


# Bacterial infection in children hospitalised with diarrhoea in the South of Vietnam

**Vu Thuy Duong**

**Kellogg College**

**Clinical medicine**

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**Bacterial infection in children hospitalised with diarrhoea in the South of Vietnam**

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## Abstract

Paediatric diarrhoeal disease is major global health problem. The aetiological diagnosis of diarrhoeal disease is often complex due to the wide array of potential infectious agents, available diagnostic techniques and local resources. Bacterial associated diarrhoeal diseases are an increasing issue due to high burden of antimicrobial resistance (AMR). As a consequence of standard treatments for diarrhoea (i.e. ORS), diarrhoea mortality has been decreased globally. However, the use of antimicrobial therapy for infectious diarrhoea has become controversial as the disease is often self-limited and the epidemiology of AMR pathogens. Here, I estimated the prevalence of bacterial infection among children hospitalised with bloody/mucoid diarrhoea using routine standard methodology (microbial culture) and multiplex real-time PCR. Information regarding antimicrobial usage and the proxy outcome of the patient were recorded. Novel diagnostics and methods (Luminex xTAG GPP and whole genome sequencing) were used in comparison to routine standard methodology and to investigate the infecting agents in more detail. In this setting non-typhoidal *Salmonella* (NTS) has become the most common bacterial cause of diarrhoea in children; followed by *Campylobacter* and *Shigella* with a substantial rate of AMR observed among these three major enteric pathogens. In a high usage setting, antimicrobial treatment for children with diarrhoea contradicted the antimicrobial susceptibility profiles and appeared to prolong the duration of hospitalisation. Diarrhoeagenic *E. coli* (DEC) were commonly detected; however, most pathotypes were found in stool samples from both diarrhoeal and non-diarrhoeal children. Only enteroinvavise *E. coli* (EIEC)/*Shigella* infections were significantly associated with diarrhoea. The xTAG GPP assay provided high sensitivity and specificity and was valuable for detecting other enteric pathogens which were not diagnosed by routine laboratory. *S*. Typhimurium was determined the most frequent NTS serovar via genoserotyping and were associated with an array of AMR genes; especially *blaTEM-95, qnrS1*.

In conclusion, future studies in Vietnam should focus on a more streamlined approach to the diagnosis of diarrhoeal disease, to provide greater clinical evidence for supporting better clinical decision making to reduce or stop empirical antimicrobial usage for paediatric diarrhoea.

## Acknowledgments

I have been working as Paediatrician in the Gastrointestinal Department at Children Hospital 1 in Ho Chi Minh City since 2008. My department usually has 150-200 child patients with the admission rate as 30-40 patients a day and two thirds of them have a diarrhoeal disease. I examined and treated children at different levels of severity every day for six years before working on infectious diarrhoeal diseases from a "scientific-research" perspective. I have enjoyed my PhD time and I have been given a chance to study hard, to work hard, to work in team and to contribute a deeper understanding of healthcare. I enjoyed every moment, even the feelings when I faced difficulties and especially when I finished each component of my project the help of my tutors, my friends, my colleagues and my family. I would like to thank Dr Phúc, who introduced me to the PhD application and supported me the whole time I worked in hospital and in research. I would like to thank Prof. Stephen, my supervisor, particularly for his trust, inspiration and passion. From him, I understand "what is a scientist?" and I want to become one.

I have learned great medical knowledge, critical thinking, work ethics, peer-to-peer communication and more during my PhD period. I have learned from everyone in Enteric infections group at OUCRU, specifically: Thanh Tuyền, James Campbell, Văn Minh, Corinne Thompson, Quỳnh Nhi, Vĩnh Phát, Phương Tú, Hoàng Như, Thế Hào, Thanh Duy, Maia Rabaa, and Ruklanthi de Alwis. I would like to send my appreciation to Dr Minh Ngọc, Dr Hồng Châu, Dr Lan Vi and research teams at Children Hospital 1, Children Hospital 2 and Nhi B ward in Hospital of Tropical Diseases for your kind support and collaboration. I also appreciate all the parents and children who understood and took apart in the research. During my period of time working in Oxford University Clinical Research Unit, I could not thank all the departments enough for their keen assistance in my research.

I had an anguished family crisis right before my PhD registration and I could not have survived without the unconditional love of my parents, Dad Ái and Mom Anh and my brother, Minh. I should say thank you also to my little son, Việt Tuấn, who seemed understand and never asked me not to go to work but kept reminding me to come home safely.

Now, while sitting down and looking back my medical career and my four-year PhD period, I thank everyone who I have worked with and had the chance to meet to make me more positive, more confident and more knowledgeable. Now to deal with the question "what do I want to do in the future"? I really want to continue working on how to improve the health of children and reduce the antimicrobial use in public and in hospitals.

## Declaration

I, Vu Thuy Duong, declare that the majority of the work presented in this thesis is on my own and was guided by Professor Stephen Baker at the Oxford University Clinical Research Unit (OUCRU), Vietnam. The detail of my contribution was as followed

Chapter 4: I worked with Dr Corinne Thompson to complete the protocol and SOPs. I presented to the ethic committees at collaborating hospitals and was granted approval. I trained the study teams, including the doctors and nurses at sites. I was involved in recruitment and enrolment patients in Gastrointestinal Department in Children Hospital 1. I followed up the process in the three collaborating sites including recruitment, enrolment, