but is a random approach that can potentially screen all human genes. It can be used to identify new or previously unknown genes that may be important for disease protection. A very large number of single nucleotide polymorphisms (SNPs) which are spread along every chromosome of the genome are genotyped. SNPs are the most common type of human genetic variation and they occur ~ 1 per 1000bp of DNA. SNPs serve as genetic markers for identifying disease genes and can lie within or between genes. The future aim is to genotype SNPs that are markers for every human gene. Currently commercial genotyping arrays are available that allow genotyping of 5 million SNPs. Our genome wide studies require genotyping up to 5 million SNPs in large sized case and control populations (>2000 cases and >2000 controls). This approach should lead to the identification of disease protective genes that classical biological and immunological methods have been unable to detect. We propose to perform both candidate gene and genome wide studies on our dysentery case and control cohort.

Gene region re-sequencing

Following candidate gene or genome wide case control association studies it may be necessary to perform gene region re-sequencing. SNPs associated with a disease in a candidate gene or genome wide study may not necessarily be the gene variant that is responsible for the disease susceptibility or protection, and may just be a marker of the true disease causative mutation which is modifying gene function or expression level. As not all gene variants are covered by commercial genotyping arrays it may be necessary to re-sequence associated genes/gene regions to identify the disease causative mutation. Such investigations could typically include 100s of case and/or control samples. Once we have

performed genome wide association study (GWAS) it may be necessary to perform re-sequencing to pinpoint the disease causative mutation.

Control populations for host genetics

The controls used in our host genetics studies are population controls. The population controls we currently have available have been collected as part of larger birth cohorts. Control DNA is extracted from the umbilical cord blood of newborn babies. This provides a population control group with allele frequencies representative of the general Vietnamese Kinh population. The collections of these control populations and their use in genetics studies has undergone separate IRB review and they are approved for use in genetics studies.

Inclusion criteria for control populations

Informed consent to collect, store and analyze samples for the purpose of genetic testing

Participant in one of the following approved studies; AN, FC, 07DX in Vietnam

Exclusion criteria for control populations

Denial of consent to perform genetic testing on stored samples

Ethical considerations

The protocol will be submitted for review to the ethical committees of: (i) Hung Vuong Hospital, HCMC, (ii) Hospital for Tropical Disease, HCMC and (iii) the Oxford University Tropical Ethics Committee (OXTREC).

Informed consent

Parents/guardians of children enrolled in the 07DXA Birth Cohort Extension will be invited to consider joining the *Shigella cohort* at their first visit of 2014. Parents/guardians will be asked to consider participation in the *Shigella cohort* separately from their current participation in the original 07DX Birth Cohort so that they do not feel pressured to join the longer *Shigella cohort*. If an interest in joining is demonstrated, parents/guardians will be provided with further information and consent procedures. They will then be enrolled in both 07DXA Birth Cohort Extension and 14EN *Shigella cohort*. The *Shigella cohort* will require a separate informed consent form.

Parents/guardians of children enrolled in the original 07DX Birth Cohort in 2013 will be invited to enrol in the *Shigella cohort* at the scheduled 07DX 12 month follow-up visit in 2014, at which point they would be exiting the 07DX study. This set of children will only be enrolled in the 14EN *Shigella cohort*.

In all cases, a dedicated and trained group of research nurses will be responsible for describing and discussing the aims, methods, possible risks and benefits of the study to eligible parents/guardians. Potential participants will be provided with an information sheet to read and keep and have the opportunity to ask questions as needed. Written informed consent from the parent/guardian and on behalf of their infant may be obtained during the first interaction with the research nurses or up until 2 days after the initial discussion of enrolment.

Household visits and messaging

Specially trained study nurses whose employment is dedicated to the study and who therefore have opportunity to develop relationships with the children and their families will conduct the household visits. All household visits will be arranged in advance with the agreement of the child's parent/guardian. Visits will take a maximum of 1 hour and study nurses will make efforts to minimise the inconvenience to the family and ensure that visits take place at times and in a fashion to protect the family's privacy.

Benefits and risks

Benefits

The potential benefits to children and families include:

When an infant/child suffers diarrhoeal illness, the family will have access to prompt medical attention either in the home or in the Hospital of Tropical Diseases, delivered by highly experienced medical professionals

Free diagnostic testing for an array of diarrhoeal pathogens

Free registration fee at the HTD at each diarrheal episode

Free health consultations every 6 months for the enrolled child with provision of nutritional and general health recommendations

Risks

The potential risks to children and families include:

Venupuncture can be painful for the child and may leave a small bruise. There is a very small risk of infection. Venupuncture may also be upsetting for the mother.

There is a risk of loss of confidentiality and inconvenience during home study visits.

Participants already enrolled to the original 07DX Birth Cohort, and recruited to this *Shigella* cohort may feel obligated to participate or burdened by too many research studies.

Financial Compensation

The cost of research laboratory tests if children are admitted to HTD, including bacterial culture of stool and full blood count analysis will be funded by the study. Travel reimbursement for routine follow-up appointments at HVH and outpatient visits at HTD will be funded at approximately USD7.5 or more for a return trip, dependent on distance travelled according to standard OUCRU policy. The cost of the standard registration and booklet fee in the outpatient department will also be paid for when the cohort member attends HTD for diarrhoeal disease.

Study withdrawal

Parents/guardians are free to withdraw their consent for their child at any time and request that study samples not be stored for further testing.

Patient confidentiality

Parents/guardians will be assured that all information generated in this study will remain confidential. The names and addresses of study participants will be recorded in the database but kept separate from the main body of data and access will be strictly controlled through password protection. Any scientific publications or reports will not identify any child by name or initials. When the research team reviews their notes, they are also bound by professional confidentiality. All genetic data resulting from this study will be de-identified using current best practices.

Financing and Insurance/Indemnity

This study will be funded by Oxford University. Indemnity insurance will be provided by Oxford University.

Publications

Data from this study will be published in peer reviewed journals. Authorship and reporting of this work will follow international guidelines. No aspect of this study will be reported without approval from all collaborating parties.

Table 2. Sample types and timepoints for collection throughout the cohort study

Sample	Enrolment	Routine visit	Diarrhoea : home visit	Diarrhoea : HTD visit
Blood*	X	X		
Stool or anal swab	X	X	X	X
Nasopharyngeal swab	X	X		

*a total of 2ml from children <36 months and 3ml for children $\geq$ 36 months

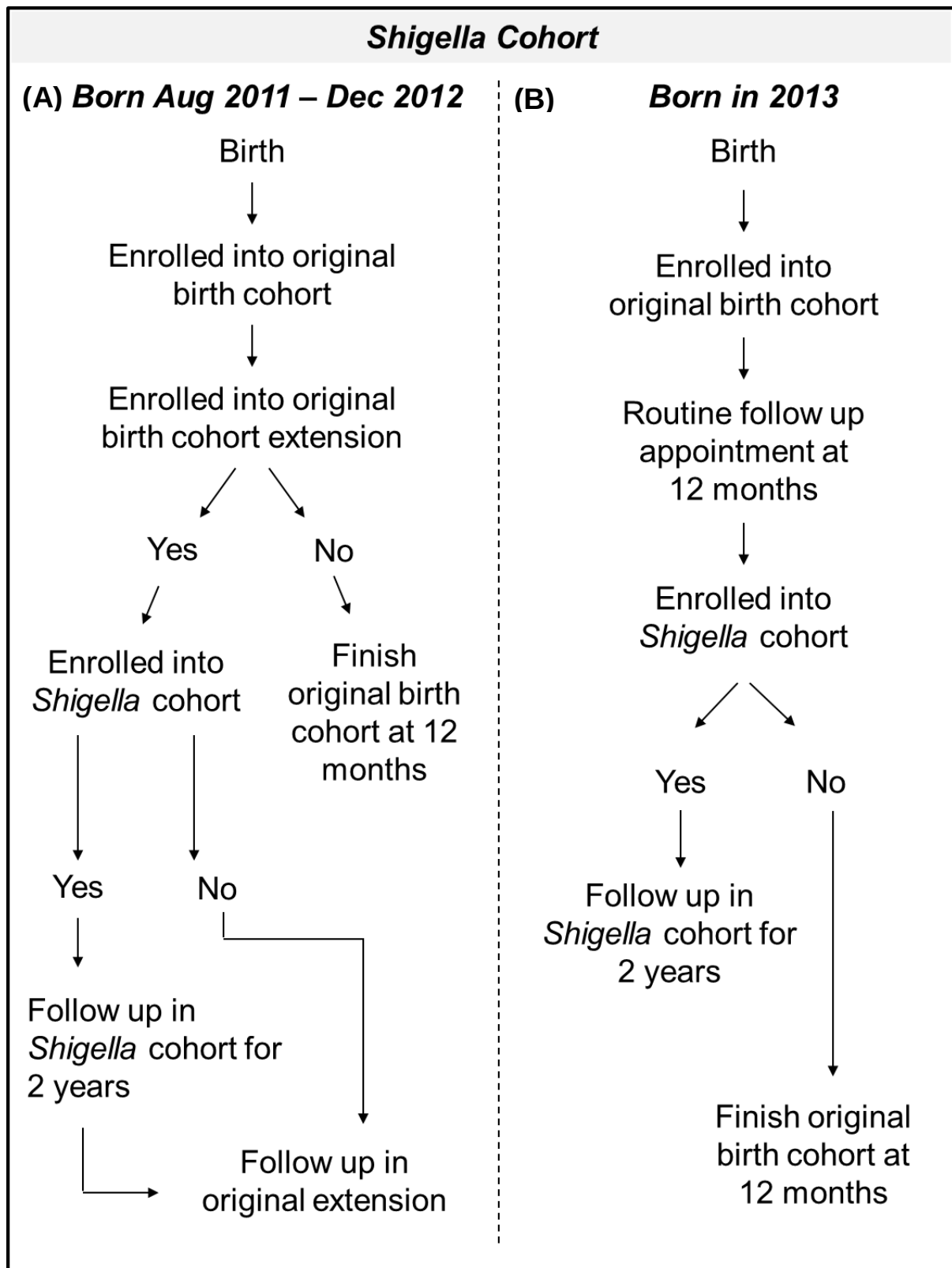


Figure 1. A schematic showing the two sets of children and respective study enrolment options. (A) Children born and enrolled into the 07DX Birth Cohort during 2012 who are

enrolled in the 07DXA extension will be invited to enrol into the *Shigella* cohort (B) Children born and enrolled in the 07DX Birth Cohort during 2013 will be asked to join the diarrhoeal extension at or soon after their final 12 month appointment of the 07DX study.

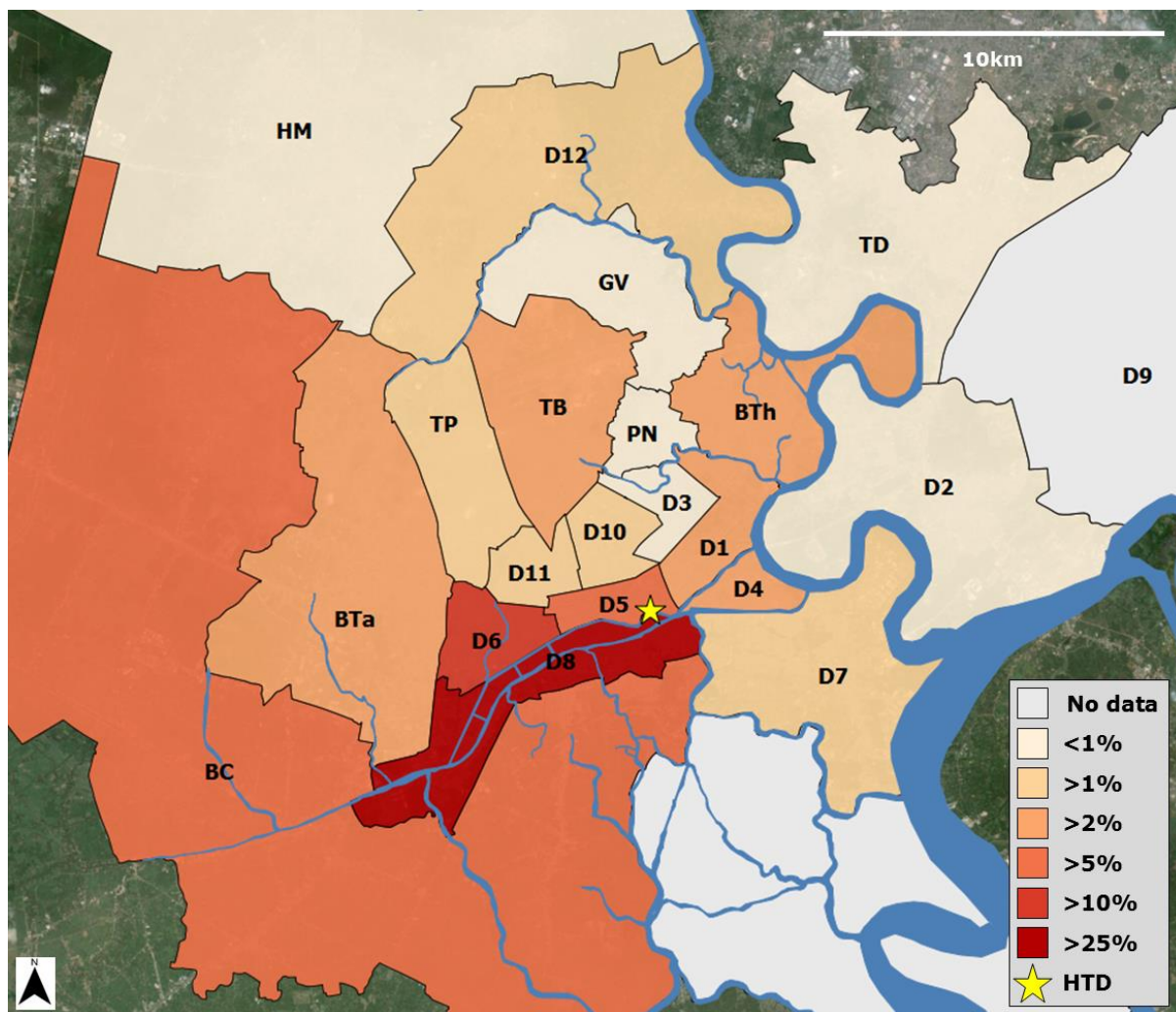


Figure 2. The distribution of the residences of cases of childhood shigellosis admitted to the Hospital for Tropical Diseases in Ho Chi Minh City. Over periods 1995-1998, 2000-2002 and 2007 – 2008, we were able to positively identify infecting *Shigella* serotypes in stools of 297 children with symptoms consistent with shigellosis. Of these patients, 228 (76.8%) children lived in the 24 districts that constitute Ho Chi Minh City. This figure represents the distribution of the homes of patients reporting to HTD with *Shigella* isolated from stool, by district. The percentage of cases reporting from each district is distinguished by gradual shading. The location of HTD is shown by a yellow star. Large waterways are shown in blue. Reproduced from ⁴⁵.

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9.5 Publications during DPhil candidature

Quantifying antimicrobial access and usage for paediatric diarrhoeal disease in an urban community setting in Asia

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Objectives: Antimicrobial-resistant infections are a major global health issue. Ease of antimicrobial access in developing countries is proposed to be a key driver of the antimicrobial resistance (AMR) epidemic despite a lack of community antimicrobial usage data.

Methods: Using a mixed-methods approach (geospatial mapping, simulated clients, healthcare utilization, longitudinal cohort) we assessed antimicrobial access in the community and quantified antimicrobial usage for childhood diarrhoea in an urban Vietnamese setting.

Results: The study area had a pharmacy density of 15.7 pharmacies/km² (a pharmacy for every 1316 people). Using a simulated client method at pharmacies within the area, we found that 8% (3/37) and 22% (8/37) of outlets sold antimicrobials for paediatric watery and mucoid diarrhoea, respectively. However, despite ease of pharmacy access, the majority of caregivers would choose to take their child to a healthcare facility, with 81% (319/396) and 88% (347/396) of responders selecting a specialized hospital as one of their top three preferences when seeking treatment for watery and mucoid diarrhoea, respectively. We calculated that at least 19% (2688/14427) of diarrhoea episodes in those aged 1 to <5 years would receive an antimicrobial annually; however, antimicrobial usage was almost 10 times greater in hospitals than in the community.

Conclusions: Our data question the impact of community antimicrobial usage on AMR and highlight the need for better education and guidelines for all professionals with the authority to prescribe antimicrobials.

Introduction

Antimicrobial resistance (AMR) is rapidly becoming a global public health issue.¹ In the past decade, many of the key antimicrobials on which we have come to rely in human medicine are in the process of losing, or have already lost, their effectiveness in treating infections caused by bacterial pathogens.^{2,3} AMR is a complex scientific, political, economic and social issue, and although developed countries are leading research efforts to tackle the problems, the greatest impact of AMR is currently being felt in low- and middle-income countries (LMICs).^{4–6} AMR is magnified in LMICs as antimicrobial access and usage, poor sanitation and limited access

to clean water are amongst the principal drivers. Therefore, unrestricted access to low-cost antimicrobials combined with a high burden of infectious disease, limited diagnostic capacity and large urbanizing populations demanding better medical care has created a favourable environment for propagating antimicrobial-resistant bacteria in LMICs.^{7,8}

Antimicrobials are frequently used indiscriminately to treat common human diseases, of which diarrhoea is a major contributor. It has been estimated that the worldwide burden of diarrhoea was >1.7 billion episodes in 2010, with the vast majority of these episodes arising in children in LMICs.⁹ The aetiological agents of diarrhoea are broad and can induce an array of symptoms and

severity, including watery, mucoid and bloody diarrhoea. Currently, the WHO recommends oral rehydration solution (ORS) and zinc for all diarrhoea, and antimicrobial treatment for those with more severe symptoms.^{10,11} A large disease burden, combined with a lack of financial and diagnostic resources in LMICs, means that the causative agent is rarely or never identified.¹² In Asia, this lack of a confirmative diagnosis results in the empirical use of antimicrobials in the community or a healthcare facility. However, many of those receiving antimicrobials for diarrhoea may not require them,¹³ as the disease is generally self-limiting and frequently of viral aetiology,¹² and AMR in diarrhoeagenic bacteria is now highly prevalent in LMICs in Asia.^{3,13}

Despite the perceived haphazard use of antimicrobials in LMICs, antimicrobial access and usage for the treatment of common infections in vulnerable populations has seldom been accurately estimated. Such data are vital for understanding how AMR may emerge, spread and be sustained, and are critical for targeting public health interventions to reduce antimicrobial usage. Vietnam is a rapidly developing LMIC in Southeast Asia with a population of >90 million people.¹⁴ An increasing population, a high infectious disease burden and a largely unregulated antimicrobial market combine to make Vietnam the ideal location for investigating antimicrobial access and usage. Diarrhoeal disease is a common childhood illness in Vietnam, a country with high recorded antimicrobial use in the healthcare system.^{13,15} By combining geospatial analysis, community and pharmacy surveys, and a longitudinal cohort study, we aimed to investigate antimicrobial access and usage for paediatric diarrhoeal disease in the community and the healthcare system in an urban setting in Vietnam.

Materials and methods

Ethics

Ethics approvals were provided by the Oxford University Tropical Research Ethics Committee (OxTREC approval 5110-16) and the institutional review board of the Ho Chi Minh City (HCMC) University of Medicine and Pharmacy (No. 220/DHYD-HD). Written informed consent was obtained from all participants (from parents and guardians if the participant was <18 years old).

Study setting and design

With an estimated population of >8 million people, HCMC is the largest city in Vietnam.¹⁶ HCMC is divided into five rural districts and 19 urban districts that cover an area of 19 km²; the urban districts had a mean population density of 13 394 people/km² in 2016.¹⁶ We selected District 8 (hereinafter referred to as the study area), with 16 wards and a population density of 22 522 people/km², as the location for sampling owing to its urban setting and central location (Figure S1, available as Supplementary data at JAC Online, and Table 1).¹⁶ To assess antimicrobial access and usage we triangulated data from a prospective paediatric cohort¹⁷ and conducted a cross-sectional study with several components: (i) geographical mapping of all pharmacies and healthcare facilities in the study area; (ii) a pharmacy practice survey to assess medication suggested by retail pharmacies for paediatric diarrhoea; and (iii) a self-reported caregiver community survey on the management and antimicrobial treatment of paediatric diarrhoea.

The coordinates of all pharmacies in the study area were recorded and entered into EpiCollect5.¹⁸ Vietnam administrative boundaries were downloaded from Global Administrative Divisions Map (https://gadm.org/download_country_v3.html). Vietnam population data rasters (population per 0.01 km²) were downloaded from WorldPop (http://www.worldpop.org.uk/data/WorldPop_data/AllContinents/130_metadata.html; 100 m resolution),

Table 1. Pharmacies and healthcare clinics in the various wards in the study area

Ward in District 8	Healthcare facilities	Pharmacies	Population ^a		Pharmacy density (per 10 000 people)	
			all	1 to <5 years	all	1 to <5 years
1	7	10	25 782	944	3.88	105.93
2	20	29	20 694	843	14.01	344.01
3	15	16	24 803	1139	6.45	140.47
4	51	36	42 200	2284	8.53	157.62
5	46	48	40 177	2538	11.95	189.13
6	15	34	30 144	1353	11.28	251.29
7	12	28	31 685	1414	8.84	198.02
8	4	5	9024	444	5.54	112.61
9	8	14	21 564	1194	6.49	117.25
10	15	14	15 841	836	8.84	167.46
11	7	5	7591	294	6.59	170.07
12	7	10	16 653	816	6.00	122.55
13	12	7	8809	480	7.95	145.83
14	4	6	20 575	1144	2.92	52.45
15	10	13	39 623	2354	3.28	55.23
16	6	26	41 010	1243	6.34	209.17
Total	239	301	396 175	19 320	7.59	142

^aData from HCMC Demographic Department, 30 June 2017.

and satellite imagery of Vietnam was downloaded from ESRI (<http://www.esri.com/en/index.html>) basemaps. All geospatial mapping was conducted using ArcGIS version 10.2 (Redlands, CA, USA). Euclidean distance to the nearest pharmacy and kernel density plots for pharmacy density (i.e. pharmacies per 0.01 km²) were estimated for the study area using the 'Euclidean distance' and 'Kernel' ArcGIS tools, respectively.

Pharmacy practice survey

We conducted a survey of pharmacy behaviour using an unbiased observational technique for assessing dispensed medication known as 'simulated client methodology'.^{19–22} Two 'mystery shoppers' with no healthcare training role-played as caregivers and approached pharmacies in a sub-region (Ward 5) of the study area to seek medical advice and treatment for two childhood diarrhoea scenarios. Scenario 1 (watery diarrhoea): a mother attending the pharmacies in the study area to buy medication for a child aged 2 years. The child has had 4–5 loose stool episodes within the previous 24 h. The child has no fever, and no blood or mucus in stools. Scenario 2 (mucoid diarrhoea with fever): a mother attending the pharmacies in the study area to buy medication for a child aged 2 years. The child has had 4–5 loose stool episodes within the previous 24 h. The child had a mild fever and mucus in the stool. Pharmacy-prescribed drugs were labelled anonymously, and then were classified by a clinician and confirmed by a qualified pharmacist (scripts in Table S1). In addition we aimed to investigate whether antimicrobials were being sold without a doctor's prescription by sending a mystery shopper to pharmacies in the study area to buy ciprofloxacin. This was a specific scenario in which the shopper requested this antimicrobial and did not provide any information about for what or whom the treatment was required.

Self-reported behaviour survey

We performed a community survey to capture the self-reported behaviour of parents and caregivers in managing diarrhoea in their children aged

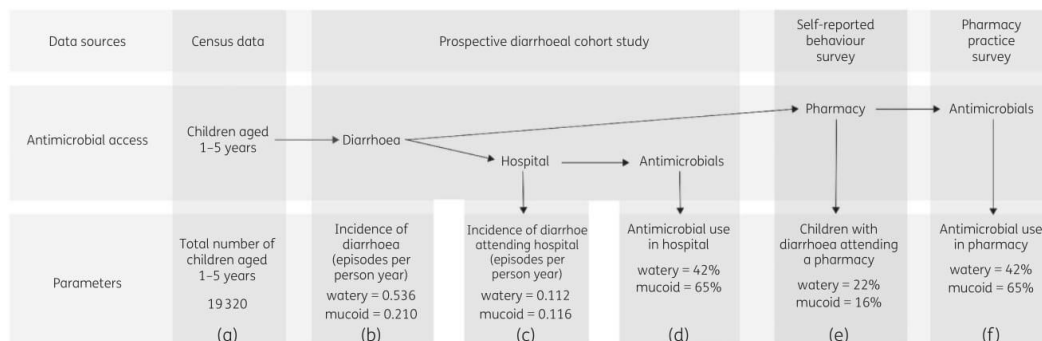


Figure 1. Estimating antimicrobial usage for diarrhoea in children aged 1–5 years in pharmacies and hospital. Diagram outlining the sources of data, parameters, and calculations used to quantify antimicrobial usage for diarrhoea in children aged 1–5 years in this population. Note each of the parameters have been given an initial (A–F), which informs the calculations in Table 3.

between 1 and 5 years. The surveyed population consisted of participants residing in the study area. We randomly sampled a representative population stratified by 16 wards and with registered children aged <5 years. Local health authorities accompanied and assisted in community visits. Mothers or caregivers were asked a short questionnaire regarding their choice of care if their child had diarrhoea in simulated scenarios similar to those described in the pharmacy behaviour study. We surveyed 396 parents or caregivers (Table S2). Results from these questionnaires are reported as means (interquartile range) and frequencies (%) for continuous and categorical variables respectively. For comparisons between groups, we used Fisher's exact test for categorical variables, and the Mann-Whitney U-test for continuous variables (significant $P \leq 0.05$).

Quantifying antimicrobial use for diarrhoea

We considered that antimicrobial usage for diarrhoeal disease was dependent on the number of diarrhoeal episodes for which medical treatment was sought, and how likely antimicrobials were to be prescribed for diarrhoeal treatment. Figure 1 outlines the parameters, the source of data and the calculations used to quantify antimicrobial usage for diarrhoea in children aged 1–5 years. The annual number of diarrhoeal episodes presenting at pharmacies was the product of (A) the total number of children aged 1–5 years in the study area, (B) the incidence rate of diarrhoea episodes per child-year, and (E) the proportion of parents or caregivers selecting to first take their child to a pharmacy. The annual number of diarrhoeal episodes presenting at hospitals was multiplied by (A) the total number of children aged 1–5 years living in the study area and (C) the incidence rate of diarrhoea episodes presenting at hospital per child-year. Parameter (A) was derived from recent census data from the HCMC Demographic department (30 June 2017). Incidence rates (B) and (C) were estimated from a prospective diarrhoeal cohort conducted between 2014 and 2016 that actively followed up 748 children residing in the study area.¹⁷ The proportion of parents or caregivers seeking pharmacies as their first choice (E) was derived from the self-reported behaviour survey of parents and caregivers. To quantify the likelihood of prescribing antimicrobials for diarrhoeal treatment at a hospital or a pharmacy, we used antimicrobial treatment data from (D) the prospective cohort study¹⁷ (from hospital files) and (F) the pharmacy behaviour study, respectively.

Results

The distribution of outlets selling antimicrobials

We mapped a total of 301 pharmacies and 243 private or public healthcare clinics in the study area (Table 1 and Figure 2a).

Figure S1 shows the geographical location of the study area. The distribution of pharmacies was not uniform and ranged from 0 to 61 pharmacies/km², with a mean density of 15.7 pharmacies/km². The majority of outlets were located on main thoroughfares in the north of the study area (Figure 2a). The population pharmacy density equated to 7.59 pharmacies/10000 people, corresponding to a pharmacy for every 1316 people. The number of pharmacies within the population varied from 0 to 38.2 pharmacies/10000 people, with four high-density areas (>30 outlets/10000 people) (Figure 2b). The high density of pharmacy shops epitomized routine access to antimicrobials in this community and equated to >75% of the study area population living within 250 m of an outlet selling antimicrobials (Figure 2c).

Pharmacy prescribing practices for diarrhoea

We surveyed 48 pharmacy shops (37 were open when visited) in a ward within the study area (Figure S1), and used two different scenarios for paediatric diarrhoea. In the first scenario (watery diarrhoea), the number of prescribed medications ranged from one to five, with the majority (65%; 24/37) of pharmacies prescribing two different medications (Figure 3a). The median total cost for each pharmacy visit was 17000 Vietnamese dong (VND) (US \$1 ≈ 23000 VND); range 7000–54000 VND. A total of 46 different types and brands of medications were sold, which could be broadly categorized into 11 classes: anti-motility, zinc, anti-emetics, anti-secretory, probiotics, ORS, antimicrobial, antipyretic, adsorbent, unlabelled and others. Unlabelled treatments were prescribed in 16% (6/37) of pharmacy visits, with probiotics (81%; 30/37) and adsorbents (68%; 25/37) being the most commonly sold treatments. Antimicrobials were prescribed by 3/37 (8%) pharmacies for watery diarrhoea (Figure 3b).

In the second scenario (mucoid diarrhoea with fever), 53 different medications were sold by the 37 pharmacies; undefined/unlabelled medications were sold in 49% (18/37) of pharmacy visits (Figure 3b). Probiotics (81%; 30/37) and adsorbents (51%; 19/37) remained the most common medications prescribed, but the proportion of pharmacies prescribing an antimicrobial increased to 22% (8/37). There was also an increase in the number of different medications sold for mucoid diarrhoea in comparison with those

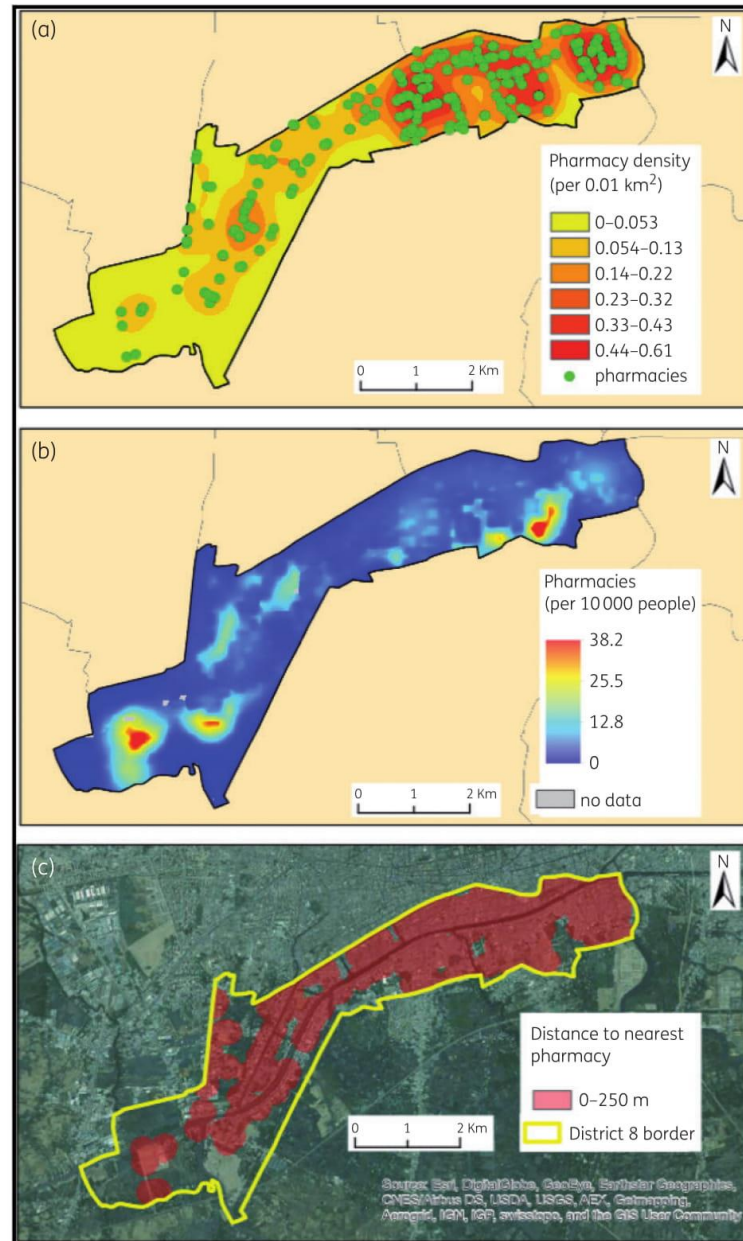


Figure 2. The geographical distribution of pharmacies in the Ho Chi Minh City study area. Maps of district 8 in Ho Chi Minh City. (a) The point locations of pharmacies and the kernel distribution of pharmacy shop density. Green points identify the pharmacy shops and pharmacy density is determined by colour intensity (see key). (b) The distribution of pharmacy shops density per 10 000 inhabitants. The greater the intensity of red the higher the number of pharmacy shops per 10 000 people (see key, areas in grey are those with no data owing to missing data in the population raster). (c) The geographical areas in District 8 that are within 250 m of a pharmacy. Red shaded areas are locations in which residents live with 250 m of a location selling antimicrobials.

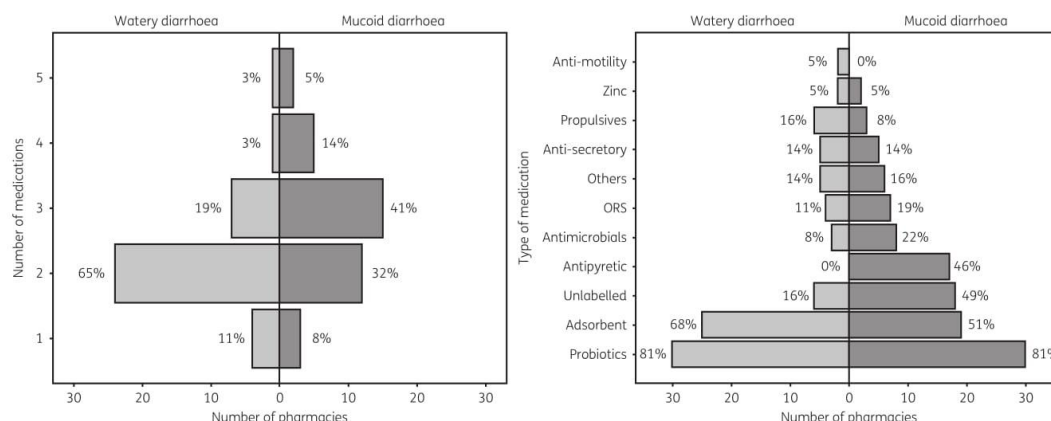


Figure 3. Medications sold by pharmacies in the study area for paediatric diarrhoea. (a) Rotated histogram showing the number and proportion (x-axis) of the 37 pharmacies visited during the 'mystery shopper' simulated client method selling between 1 and 5 medications (y-axis) for watery and mucoid diarrhoea, respectively. (b) Rotated histogram showing the number and proportion (x-axis) of the 37 pharmacies visited during the 'mystery shopper' simulated client method selling different medications (y-axis) for watery and mucoid diarrhoea, respectively.

sold to treat watery diarrhoea (Figure 3a), with a higher proportion of pharmacies selling a combination of three (41%; 15/37), four (14%; 5/37) or five (5%; 2/37) medications. Despite there being a greater number of medications sold, the cost to caregivers in this scenario was not substantially greater than with watery diarrhoea (median 18000 VND; range 3000–31 000 VND).

There was considerable disparity in the type of antimicrobials sold in the 37 surveyed pharmacies, with pharmacies prescribing trimethoprim sulphate, sulphonamides, fluoroquinolones, macrolides and cephalosporins. When the mystery shopper returned to the same pharmacies a week later and asked to buy ciprofloxacin, 100% (37/37) of the vendors sold the requested antimicrobial. This medication was consistently sold without dosing advice and without the caregiver having to provide information as to why the antimicrobial was required. Generic ciprofloxacin was sold in a range of different packaging from 14 different manufacturers in two different concentrations.

Antimicrobial usage surveys in caregivers

To evaluate how local residents in the study area access antimicrobials and to understand their knowledge, attitudes and practices regarding antimicrobial usage, we surveyed 396 individuals (Table S2). The majority (82%; 325/396) of the interviewees were women and the median age was 39 years (range 19–87 years). Ten percent of participants (39/381) indicated some background in healthcare. The majority (69%; 243/351) had an average household income ($\geq 1\,300\,000$ and $\leq 9\,000\,000$ VND/household/month), with 21% (74/351) reporting being in a poor financial situation ($< 1\,300\,000$ VND/household/month). Most caregivers/parents (60%; 232/386) had ≥ 2 children living in the household. Many (59%; 231/394) reported that their children had received medication for a medical condition within the last month, of which approximately half (47%; 109/231) were reported to be an antimicrobial.

We found that a majority of interviewed parents and caregivers had limited knowledge about antimicrobials. Many assumed that antimicrobials could be generically used to treat coughing (46%; 181/396), fever (35%; 137/396), colds (30%; 118/396), headaches (21%; 81/396) and diarrhoea (19%; 76/396). Approximately half (51%; 203/396) of the parents and caregivers agreed with the statement 'some infections can be difficult to treat if there is antimicrobial resistance', and 59% (233/396) agreed with the statement 'resistance can occur if you do not take a sufficient dose'. In self-reported prescribing, more than half of parents and caregivers (54%; 213/394) said they had at some time purchased antimicrobials from pharmacies, with many of them (85%; 182/213) buying antimicrobials within the 30 days prior to the interview (Table S3).

Diarrhoeal treatment-seeking behaviour

Figure 4 shows the top three preferences for places where parents and caregivers would seek healthcare advice if their children had either of the two previously described diarrhoeal disease scenarios. In both scenarios, the most common first choice was to treat their children at home. However, a specialized hospital was the most common option, with 81% (319/396) and 88% (347/396) of responders selecting this as one of their top three preferences for treatment of watery and mucoid diarrhoea, respectively. Visiting a local pharmacy as a first choice ranked third for watery diarrhoea and second for mucoid diarrhoea. Overall, visiting a pharmacy was selected as one of the top three preferences by 45% (180/396) and 34% (134/396) of caregivers for watery and mucoid diarrhoea, respectively (Figure 4). We observed that parents (rather than a caregivers) and those of younger age were more likely to take their children to a pharmacy than a hospital for watery diarrhoea ($P = 0.015$ and $P = 0.051$, respectively). Similarly, parents (rather than caregivers) were significantly more likely to take their children to a pharmacy than a hospital for mucoid diarrhoea (Table 2).

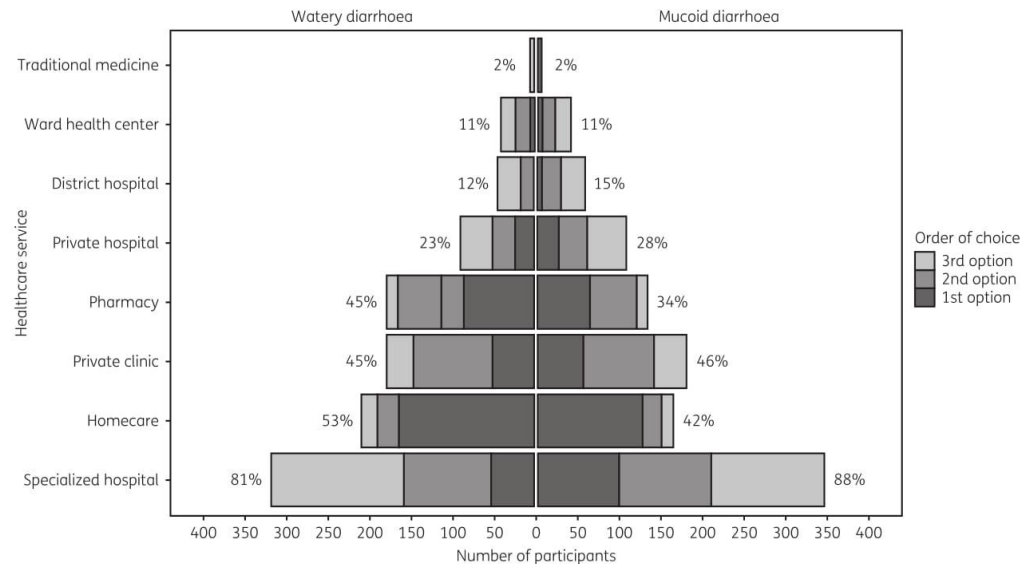


Figure 4. Healthcare utilization for paediatric diarrhoea by caregivers. Pyramid plot showing the number and proportions of 396 individual caregivers or parents of children aged <5 years residing in the study area (x-axis) selecting the various healthcare services (y-axis) as first, second or third choices (see key for shading) for watery and mucoid diarrhoea.

Quantifying antimicrobial use for diarrhoea

We lastly aimed to calculate antimicrobial usage in the community and in hospitals for diarrhoeal disease in children aged between 1 and 5 years (Figure 1). From following 748 children and quantifying episodes of diarrhoea over a 2 year period in the diarrhoea cohort, we estimated the total annual incidence of watery and mucoid diarrhoea to be 536 (95% CI 463–609) and 210 (95% CI 154–267) per 1000 child-years, respectively (Table 3). The annual incidences of watery and mucoid diarrhoea resulting in hospital attendance were 112 (95% CI 83–141) and 116 (95% CI 83–149) per 1000 child-years, respectively. Among the diarrhoeal episodes resulting in hospital attendance, 66/159 (42%) watery episodes and 107/164 (65%) mucoid episodes received antimicrobial treatment in hospital. We therefore estimated the total number of diarrhoeal episodes within the study area in this age group to be 10355 (95% CI 8945–11765) and 4057 (95% CI 2975–5158) per year for watery and mucoid diarrhoea, respectively.

Combining data from the surveys of the self-reported behaviour and the pharmacy practice, we estimated that the annual number of diarrhoeal episodes in children aged 1–5 years that would receive antimicrobial treatment at pharmacies were 184 (95% CI 159–209) for watery diarrhoea and 144 (95% CI 105–183) for mucoid diarrhoea (Table 3). However, data from the diarrhoea cohort indicated that the number of paediatric diarrhoeal cases that resulted in an antimicrobial administration in a hospital was approximately 5 [898 (95% CI 673–1130)] and 10 times higher [1462 (95% CI 1046–1878)] for watery and mucoid diarrhoea, respectively (Table 3). In total, we estimated that at least 19% (2688/

14 427) of diarrhoeal episodes in children aged 1–5 years in the study area would receive an antimicrobial annually.

Discussion

AMR infections are increasingly common throughout Asia, a trend that may be driven in part by access to antimicrobials.^{23–25} Vietnam illustrates this trend; it is the world's 15th most populous country, urbanization has increased from 28% in 2014 to 34.9% in 2017 and it is predicted to have a population of >100 million people by 2025.²⁶ Antimicrobial dispensing in Vietnam is known to be poorly regulated, leading to inappropriate prescribing and self-medication for a number of common infections, including fever and acute respiratory infections.^{27,28} We selected childhood diarrhoea, which is common in Vietnam, and among the top motives for seeking healthcare in HCMC, as a syndrome for which to assess antimicrobial use.^{12,29–31}

The overall density of pharmacies in the community in HCMC is high (7.59/10000 people), and outnumbers the number of pharmacy staff that have been trained at any level (assistant or university degree) in the city (5.98/10000 people).³² This density of pharmacies in HCMC is reflective of the general pattern in LMICs, for example Sabde *et al.*³³ reported a comparable ratio in an urban area of India (5.84/10000 people). However, the number of pharmacy outlets in HCMC is more than double the mean density of pharmacies in other areas of the world, such as Europe (3.06/10000 people)³⁴ and other parts of Southeast Asia (3.02/10000 people),³⁴ and three times higher than that of some countries in the Western Pacific (2.28/10000 people)³⁴ and the

Table 2. The demographic characteristics of parents and caregivers who chose a pharmacy or a hospital as first choice for a child with diarrhoea

	Watery diarrhoea			Mucoid diarrhoea		
	pharmacy (n = 87)	hospital (n = 89)	P value ^a	pharmacy (n = 65)	hospital (n = 142)	P value ^a
Female sex	73/87 (84)	72/89 (81)	0.693	55/65 (85)	113/142 (80)	0.448
Role of parent (not caregivers)	54/86 (63)	39/89 (44)	0.015	43/64 (67)	69/142 (49)	0.015
Age in years, mean (IQR)	38 (30–50) ^b	40 (32–57)	0.051	38 (31–50) ^c	40 (32–56) ^d	0.198
Healthcare trained	7/83 (8)	11/83 (13)	0.461	4/63 (6)	16/138 (12)	0.316
Employment	87/87 (100)	89/89 (100)	0.661	65/65 (100)	142/142 (100)	0.943
worker	10/87 (11)	10/89 (11)		6/65 (9)	16/142 (11)	
officer	6/87 (7)	6/89 (7)		5/65 (8)	10/142 (7)	
health staff	2/87 (2)	6/89 (7)		1/65 (2)	6/142 (4)	
business owner	15/87 (17)	10/89 (11)		11/65 (17)	23/142 (16)	
housewife	35/87 (40)	34/89 (38)		29/65 (45)	56/142 (39)	
other (retired)	19/87 (22)	23/89 (26)		13/65 (20)	31/142 (22)	
Education ≤12 years	75/87 (86)	68/88 (77)	0.171	55/65 (85)	115/141 (82)	0.695
Monthly income (VND) ^e			0.406			0.739
≤1.3 million	15/78 (19)	14/80 (18)		11/57 (19)	20/127 (16)	
1.3–9 million	59/78 (76)	57/80 (71)		42/57 (74)	95/127 (75)	
>9 million	4/78 (5)	9/80 (11)		4/57 (7)	12/127 (9)	
Only 1 child	36/84 (43)	37/84 (44)	1.000	21/65 (32)	61/134 (46)	0.091
Only 1 child aged <5 years	68/85 (80)	71/86 (83)	0.699	49/65 (75)	105/134 (78)	0.718
Age of first child, years, mean (IQR)	3 (2–4)	2/87 (2–4) ^f	0.486	3 (2–4)	3/140 (2–4) ^g	0.553
Infections in previous month	57/86 (66)	44/86 (51)	0.063	43/65 (66)	73/139 (53)	0.071
Medication in previous month	60/87 (69)	51/87 (59)	0.207	45/65 (69)	83/141 (59)	0.167
Antimicrobials in previous month	24/60 (40)	26/70 (37)	0.857	18/41 (44)	46/118 (39)	0.585

All values shown are n/N (%) unless otherwise indicated.

^aP values calculated using Fisher's exact test for categorical variables and the Mann-Whitney U-test for continuous variables. Significant P values are highlighted in bold.

^bNumber of respondents = 81.

^cNumber of respondents = 60.

^dNumber of respondents = 139.

^eMonthly income <1300000 VND/month is determined to be poor and below average (as suggested by the Vietnamese government), whereas income ≥9000000 VND/month is considered a good monthly income and above average.

^fNumber of respondents = 87.

^gNumber of respondents = 140.

Table 3. The burden of diarrhoea and antimicrobial treatment for diarrhoea in children aged 1–5 years

Parameter	Diarrhoea episodes per year (95% CI)				
	total	attending hospital	receiving an antimicrobial in hospital	attending a pharmacy	receiving an antimicrobial at a pharmacy
Calculation ^a	(A × B)	(A × C)	(A × C × D)	(A × B × E)	(A × B × E × F)
Diarrhoea type					
watery	10355 (8945–11765)	2163 (1622–2724)	898 (673–1130)	2275 (1965–2584)	184 (159–209)
mucoid	4057 (2975–5158)	2241 (1603–2878)	1462 (1046–1878)	667 (489–848)	144 (105–183)
Total	14427 (12636–16219)	4404 (3560–5249)	2359 (1884–2834)	2945 (2585–3305)	329 (282–375)

^aCalculation derived from parameters described in Figure 1.

USA (2.11/10000 people).³⁵ This ratio of pharmacies is six times higher than in LMICs in Africa.³⁴ Ease of access to retail pharmacies may improve equity in the use of medication but it allows access to treatments that should be better controlled, especially

when pharmacy staff are underqualified and/or fail to follow appropriate guidelines.

Our study demonstrated the common sale of antimicrobials without prescription for paediatric diarrhoeal disease in the

community in HCMC. Notably, we estimated that antimicrobial sales for paediatric diarrhoeal disease in the community in HCMC were lower than in several other LMICs, such as Ethiopia (26%; 58/223),²⁰ and other Asian countries, such as Pakistan (14%)³⁶ and Thailand (52%).³⁷ The low proportion of antimicrobials dispensed for acute watery diarrhoea in HCMC corresponds with a declining trend in antimicrobials being used for watery diarrhoea in Vietnam. Limited available data suggest that the proportion of antimicrobials dispensed for acute watery disease has decreased over the last 20 years, from 45% of cases in 1997,³⁸ to 14% in 2013,³⁹ to 8% (3/37) here. This trend may result from a substantial improvement in practices in pharmacies to alleviate antimicrobial prescribing and the impact of resistance. However, despite an array of public health engagement and intervention projects, antimicrobials still remain the most inappropriate drugs dispensed from retail pharmacies in Vietnam.⁴⁰ It is additionally worth noting that previous studies have suggested that antimicrobial usage is greater in rural areas than in urban areas.²⁸

We predicted that the diarrhoeal disease management in the community in HCMC would be comparable with that of other LMICs, where antimicrobials are dispensed without further consideration.^{36–38,41,42} However, we found that the total number of diarrhoeal episodes that would receive an antimicrobial in a hospital was 5–10 times higher than in retail pharmacies. Our findings are consistent with a recent finding in this setting, where 85% of paediatric diarrhoeal patients admitted to hospital were prescribed an antimicrobial.¹³ It has been estimated that one-third of antimicrobial prescriptions in Vietnamese hospitals may be inappropriate,⁴³ a level that could promote AMR in various bacterial pathogens. It is apparent that in a setting with limited-to-no diagnostic testing, this clinical approach is considered optimal for preventing complications and saving lives. There is a large demand for hospital care in HCMC, and as doctors have limited time to make clinical decisions, such an approach is deemed effective and less time consuming. Therefore, there is a clear requirement for tests that can identify children at the more severe end of the disease spectrum and can distinguish between a viral and a bacterial infection.

Our study has some limitations. We likely underestimated the overall antimicrobial use as our calculation did not take into account antimicrobial use in facilities other than specialized hospitals. Additionally, relying on the choice of attending pharmacy as the first option only and the practice of selling antimicrobials in a small number of pharmacies from two scenarios may underestimate antimicrobial sales in pharmacies. Even though we employed a thorough process to quantify antimicrobial use for diarrhoea, our data extrapolation may contain errors as we compiled data from several sources. In addition, as we aimed to assess antimicrobial dispensing only, we did not record or evaluate the retailer's consulting abilities for using these treatments, which may reflect local antimicrobial handling practices. Lastly, our combined studies focused on diarrhoeal diseases in young children only, and we recognize that our approach offers limited understanding of antimicrobial usage in other populations and other presentations.

We conclude that the density of pharmacy retailers is extremely high in urban HCMC. Antimicrobial usage for diarrhoeal disease in children is more common in hospitals than via community pharmacies. Given these findings, combined with the general low level

of knowledge regarding antimicrobials among parents and caregivers, we predict a high burden of antimicrobial overuse in Vietnam. We advocate better education, training and guidelines for antimicrobial usage and AMR for all health professionals prescribing antimicrobials in Vietnam.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Tables S1–S3 are available as Supplementary data at JAC Online.

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Excess body weight and age associated with the carriage of fluoroquinolone and third generation cephalosporin resistance genes in commensal Escherichia coli from a cohort of urban Vietnamese children Le Thi Quynh Nhi; Ha Thanh Tuyen; Pham Duc Trung; Tran Do Hoang Nhu; Pham Thanh Duy; Hao Chung The; Nguyen Thi Thanh Nhan; Lu Lan Vi; Hoang Thi Diem Tuyet; Tran Thi Thuy Tien; Nguyen Van Vinh Chau; Phung Khanh Lam; Guy Thwaites; Stephen Baker

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1 **Excess body weight and age associated with the carriage of fluoroquinolone and third generation**
2 **cephalosporin resistance genes in commensal *Escherichia coli* from a cohort of urban Vietnamese**
3 **children**

4

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19 **Keywords**

20 Antimicrobial resistance; fluoroquinolones; third generation cephalosporins; cohort; Vietnam; EBSL;
21 Plasmid Mediated Quinolone resistance; co-selection

22

23 **Abstract**

24 Antimicrobial resistant bacterial infections in low and middle-income countries (LMICs) are well-
25 established global health issue. We hypothesised that antimicrobial resistant commensal organisms in the
26 community in LMICs is a major reservoir of antimicrobial resistance genes that can be transferred to

27 pathogen. Therefore, we aimed to assess the prevalence and epidemiological factors associated with the
 28 carriage of ciprofloxacin and ceftriaxone resistant *Escherichia coli* and associated resistance genes in a
 29 cohort of healthy children residing in urban Ho Chi Minh City, Vietnam. We cultured rectal swabs from
 30 498 children onto MacConkey agar independently supplemented with resistant concentrations of
 31 ciprofloxacin and ceftriaxone. Additionally, we screened the individual *E. coli* populations by
 32 conventional PCR from samples to detect a panel of plasmid mediated quinolone resistant (PMQR) and
 33 extended spectrum β -lactamase (ESBL) encoding genes. Associations between phenotypic/genotypic
 34 resistance and demographic characteristics we measured using univariate and multivariable logistic
 35 regression. Ciprofloxacin and ceftriaxone resistant *E. coli* were cultured from the faecal samples of 67.7%
 36 (337/498) and 80.3% (400/498) of children, respectively. The prevalence of any associated resistance
 37 marker was 86.7% (432/498) for PMQR genes and 90.6% (451/498) for β -lactamase genes. Of these, *qnr*
 38 and CTX-M were the most dominant gene families identified (83.9%; 418/498 and 75.1%; 374/498,
 39 respectively. Overweight and obese children were significantly more likely to carry *qnr* genes than
 40 children with lower weight for height z-scores (OR: 1.24; 95%CI: 10.5-1.48 *for each unit increase in*
 41 *weight for height z-score; p=0.01*). Additionally, younger children were significantly more likely to
 42 carry ESBL CTX-M genes alone, and in combination with *qnr* genes, than other children (OR: 0.97,
 43 95%CI: 0.94 -0.99 for each year increase in age, *p=0.01*). We conclude that the carriage of genotypic and
 44 phenotypic resistance against fluoroquinolones and third generation cephalosporins are highly prevalent
 45 among commensal *E. coli* in healthy children in the urban community in Vietnam. Future investigations
 46 on the carriage of antimicrobial resistant organisms in LMICs should focus on the progression of carriage
 47 from birth and structure of the microbiome in obesity.

48

49 **Introduction**

50 Antimicrobial resistance (AMR) has become an increasingly recognised global health problem. The social
 51 complexities associated with AMR are multifaceted; however, a major driver of AMR is thought to be the

52 unsolicited use of antimicrobials in the community, which is particularly common in many low and
53 middle-income countries (LMICs)¹. It is generally accepted that the more a community is exposed to
54 antimicrobials, the more likely AMR bacteria will emerge and be maintained². Multiple studies have
55 demonstrated a recent dramatic rise in pathogenic bacteria exhibiting resistance against fluoroquinolones
56 and third-generation cephalosporins³⁻⁵; two of the most commonly used classes of antimicrobials
57 globally⁶. Consistently, cephalosporins and fluoroquinolones are most consumed antimicrobials classes
58 in Vietnam, with prescribed consumption from hospitals equal to that from retail pharmacies^{7,8}.

59

60 *Escherichia coli* (*E. coli*) is an Gram-negative bacterium, which is abundant in the intestine of healthy
61 people and can be used to assess the composition of AMR in the human gut⁹⁻¹¹. *E. coli* are proficient
62 vehicles of AMR gene circulation, and are commonly co-resistant to several clinically important
63 antimicrobials including cephalosporins, fluoroquinolones, and sulfamethoxazole/trimethoprim^{12,13}.
64 Therefore, commensal *E. coli* are sentinel Gram-negative organisms that play a principal role in the
65 maintenance and spread of plasmid associated AMR genes within the Enterobacteriaceae^{13,14}.
66 Specifically, *E. coli* are known to be a major reservoir of plasmids carrying both Extended Spectrum β -
67 lactamases (ESBLs) and plasmid-mediated quinolone resistance (PMQR) genes^{15,16}.

68

69 The expression of ESBLs genes is a key resistance mechanism against later generation beta-lactams; the
70 circulation of genes encoding these enzymes is of critical importance in human health¹⁵. ESBLs, which
71 can hydrolyse a wide variety of β -lactamases, are presently classified into several sub groups, the most
72 frequently identified include TEM, SHV, and CTX-M^{17,18}. TEM and SHV are historic β -lactamase genes
73 that have evolved to exhibit ESBL activity; TEM and SHV variants that have ESBL activity can be
74 commonly found in members of the Enterobacteriaceae associated with infection. CTX-M genes were
75 first described in the 1980s and found to have greater activity against cefotaxime than existing ESBL
76 genes. The *bla*_{CTX-M} genes have been extremely successful, becoming globally ubiquitous and undergoing
77 extensive diversification (>100 variants have been identified). This important group of ESBL genes is

78 broadly divided into five phylogroups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. The
79 most dominant global lineage is *bla*_{CTX-M-1}, which incorporates *bla*_{CTX-M-15}, a particularly successful and
80 persistent variant found commonly in Asia ¹⁷.

81

82 Fluoroquinolone resistance generally occurs as a result of mutations in the chromosomal drug target genes
83 encoding the DNA gyrase (*gyrA* and *gyrB*) and the topoisomerase IV (*parC* and *parD*) ¹⁹. However,
84 fluoroquinolone resistance can also be induced and enhanced by the PMQR genes. The most common
85 PMQR genes are the *qnr* family (*qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*), which act by altering the DNA
86 gyrase and topoisomerase IV, thus reducing the interaction between drug and target ^{20,21}. The *qnr* genes
87 have been found on a broad range of plasmid structures in various pathogens within the
88 Enterobacteriaceae. Alternative PMQRs include *aac*-(6')-Ib-cr, which encodes a modified
89 aminoglycoside acetyltransferase that inactivates specific fluoroquinolones, and the efflux pump encoding
90 genes, *qepA* and *qqxAB* ^{19,22}. Notably, PMQR genes frequently co-exist on the same plasmid backbones as
91 ESBL genes, which can be co-transferred during horizontal gene exchange ^{20,23,24}.

92

93 Several studies have focused on characterising the carriage of AMR organisms in hospitalized patients
94 and healthy individuals in the community in LMICs ^{4,13,25}. The majority of these investigations have
95 isolated *E. coli* and then performed conventional antimicrobial susceptibility testing ²⁶. In addition,
96 various studies have measured the prevalence of ESBL producing commensal *E. coli* in Vietnamese
97 individuals ^{27,28}. However, less is known about the prevalence of AMR bacteria and the diversity of
98 associated AMR genes in the commensal microbiota of healthy children living in an urban community.
99 Furthermore, the potential risk factors associated with the carriage of AMR organisms of AMR genes
100 have not been defined. Here, we used a direct plating method on selective media to measure phenotypic
101 and genotypic resistance against fluoroquinolones and third generation cephalosporins among a cohort of
102 498 healthy children resident in Ho Chi Minh City (HCMC) ²⁹. Further, we aimed to identify
103 epidemiological risk factors associated with the carriage of phenotypic and genotypic resistance in these

104 urban children. Such data from an urbanizing LMIC is important for a better understanding the circulation
105 of AMR bacteria in the community and to quantify the key populations associated with the global AMR
106 crisis.

107

108 **Methods**

109 *Ethical approval*

110 The Ethics Committee of the Hospital for Tropical Diseases (HTD) in Vietnam and the Oxford Tropical
111 Research Ethics Committee (OxTREC) of the United Kingdom provided ethical approval for this study.
112 Written informed consent was provided by parents and care-givers of children.

113

114 *Study population*

115 All rectal swabs were collected from healthy children who were participants of a previously described
116 prospective longitudinal study³⁰. In brief, infants were recruited into a birth cohort at Hung Vuong
117 hospital (HVH) in HCMC, the second largest obstetric hospital in south Vietnam, and followed for 12
118 months for febrile disease through passive surveillance³¹. A subset of these children were followed
119 beyond 12 months and recruited into a continuous cohort conducting active surveillance for diarrheal
120 disease. The children attended scheduled routine follow up visits at HVH every six months. Information
121 on demographics, household water sources, sanitation, health issues in the intervening six months
122 between visits, and food consumption habits in the three days prior to the visit were collected. A rectal
123 and nasopharyngeal swab was collected at the time of the visit. There were 748 children enrolled from
124 June 2014 to December 2014 and followed to December 2016. To actively monitor diarrheal disease
125 episodes, fortnightly SMS messages were sent to the phone of the children's parent/care provider. During
126 diarrhoeal episodes nurses visited their homes to confirm the case, collect samples and provide supportive
127 care. Cases were treated according to local standard of care and were required to attend the Hospital for
128 Tropical Diseases (HTD) in central HCMC for medical care.

129

130 *Identification of antimicrobial resistant E. coli*

131 From March to August 2016, we collected 498 rectal swabs from healthy children (no diarrhea or other
132 symptoms of illness) at routine follow up visits for the purposes of this investigation. At routine follow up
133 visits, we recorded information regarding their general well-being and regarding their antimicrobial
134 practices in the prior six months. All rectal swabs were inoculated onto MacConkey agars to pick all
135 suspected *E. coli* colonies (e.g. lactose fermenting and non-lactose fermenting, circular, elevated, entire
136 margins, smooth texture, pink or colourless, dry, and shiny). These meta *E.coli* population were then
137 inoculated into brain heart infusion (BHI) media supplemented with 20% glycerol and stored at -80°C
138 until being subjected to inoculation onto Mac Conkey agars supplemented with either ciprofloxacin
139 (4mg/l) or ceftriaxone (6mg/l) to select for resistant organisms against these antimicrobials. *E. coli* strain
140 ATCC 25922 (MIC against ciprofloxacin and ceftriazone 0.008 mg/l and 0.094 mg/l, respectively) and a
141 clinical *E. coli* strain 03_0334 (MIC against ciprofloxacin and ceftriazone with >256mg/l) were used as
142 negative and positive controls for testing the culture media. All suspected *E.coli* colonies of each plate
143 were then confirm by MALDI-TOF.

144

145 *Detection of antimicrobial resistant genes*

146 The meta *E.coli* population in frozen BHI were subjected to nucleic acid extraction and multiplex
147 polymerase chain reaction (PCR) amplification. Approximately 200µl of each meta-*E. coli* in frozen BHI
148 was removed and aliquoted into a 96-well plate and centrifuged for 10 minutes at 4,000rpm. The
149 supernatant was removed and discarded. The remains in the wells were added 200µl sigma water and the
150 bacterial solution was used to gentle agitation to ensure mixing. Nucleic acid was extracted by heating the
151 solution to 100°C on a heating block for 10 minutes, then cooling to ambient temperature. The solution
152 was subjected to centrifugation for 10 minutes at 4,000 rpm in a microfuge. The supernatant was removed
153 and used as template DNA in all PCR amplifications. We conducted a series of multiple PCR
154 amplifications aiming to detect PMQR genes (*aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, and *qnrS*), ESBL genes

155 (*bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, and *bla*_{OXA}) , and AmpC lactamase genes (MOX, DHA, EBC, FOX, ACC,
 156 CIT). The primer sequences and the predicted amplification sizes are shown in Table S1. The conditions
 157 were as previously described ^{14,16,32,33} a positive PCR result was determined by the detection of a PCR
 158 amplicon of an appropriate size (using a UV transilluminator and gel documentation system) for each
 159 target in the presence of appropriate positive and negative controls.

160

161 *Statistical analyses*

162 Categorical and continuous variables were described using frequencies (%) and medians (interquartile
 163 range), respectively. Bar plots were used to describe the prevalence of ciprofloxacin and ceftriaxone
 164 resistant *E. coli* and the various ESBL and PMQR genes. We used univariate and multivariable logistic
 165 regression to assess the relationship between participants' characteristics and the presence of AMR *E. coli*
 166 and AMR associated genes. These relationships were described in odds ratios (OR) and their
 167 corresponding 95% confidence intervals (CI). The significance level for statistical tests was 0.05. All
 168 analyses were performed in R v3.4.0 (R Foundation, Vienna, Austria) and companion packages.

169

170 **Results**

171 *Baseline characteristics*

172 Between March and August 2016, we collected rectal swabs from 498 children without diarrhoea
 173 attending a routine health check at the designated healthcare facility. All children were from a single
 174 district (eight) in HCMC (the same study area assessed for access to pharmacies in this age group ⁸) and
 175 enrolled in a longitudinal cohort study of 748 individuals. This population was considered to be generally
 176 representative of the child population in HCMC. The baseline characteristics of those sampled are in
 177 Table 1; 269/498 (54%) were male, 211/498 (42.4%) were born by Caesarean section, and 64/498
 178 (12.9%) were preterm deliveries (Table 1). The median age of the children at the point of sampling was
 179 46 months. At the time of the health check in which they were sampled, the majority of children were
 180 attending nursery/school (67.9%; 338/498), and only a small fraction of children were underweight,

181 stunted or wasted. However, more than a quarter of children were overweight (27.5%; 137/498), with a
 182 similar proportion (136/498; 27.3%) having a minor non-diarrheal illness at the time of visit. A
 183 substantial proportion (42.8%; 213/498) of children had received antimicrobials in the six months prior to
 184 sampling and 26.9% (134/498) regularly consumed some form of probiotic as part of their diet (Table 1).
 185 A third (29.1%; 145/498) of the children had taken oral vitamin A supplementation, provided via a
 186 government public health nutrition program. The children were a broad cross-section of the community in
 187 this urban district of HCMC and had correspondingly consumed a wide variety of animal products in the
 188 three days prior to sampling, which included pork (89.6%, 446/498), shrimp (85.1%; 424/498), eggs
 189 (77.7%; 387/498), beef (75.9%; 378/498), and poultry (64.5%; 321/498) (Table 1).

191 *Ciprofloxacin and ceftriaxone resistant E. coli*

192 Aiming to assess the prevalence of antimicrobial resistant *E. coli* carriage this population we
 193 independently plated suspected *E. coli* colonies onto MacConkey agar supplemented with ceftriaxone and
 194 ciprofloxacin to isolate resistant organisms. We cultured *E. coli* colonies that were resistant to ceftriaxone
 195 (according to current CLSI guidelines ³⁴) from 80.3% (400/498) of the rectal swabs and *E. coli* colonies
 196 that were resistant to ciprofloxacin (according to current CLSI guidelines ³⁴) from 67.7% (337/498) of the
 197 rectal swabs. As shown in Figure 1, resistant *E. coli* colonies were cultured from at least one of the
 198 antimicrobial supplemented plates (i.e. either ciprofloxacin or ceftriaxone) in 84.7% (422/498) samples;
 199 no antimicrobial resistant *E. coli* colonies were isolated on either plate from the remainder of samples
 200 (76/498; 15.3%). From the 422 samples from which we isolated resistant *E. coli* colonies on at least one
 201 plate, 315 produced *E. coli* colonies on both the ceftriaxone supplemented media and the ciprofloxacin
 202 supplemented media. We aimed to identify epidemiological characteristics associated with the detection
 203 of fluoroquinolone and cephalosporin resistant *E. coli* in the rectal swabs. However, after investigating
 204 multiple factors including age, sex, antimicrobial usage, and other household variables, we found that no
 205 factors were significantly associated with their carriage resulting from either the limit of our sample size
 206 or from an indication that antimicrobial resistant *E. coli* were so ubiquitous (Table S2).

207 *The prevalence of antimicrobial resistant genes in rectal swabs from healthy children*

208 The 498 paired MacConkey plates were scraped and aliquoted; DNA was extracted from the mixed *E.*
209 *coli* populations and all were subjected to PCR amplification to identify a panel of genes associated with
210 resistant to fluoroquinolones and cephalosporins. The overall prevalence of any plasmid mediated
211 quinolone resistant (PMQR) gene (*aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, and *qnrS*) in the samples was 86.7%
212 (432/498); the corresponding prevalence of a β -lactamase genes was 90.6% (451/498). Notably,
213 individual *qnr*-determinants were the most commonly detected (83.9%; 418/498) resistance genes,
214 especially *qnrB* (67.3%; 335/498) and *qnrS* (61.4%; 306/498) (Figure 2A). The prevalence of ESBL
215 genes (*bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, and *bla*_{OXA}) and AmpC genes (MOX, DHA, EBC, FOX, ACC, CIT)
216 were 83.7% (417/498) and 67.5% (336/498), respectively. Alarming, the CTX-M class of ESBL gene
217 was the most commonly detected (75.1%; 374/498), followed by *bla*_{TEM} (36.9%; 184/498) (Figure 2B).
218 Generally, we found that *qnrB*, *qnrS*, *bla*_{CTX-M9}, and DHA were sequentially the most frequently detected
219 genes, and typically identified in the same sample (Figure 3A). More than half (52.2%; 260/498) of
220 samples had a combination of at least one *qnr*, one AmpC, and one *bla*_{CTX-M} (Figure 3B).

221

222 Not all antimicrobial resistant samples were associated with a corresponding antimicrobial resistance gene
223 marker. From the ciprofloxacin resistant *E. coli*, 37/337 (11%) samples did not generate an amplicon
224 associated with a PMQR genes; potentially explained by chromosomal DNA gyrase mutations.
225 Correspondingly, from the ceftriaxone resistant *E. coli*, 20/400 (5%) did not produce an ESBL or AmpC
226 amplicon. Conversely, from the 161 samples with no ciprofloxacin resistant *E. coli*, 73% (118/161)
227 generated an amplicon for at least one *qnr* gene. From the 98 rectal swabs containing no *E. coli* exhibiting
228 phenotypic ceftriaxone resistance, a large proportion (72%; 71/98) generated at least one ESBL or an
229 AmpC amplicon.

230

231 *Risk factors for PMQR and β -lactamase genes carriage*

232 We next explored potential epidemiological associations between the participants’
 233 characteristics/behaviour and the most common PMQR genes (*qnr*-family: 83.9%; 418/498)) (Table 2)
 234 and β -lactamase genes (*bla*_{CTX-M} (75.1%; 374/498) (Table 3). We found that the detection of *qnr* genes in
 235 commensal *E. coli* were significantly more common in children with higher weight-for-height z-scores
 236 (OR: 1.24, 95%CI: 1.05 -1.48, for each unit increase in weight for height z-score, $p=0.01$). However, we
 237 did not find any significant association between the age of the children when sampled and the presence of
 238 any *qnr* gene ($p>0.05$). Conversely, we did not identify a relationship between weight-for-height z-score
 239 and the presence of *bla*_{CTX-M} genes ($p>0.05$). However, *bla*_{CTX-M} genes were more commonly detected in
 240 the commensal *E. coli* in children of a younger age (OR: 0.97, 95%CI: 0.94 -0.99 for each year increase
 241 in age, $p=0.01$). Additionally, we investigated potential epidemiological associations between
 242 participants’ features and the presence of the most common genes in combination, (*bla*_{CTX-M} and *qnr*). We
 243 found a consistent relationship, whereby the co-existence of these genes was significantly higher among
 244 younger children (OR: 0.95, 95%CI: 0.9 – 1.00, $p=0.03$) (Table S3).

245

246 Discussion

247 Commensal *E. coli* are important vehicles for the transmission of antimicrobial resistance determinants
 248 ^{11,13,14}. Here, we investigated both the phenotypic and genotypic characteristics of AMR in a cohort of
 249 urban children in Vietnam using direct plating and conventional PCR, a method which is sensitive,
 250 specific, inexpensive, and suitable for screening in LMICs ^{27,28}. Our study outlines several new insights
 251 into this potential reservoir of AMR genes and their association with demographic characteristics. We
 252 found an exceptionally high prevalence of fluoroquinolone and third generation cephalosporin resistant *E.*
 253 *coli* and their associated resistance genes in young children in this location. We report that the most
 254 commonly identified PMQR genes were within *qnr* family (including *qnrB* and *qnrS*) and the most
 255 commonly detected class of ESBL gene was *bla*_{CTX-M}. We additionally found that the carriage of *qnr*
 256 genes was associated with higher weight for height z-scores and that younger children were more likely to
 257 carry *bla*_{CTX-M} ESBL genes in combination with *qnr* PMQR genes.

258

259 Previous comparable studies in Vietnam demonstrated a low prevalence of quinolone resistant commensal
260 *E. coli*^{27,35}. These two studies, conducted in Northern Vietnam, suggested that even though the overall
261 prevalence of AMR *E. coli* was low, the isolation of ciprofloxacin resistant organisms was higher in the
262 urban area than in the rural area. Here we show a trend towards an increasing prevalence of ciprofloxacin
263 resistant commensal *E. coli*. This trend may reflect a generic higher degree of resistance to this group of
264 antimicrobials over time in combination with dramatic increases in antimicrobial usage within Vietnam,
265 principally in urban areas. Using direct plating, we show a higher prevalence of ciprofloxacin resistant
266 *E.coli* in the commensal microbiota of children in Vietnam compared to those described in Bolivia and
267 Peru (18% in 2002 and 33% in 2005)^{36,37}. Additionally, in comparison to a recent study conducted in
268 HCMC among healthy adults³⁸, we also show a higher prevalence of ciprofloxacin resistant *E. coli*
269 (15.5%, 16/103 *E. coli* isolates vs. 67.7% 337/498 rectal swab samples). However, these differences may
270 be derived from the method of resistance detection; the previous study exploited routine bacterial culture
271 and antimicrobial susceptibility testing, or the population sampled.

272

273 Here, we additionally detected a substantially higher prevalence of PMQR and ESBL genes than observed
274 in previous investigations in human populations. Our data illustrate a much higher prevalence of PMQR
275 genes among the healthy paediatric population (86.7%; 432/498), than that have been described in other
276 countries. For example, in Mexico in 2013, the prevalence of PMQR genes among Enterobacteriaceae
277 isolates from children was 32%³⁹. The global meta-analysis estimated that ESBL positivity rate for
278 Enterobacteriaceae among healthy community reported in 2016 was 14%⁴⁰. The ESBL producing
279 Enterobacteriaceae among paediatric community varied according to level of development; 2.9%
280 (Sweden)⁴¹, 11.6%(Tanzania)⁴², 23% (Lao)⁴³, 24.8% (Lebanon)⁴⁴ and 59% (Bangui). Potential
281 explanations for this discrepancy may include the use of differing methods for detecting PMQR and
282 ESBL determinants (i.e. PCR methods) and a temporal difference of five years, which may reflect a
283 natural increase in the circulation of organisms and people carrying such AMR genes. Our data indicate

284 that a high proportion of children in urban Vietnam carry multiple AMR genes, implying high exposure to
285 a range of antimicrobials in this local setting⁷, is comparable to other reports in Southeast Asia^{43,45-47}.
286

287 Notably, we found that the prevalence of carrying the ESBL *bla*_{CTX-M} gene was 75.1% (374/498), of
288 which the majority (62.9%; 313/498) were *bla*_{CTX-M-9}; the next most common were *bla*_{TEM} (36.9%;
289 184/498). This distribution is comparable to patterns observed in a small sample of healthy adults residing
290 in HCMC, where the *bla*_{CTX-M} class of genes was the most dominant among ESBL producing *E.coli*
291 isolates (100%; 10/10), followed by *bla*_{TEM} (50%; 5/10)⁴⁸. We found a relatively consistent prevalence
292 of *qnr* genes as previously reported in this setting¹⁴. We further established that *qnrB* and *qnrS* were the
293 most prevalent PMQR genes (67%; 335/498 and 61%; 306/498, respectively). This prevalence was higher
294 in comparison to data from China and some settings in Latin American³⁹. However, this disparity may
295 again be associated by a difference in the population sampled. In a recent study, the prevalence of *qnrB*
296 was 94% (16/17) but this result was recovered from *E. coli* isolated only, while our study reveal the
297 prevalence of a whole population of bacteria in the fecal samples of healthy children. However, while
298 *qnrS* was not commonly detected in other locations³⁹, our results shows that *qnrS* is among the most
299 common AMR genes in children within HCMC.

300

301 We found a significant association between children carrying *qnr* genes and a higher weight for height z-
302 score. An explanation for this association could be that overweight and obese children are more likely to
303 be treated with antimicrobials. In this setting, children that are overweight are more commonly from
304 families with a higher socio-economic status, which in turn may be more likely to seek healthcare and,
305 therefore receive antimicrobials. Besides, childhood obesity is known to trigger chronic low-grade
306 inflammation⁴⁹, which may lead to their vulnerability to gastrointestinal diseases and diarrhea⁵⁰. This in
307 turn results in a higher likelihood of antimicrobial consumption prior to hospitalization in case of a
308 diarrheal episode⁵¹. Furthermore, quantitative microbiological examination has reported a significant
309 expansion of *E. coli*, coupled with the contraction of *Bifidobacterium*, in obese children in China when

310 compared to age-match controls⁵². As we screened for PMQR genes, which chiefly circulate within the
311 Enterobacteriaceae, we speculate a link between shifts in the gut microbiome during obesity and the
312 selection of genotypic resistance. Fluoroquinolones are amongst the most commonly used antimicrobials
313 worldwide, and their use is particularly common in Vietnam^{6,53}. The association of the carriage of *bla*_{CTX-}
314 _M genes and younger children may be induced by reducing antimicrobial usage with age. It is difficult to
315 assess the nature of the selective pressure for specific AMR genes during therapeutic use, but we predict
316 that selection would reduce as children mature.

317

318 Our study has limitations. First, the point prevalence of phenotypic and genotypic resistance could
319 identify non-causal associations with the variable assessed in the children. In addition, the interpreting of
320 resistance commensal *E.coli* should be in caution because *E.coli* we have detected from rectal swabs from
321 healthy children may either commensal or remained pathogenic *E.coli* that healthy children carried.
322 Second, we investigated associations between the carriage of various AMR genes and a wide range of
323 heterogeneous epidemiological variables among a healthy population, which may explain the limited
324 number of significant epidemiological associations. Furthermore, by screening for the presence of PMQR
325 and β -lactamase genes directly from rectal swabs using conventional PCRs we were unable to assess the
326 direct association between resistant phenotypes and associated genotypes. Notwithstanding these
327 limitations, these results from this first of its type study in Vietnam will provide an important step in
328 understanding the role of the microbiome in maintaining AMR genes in the community.

329

330 We conclude that there is a high prevalence of commensal *E. coli* in children aged under five years in an
331 urban setting within Vietnam exhibiting resistance against fluoroquinolones and third generation
332 cephalosporins. In addition, we have determined the prevalence of PMQR and β -lactamase encoding
333 genes is extremely high among healthy children. We highlight high weight to height z-scores as a major
334 risk for carrying *qnr* genes and that younger children we more likely to carry CTX-M genes in
335 combination with *qnr*. We advocate future longitudinal studies in Asia assess the maturing of AMR

336 component of the human microbiome. These data are imperative for understating the ecology of AMR
337 gene carriage and transmission within human. Our work emphasizes the role of the commensal bacteria,
338 especially among children, as being key for the transmission and maintenance of AMR organisms/genes.

339

340 **Declaration of interests**

341 The authors declare no competing interests.

342

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347

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486

Table 1. Baseline characteristics of the 498 study participants

Characteristics	Frequency (%) or Median (IQR)
Male	269 (54.0%)
Age at visit (month)	46.4 (35.6, 52.5)
Preterm at birth	64 (12.9%)
Low birth weight	20 (4.0%)
Caesarean section	211 (42.4%)
The only child in the family	271 (54.4%)
Early breastfeeding at birth	340 (68.3%)
Feeding at birth	
Only formula	42 (8.4%)
Exclusive breastfeeding	20 (4.0%)
Breast milk plus formula	436 (87.6%)
Breastfeeding duration (month)	4.0 (3.0, 6.0)
Breastfeeding up to 12 month	60 (12.0%)
Attending school	338 (67.9%)
Nutritional status *	
Underweight (weight for age)	2 (0.4%)
Stunting (height for age)	10 (2.0%)
Overweight (weight for height)	137 (27.5%)
Wasting (weight for height)	1 (0.2%)
Probiotic using prior 6 months	134 (26.9%)
Antimicrobials using prior 6 months	213 (42.8%)
Oral vitamin A supplementation prior 6 months	145 (29.1%)
Food consumption prior 3 days	
Pork	446 (89.6%)
Beef	378 (75.9%)
Shrimp	424 (85.1%)
Fish	339 (68.1%)
Egg	387 (77.7%)
Shellfish	108 (21.7%)
Pate (made from live liver)	53 (10.6%)
Current sickness	136 (27.3%)
Hospital admission prior 6 months	19 (3.8%)
Low maternal education (<= 9yrs)	302 (60.6%)
In-paid employment mothers	342 (68.7%)
Household crowding **	348 (69.9%)
Main water use at home	
Pipped to residence	331 (66.5%)
Bottled water	163 (32.7%)
Others	4 (0.8%)
Ice using at home	82 (16.5%)
Exposure to flooding	107 (21.5%)
Exposure to animal around home	300 (60.2%)
Presence of toilet soap at home	498 (100.0%)
Handwashing behavior after toilet	
Always	269 (54.0%)
Sometimes	227 (45.6%)
Never	2 (0.4%)
Handwashing after handling diaper	
Always	420 (84.3%)
Sometimes	78 (15.7%)
Never	0 (0.0%)

* Based on the WHO Child Growth Standards: Underweight: weight for age < -2 standard deviations (SD); Stunting: height for age < - 2SD; Wasting: weight for height < - 2SD; Overweight: weight for height >+2SD; **Two or more people per room

Table 2. Associations between the presence of *qnr* genes and characteristics of the study participants

Characteristic	Presence of any <i>qnr</i> gene		Univariate analysis			Multivariable analysis		
	Yes (N=418)	No (N=80)	OR	(95% CI)	p value	OR	(95% CI)	p value
Low maternal education	249 (59.6%)	53 (66.2%)	0.75	(0.45, 1.23)	0.259	0.77	(0.45, 1.29)	0.320
In-paid employment mother	286 (68.4%)	56 (70.0%)	0.93	(0.54, 1.55)	0.780	0.74	(0.42, 1.29)	0.292
Male	229 (54.8%)	40 (50.0%)	1.21	(0.75, 1.96)	0.432	1.10	(0.66, 1.83)	0.700
Age * (month)	46.4 (35.6, 52.4)	47.2 (35.8, 53.1)	0.99	(0.96, 1.02)	0.512	0.99	(0.96, 1.02)	0.419
Preterm at birth	54 (12.9%)	10 (12.5%)	1.04	(0.52, 2.25)	0.918	0.99	(0.49, 2.20)	0.988
Caesarean section	184 (44.0%)	27 (33.8%)	1.54	(0.94, 2.58)	0.086	1.48	(0.88, 2.53)	0.138
Breastfeeding duration * (month)	4.0 (3.0, 6.0)	4.0 (2.8, 6.0)	1.04	(0.99, 1.09)	0.162	1.04	(0.99, 1.10)	0.117
Weight-for-height z score *	1.0 (-0.1, 2.3)	0.4 (-0.4, 1.5)	1.22	(1.04, 1.44)	0.012	1.24	(1.05, 1.48)	0.011
Antimicrobials use in the prior 6 months	180 (43.1%)	33 (41.2%)	1.08	(0.67, 1.76)	0.764	0.92	(0.54, 1.57)	0.753
Probiotic use in the prior 6 months	116 (27.8%)	18 (22.5%)	1.32	(0.76, 2.39)	0.324	1.30	(0.72, 2.41)	0.388
Pork or beef consumption in the prior 3 days	412 (98.6%)	79 (98.8%)	0.87	(0.05, 5.18)	0.896	0.90	(0.05, 6.13)	0.929
Poultry or egg consumption in the prior 3 days	385 (92.1%)	70 (87.5%)	1.67	(0.75, 3.42)	0.200	1.72	(0.75, 3.68)	0.193
Ice using in the prior 6 months	65 (15.6%)	17 (21.2%)	0.68	(0.38, 1.27)	0.221	0.69	(0.37, 1.32)	0.250
Piped water to residence	278 (66.5%)	53 (66.2%)	1.01	(0.60, 1.66)	0.964	0.92	(0.53, 1.58)	0.769
Exposure to flooding	89 (21.3%)	18 (22.5%)	0.93	(0.53, 1.69)	0.810	0.91	(0.50, 1.72)	0.773
Exposure to animal	251 (60.0%)	49 (61.3%)	0.95	(0.58, 1.55)	0.840	1.03	(0.61, 1.72)	0.917
Attending school	286 (68.4%)	52 (65.0%)	1.17	(0.70, 1.92)	0.551	1.30	(0.72, 2.30)	0.383
Household crowding	293 (70.1%)	55 (68.8%)	1.07	(0.63, 1.77)	0.811	1.03	(0.59, 1.75)	0.922
Hospital admission in the prior 6 months	18 (4.3%)	1 (1.2%)	3.55	(0.72, 64.42)	0.139	3.47	(0.66, 64.31)	0.165
Current sickness	117 (28.0%)	19 (23.8%)	1.25	(0.73, 2.23)	0.430	0.99	(0.54, 1.85)	0.970

*Median (IQR); OR: odds ratio of presence of any *qnr* gene; CI: confidence interval

Table 3. Associations between the presence of CTX-M genes and characteristics of the study participants

Characteristic	Presence of any CTX-M resistant genes		Univariate analysis			Multivariable analysis		
	Any CTX-M genes(N=374)	None CTX_M genes (N=124)	OR	(95% CI)	p value	OR	(95% CI)	p value
Low maternal education	224 (59.9%)	78 (62.9%)	0.88	(0.58, 1.33)	0.551	0.92	(0.59, 1.42)	0.694
In-paid employment mother	259 (69.3%)	83 (66.9%)	1.11	(0.72, 1.71)	0.631	1.12	(0.70, 1.77)	0.643
Male	204 (54.5%)	65 (52.4%)	1.09	(0.72, 1.64)	0.681	1.07	(0.70, 1.64)	0.749
Age * (month)	41.8 (35.6, 52.3)	47.3 (36.0, 53.3)	0.97	(0.94, 0.99)	0.006	0.97	(0.94, 0.99)	0.014
Preterm at birth	45 (12.0%)	19 (15.3%)	0.76	(0.43, 1.38)	0.351	0.70	(0.39, 1.30)	0.253
Caesarean section	158 (42.2%)	53 (42.7%)	0.98	(0.65, 1.48)	0.923	0.98	(0.64, 1.51)	0.937
Breastfeeding duration * (month)	4.0 (2.0, 6.0)	4.0 (3.0, 6.0)	1.01	(0.98, 1.06)	0.499	1.01	(0.97, 1.05)	0.704
Weight for height z - score	0.8 (-0.2, 2.1)	1.0 (-0.2, 2.2)	0.96	(0.85, 1.08)	0.480	0.95	(0.82, 1.09)	0.463
Antimicrobials use in the prior 6 months	167 (44.7%)	46 (37.1%)	1.37	(0.90, 2.09)	0.139	1.26	(0.81, 1.98)	0.314
Probiotic use in the prior 6 months	102 (27.3%)	32 (25.8%)	1.08	(0.68, 1.73)	0.749	0.94	(0.58, 1.54)	0.789
Pork or beef consumption in the prior 3 days	369 (98.7%)	122 (98.4%)	1.21	(0.17, 5.69)	0.824	1.17	(0.16, 5.73)	0.854
Poultry or egg consumption in the prior 3 days	342 (91.4%)	113 (91.1%)	1.04	(0.49, 2.07)	0.914	1.04	(0.48, 2.13)	0.914
Ice using in the prior 6 months	63 (16.8%)	19 (15.3%)	1.12	(0.65, 2.00)	0.690	1.18	(0.67, 2.15)	0.581
Piped water to residence	247 (66.0%)	84 (67.7%)	0.93	(0.60, 1.42)	0.728	0.85	(0.53, 1.33)	0.473
Exposure to flooding	76 (20.3%)	31 (25.0%)	0.77	(0.48, 1.25)	0.277	0.70	(0.42, 1.18)	0.181
Exposure to animal	225 (60.2%)	75 (60.5%)	0.99	(0.65, 1.49)	0.949	1.03	(0.67, 1.60)	0.883
Attending school	251 (67.1%)	87 (70.2%)	0.87	(0.55, 1.34)	0.527	0.97	(0.59, 1.59)	0.899
Household crowding	260 (69.5%)	88 (71.0%)	0.93	(0.59, 1.45)	0.760	0.93	(0.58, 1.47)	0.757
Hospital admission in the prior 6 months	16 (4.3%)	3 (2.4%)	1.80	(0.59, 7.84)	0.326	1.48	(0.46, 6.67)	0.535
Current sickness	105 (28.1%)	31 (25.0%)	1.17	(0.74, 1.88)	0.503	1.28	(0.76, 2.22)	0.354

*Median (IQR); OR: odds ratio of presence of any CTX-M gene; CI: confidence interval

Figure legends

Figure 1. The prevalence of selected antimicrobial resistance genes in commensal *E. coli* in a cohort of 498 Vietnamese children.

Bar charts showing the prevalence of plasmid mediated quinolone resistant (PMQR) genes (A) and β -lactamase genes (B) in commensal *E. coli* isolated from faecal samples of Vietnamese children.

Figure 2. The co-selection of specific antimicrobial resistant genes in commensal *E. coli* in a cohort of 498 Vietnamese children.

(A) Heat-map showing prevalence of co-selection screened antimicrobial resistant genes; the greater the intensity the more common the combination (see key). (B) Two-dimensional bar plots showing the prevalence of the co-existence of various combinations *qnr*, AmpC, and CTX-M genes.

Figures

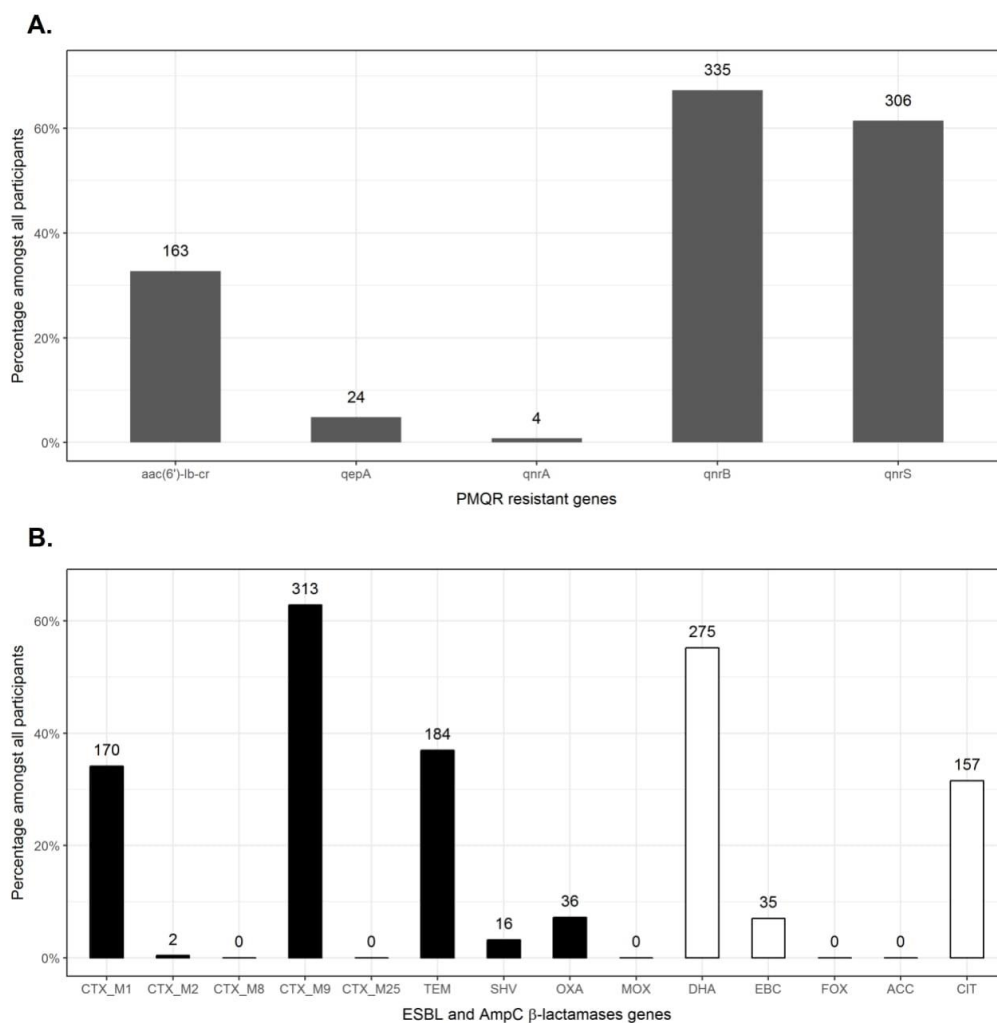


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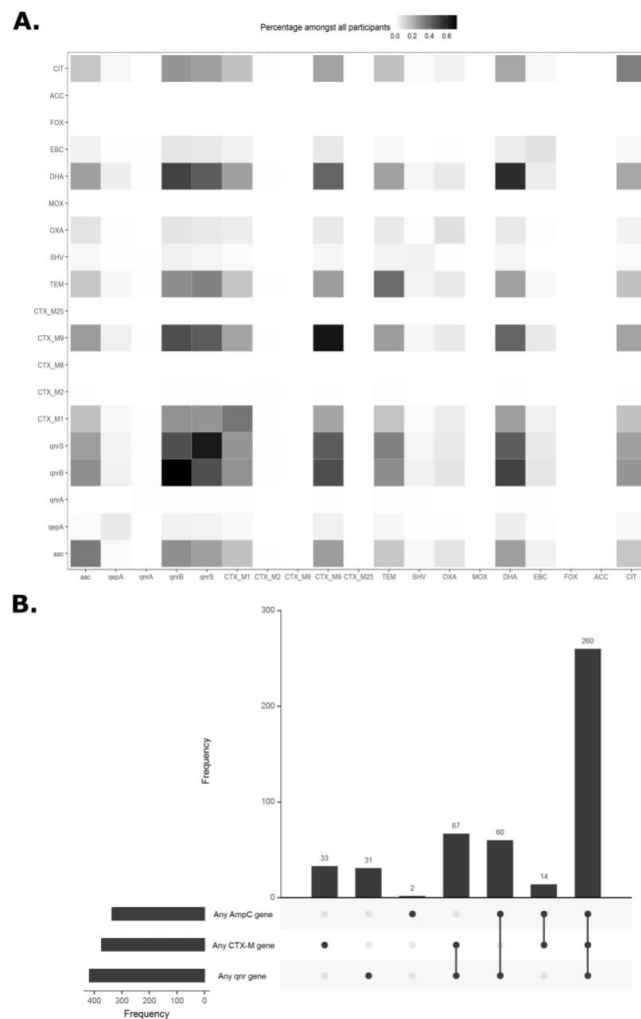


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STUDY PROTOCOL

Open Access

A cohort study to define the age-specific incidence and risk factors of *Shigella* diarrhoeal infections in Vietnamese children: a study protocol

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Abstract

Background: *Shigella* spp. are one of the most common causes of paediatric dysentery globally, responsible for a substantial proportion of diarrhoeal disease morbidity and mortality, particularly in industrialising regions. Alarming levels of antimicrobial resistance are now reported in *S. flexneri* and *S. sonnei*, hampering treatment options. Little is known, however, about the burden of infection and disease due to *Shigella* spp. in the community.

Methods/Design: In order to estimate the incidence of this bacterial infection in the community in Ho Chi Minh City, Vietnam we have designed a longitudinal cohort to follow up approximately 700 children aged 12–60 months for two years with active and passive surveillance for diarrhoeal disease. Children will be seen at 6 month intervals for health checks where blood and stool samples will be collected. Families will also be contacted every two weeks for information on presence of diarrhoea in the child. Upon report of a diarrhoeal disease episode, study nurses will either travel to the family home to perform an evaluation or the family will attend a study hospital at a reduced cost, where a stool sample will also be collected. Case report forms collected at this time will detail information regarding disease history, risk factors and presence of disease in the household. Outcomes will include (i) age-specific incidence of *Shigella* spp. and other agents of diarrhoeal disease in the community, (ii) risk factors for identified aetiologies, (iii) rates of seroconversion to a host of gastrointestinal pathogens in the first few years of life. Further work regarding the longitudinal immune response to a variety of *Shigella* antigens, host genetics and candidate vaccine/diagnostic proteins will also be conducted.

Discussion: This is the largest longitudinal cohort with active surveillance designed specifically to investigate *Shigella* infection and disease. The study is strengthened by the active surveillance component, which will likely capture a substantial proportion of episodes not normally identified through passive or hospital-based surveillance. It is hoped that information from this study will aid in the design and implementation of *Shigella* vaccine trials in the future.

Keywords: Diarrhoea, *Shigella*, Active surveillance, Vietnam, Cohort

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Background

Diarrhoea remains a major cause of childhood morbidity and mortality globally [1,2], with the vast majority of the 1.7 billion annual infections and 0.7 million deaths occurring in low and middle-income countries [3]. The four species of the Gram-negative bacterial genus *Shigella* (*S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae*) are amongst the most common causes of dysenteric diarrhoea worldwide, with >164 million infections estimated annually in 1999 [4]. There is a growing necessity to prevent and control *Shigella* infections due to a dramatic emergence of highly resistant strains in not only southeast Asia [5-7], but in many other countries in both high and low income settings [8-16]. There are also extensive knowledge gaps regarding the distribution, incidence and exposure to *Shigella* spp., including a paucity of information on age-specific incidence, which is essential for assessing disease burden and evaluating the benefits of any future vaccines.

To address the outstanding epidemiological questions related to *Shigella* infections, we are conducting a longitudinal cohort study focusing on diarrhoeal disease with both routine and active surveillance components in Ho Chi Minh City (HCMC) in southern Vietnam. HCMC is a densely populated, rapidly urbanizing setting home to over 7.5 million people with a per-capita annual income of \$1,220 [17]. The burden of *Shigella* infections is known to be substantial in children in Vietnam [5,18]. A previous cohort study conducted in the early 2000s in the coastal province of Khanh Hoa in central Vietnam estimated an incidence rate of 490/100,000/year in children under the age of five years through passive surveillance of diarrhoeal cases [19]. Although several longitudinal, community-based cohorts in Southeast Asia have been established within the last two decades to investigate diarrhoeal disease, very few have included an active disease surveillance component [19-24]. Active surveillance is challenging for obvious reasons, including the substantial cost, intensive staff and resource needs, difficulty in ascertainment and confusion on what is considered to be an 'episode' of diarrhoea by parents or guardians.

The overall aim of this study is to describe the epidemiology of *Shigella* infections in HCMC to inform the development and introduction of *Shigella* vaccines. The primary objective of this study will be to define the age-specific incidence and exposure of *Shigella* infection in children ≤ 60 months of age in the community in HCMC. Secondary objectives include (1) determination of the age-specific incidence of other agents of diarrhoeal infection in the community (2) a description of risk factors for both *Shigella* as well as other diarrhoeal aetiologies (3) evaluation of seroconversion rate (presence of antibody) to diarrhoeal pathogens at six month intervals in the first five

years of life (4) exploration of the relationship between maternal and infant IgG and protection against infection and (5) an investigation into potential host genetic susceptibilities for diarrhoeal disease.

Methods/Design

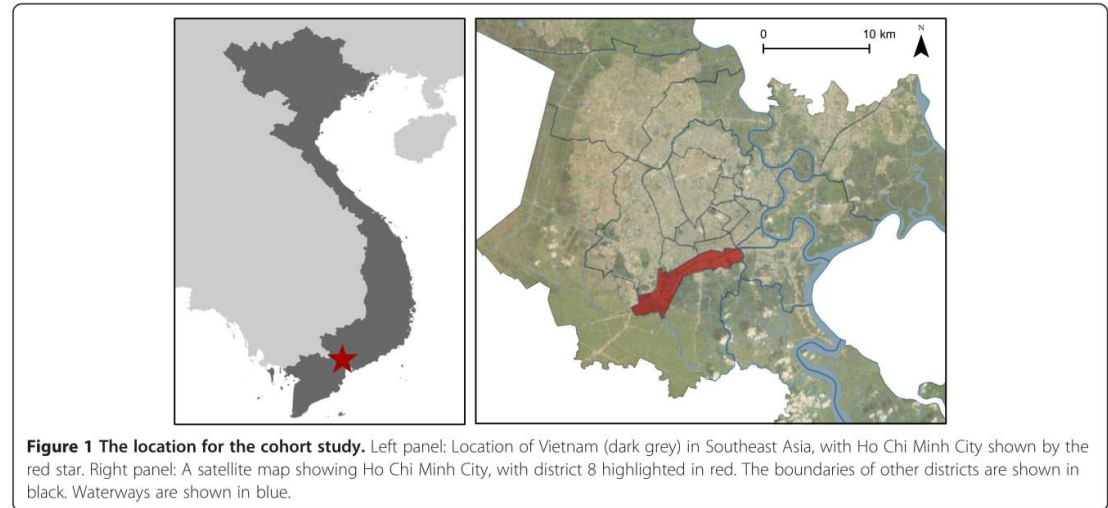
In order to achieve our objectives, we have designed a longitudinal cohort study with routine and active surveillance for diarrhoeal disease in district eight of HCHC (Figure 1). This district is a densely populated district in the center of the city with a large number of canals and waterways and is known to contribute proportionally more hospitalized pediatric shigellosis patients than any of the other districts in the city [5].

Cohort population

Children enrolled into our cohort will be recruited from an existing birth cohort conducted to investigate the incidence of dengue and other viral infections across our study site [24]. Briefly, pregnant mothers living in district eight were enrolled prior to delivery at Hung Vuong Hospital (HVVH), a large obstetric hospital in HCMC delivering approximately 44,000 babies annually. Infants were followed up routinely for the first 12 months of life for the purposes of investigating the epidemiology, aetiology and risk factors of a variety of viral infections in infancy. Clinical samples were collected at routine visits corresponding to Expanded Programme on Immunization (EPI) visits at 0, 4, 9 and 12 months of age, including throat and nasopharyngeal swabs as well as a small blood sample. A subset of these children have been followed beyond 12 months of age and are still undergoing routine follow up every six months.

Enrolment & routine follow up

Children who had been enrolled in the existing birth cohort will be approached for entry into the diarrhoeal disease cohort between 12–36 months of age at a routine birth cohort follow up visit. An enrolment questionnaire will be administered, detailing information on demographic and socioeconomic characteristics of the household, with a particular focus on water sources, water treatment and toilet practices. Once enrolled, children are requested to attend HVVH every six months for a routine health check, as shown in Figure 2. A blood sample (<2 ml), a nasopharyngeal swab and an anal swab or stool sample (if available) is collected by study nurses at these routine visits. Additionally, a questionnaire regarding growth and disease episodes of the child in the preceding six months will be administered. Routine follow up will take place for a total of two years for each child.

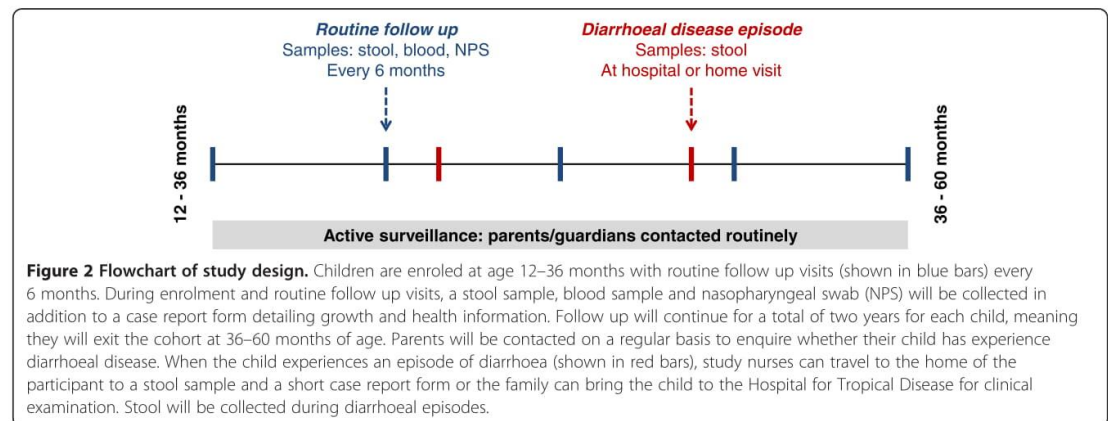


Active & passive case detection

Parents/guardians will be contacted through a text message or phone call on a regular basis to request information on whether the enrolled child has had an episode of diarrhoea in the preceding seven days. Diarrhoea is defined according to the guidelines outlined by the World Health Organization, which is three or more loose stools in a 24 hour period or at least one bloody/mucoid stool [25]. If the parent cannot be reached on the initial attempt, the study nurse will make every effort to establish contact to ensure complete data collection. Once a parent alerts a study nurse to an episode of diarrhoea, the nurse will travel to the participant's home within a 24-hour window. At the home visit, a stool sample will be collected. If no fresh stool is available at the time of the visit, a sterile stool collection pot will be left with the parents/guardians with instructions for

sample collection. Stool pots left at the home will be collected within 24 hours. Furthermore, a short case report form (CRF) will be collected detailing clinical details of the diarrhoeal episode and information on any other known diarrhoeal infections in the household. The parents will also be given oral rehydration packets, probiotics containing *Lactobacillus acidophilus* as well as zinc supplements for the child [26,27].

If at any point during follow up a parent feels that their child requires medical attention for diarrhoeal disease and feels that a home visit by a nurse is insufficient, they may attend the Hospital for Tropical Disease (HTD) at a subsidised cost (Figure 2). Upon arrival at the hospital the children will be escorted through registration processes by a study nurse who will then lead them to the gastrointestinal ward for clinical evaluation (both inpatient and outpatients). A stool sample will be collected, and a



blood sample if clinically indicated. A detailed CRF will be administered collecting clinical, laboratory and haematological information if available.

Clinical and laboratory investigations

All samples will be labeled with a study number to ensure anonymity and transported to the microbiology laboratory at the Oxford University Clinical Research Unit (OUCRU) for analysis and storage on the same day as collection. Samples from disease episodes will undergo microscopy for blood cells and parasites (*Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium*) as well as microbiology culture for *Shigella* spp., *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli*. Antimicrobial susceptibility testing of the cultured and identified bacterial pathogens will be performed by the disc diffusion method using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [28]. Results will be reported back to the treating clinician as soon as they are available.

Aliquots of stool from both healthy routine visits and from disease episodes will be stored at -80°C . Total nucleic acid will be extracted from faecal specimens using the QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany) or using the Roche MagNA pure 96 automated nucleic acid extraction machine (Roche). RNA will be converted to complementary DNA (cDNA) by reverse transcription (RT) and an aliquot of RNA will be stored -80°C . For RT, extracted RNA will be reverse-transcribed by SuperScript Reverse Transcriptase III and RNase Inhibitor (Invitrogen) combined with a random hexamer (Roche Diagnostics, UK) according to manufacturer's instructions. The resulting cDNA will be stored at -80°C . Bacterial PCR will be performed on extracted nucleic acids to potentially increase the diagnosis rate of *Shigella* spp., *Salmonella* spp. and *Campylobacter* spp. Viral pathogens will be identified through batch multiplex realtime PCR procedures to identify rotavirus and norovirus [29]. Additionally, blood samples will be separated into plasma for serology purposes and cells will be stored for future host genetics studies. Specifically, ELISAs will be used to measure IgA, IgM and IgG subtypes to *S. sonnei* O-antigen to evaluate seroconversion rates [30].

Sample size

We estimate that we will enroll between 650–750 children based on the available population and current attrition rates from the original birth cohort [24]. It is known that the median age of hospitalized *Shigella* infections in HCMC is 24 months [5]. Additionally, the annual incidence of diarrhoea in Vietnam in children under five years of age is 1.5 events/child/year [19]. Therefore, we estimate that we will have 2,100 diarrhoeal episodes in our

cohort during two years of follow up. From a cohort study in Hanoi, the *Shigella* positivity rate is approximately 6% of passively detected diarrhoeal cases in children under 5 years [20]. Therefore, we conservatively estimate that we will detect approximately 125 *Shigella* diarrheal episodes over the study period through both passive and active surveillance activities.

Data management

Each patient will have a unique identifying study code such that sample and documents will not be labeled with any identifying information. Data will be collected electronically whenever possible, including on laptops and on handheld tablets provided to the study nurses and hospital wards. Electronic data entry devices will be password protected and accessible only by authorised users. Paper CRFs will be used in the event of power failure or technical difficulty. A central database has been developed to ensure secure and confidential data management.

Ethical approval & ethical considerations

Ethical approval for this study has been obtained from the Oxford University Tropical Research Ethics Committee (OxTREC approval 1058–13) as well as from local partners including the Institutional Review Board (IRB) at the Hospital for Tropical Diseases (HTD) and the IRB at Hung Vuong Hospital (HVVH). Written informed consent will be obtained from parents/guardians of children at the time of enrolment for both participation as well as storage and future use of pathogenic and human samples. Parents/guardians will be assured that all information generated in this study will remain confidential. Participant families are reimbursed for travel costs to our study sites for routine or disease visits as well as tests involved in the standard of care (full blood count, stool microscopy and microbiology) of diarrhoeal disease at HTD. Furthermore, parents/guardians are free to withdraw their consent for their child at any time and request that study samples not be stored for further testing.

Discussion

In this work we have described a longitudinal cohort designed to estimate the incidence of diarrhoea due to *Shigella* in the community in HCMC, Vietnam. This study will follow an estimated 650–750 children under the age of five for two years each, collecting information at routine follow up visits and during diarrhoeal disease events through both passive and active surveillance. We aim not only to estimate the incidence of *Shigella* but also to examine the immune response and risk factors for infection and disease.

Study strengths

The burden of diarrhoeal disease is difficult to accurately estimate [31], and hospitalized illness is generally not representative of infection and disease in the community [32]. Therefore, the most important strength of the study is the active surveillance component as we will be able to estimate the true burden of diarrhoea due not only to *Shigella*, but due to a variety of other diarrheal aetiologies in the community as well. Longitudinal blood samples will also provide for the ability to estimate rates seroconversion to *Shigella sonnei* O-antigen [33], which will capture potential *Shigella* infections that we do not identify through our active and passive surveillance. An additional strength of this study is the collaboration with the existing birth cohort established at OUCRU [24]. Such a partnership will allow for data and sample sharing for all participants from birth through at least the first three years of life, providing for the opportunity to explore dynamics of infection and immunity from infancy.

Limitations

The major, predicted limitations of this study are loss to follow up and incomplete diarrhoeal disease ascertainment. Differential loss to follow up would introduce bias into the results as participants who remain are likely different than those who were lost. Therefore, we will ensure that the rapport between study nurses and participants remains strong through continuous training and maintenance of motivation of the nurses. We have made a significant effort to provide the families with a high level of comprehensive care for diarrhoeal disease in hopes that they will value the overall research programme and continue to participate through the follow up period. Additionally, we are investing heavily in terms of time and personnel to ensure that we capture as many diarrhoeal disease episodes as possible through intensive active surveillance and follow up.

Future work

The humoral immune response to *Shigella* is partly induced by the O-antigen and is thought to be serotype specific [34,35]. However, the immune response to the O-antigen as well as other non-polysaccharide *Shigella* antigens in children in endemic areas after symptomatic infection or exposure is extremely ill-defined [36]. We will explore the longitudinal immune response to a range of *Shigella* antigens in infected individuals in an endemic setting with a view of identifying novel vaccine and diagnostic candidates and their serological relationship to the O-antigen. To interrogate the diversity, specificity and longevity of the humoral immune response a range of *Shigella* antigens, a *Shigella* antigen-array will be constructed in collaboration with the Wellcome Trust Sanger Institute. Similar approaches have been used for

development of other bacterial vaccines and diagnostics such as *Salmonella* and *Brucella melitensis* [37,38].

Additionally, we hope to explore the relationship between genetic variation and disease susceptibility through genetic association, candidate gene and genome wide association studies in the future. Host DNA samples will be stored and used for genotyping to investigate potential genetic associations with diarrhoeal disease. Additionally, the development of gastrointestinal and respiratory tract flora will be measured by performing metagenomic analysis from the anal and nasopharyngeal swabs.

Conclusions

In conclusion, we have designed one of the largest longitudinal, active surveillance cohort studies to study *Shigella* infection and disease in children in Southeast Asia. Through this study, we will be able to estimate an incidence of *Shigella* infection and disease and define the epidemiology of the bacteria in this community, which is representative of many densely crowded, industrializing cities globally. We also hope to eventually more clearly define the longitudinal immune response to this emergent, highly antimicrobial resistant pathogen [12], as well as begin exploration into host susceptibility to infection. Samples, data and analyses from this work are hoped to be a valuable resource to international as well as local medical and public health communities for informing the development and deployment of a *Shigella* vaccine in the future.

Abbreviations

CLSI: Clinical laboratory standards institute; CRF: Case report form; EPI: Extended programme on immunization; HCMC: Ho Chi Minh City; HTD: Hospital for tropical disease; HVH: Hung Vuong Hospital; NPS: Nasopharyngeal swab; OUCRU: Oxford university clinical research unit; RT: Reverse-transcribed.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Designed the study: CNT, KLA, CS, SB, NVVC, GT. Microbiology and laboratory support: HTT, PVM, LTPT, TDHN, JIP. Coordination of study at OUCRU and study sites: LTQN, VTD, LLV, NTVT, HNT. Study nurse: NTTN, TTTL. Wrote the manuscript: CNT, SB. All authors read and approved the final manuscript.

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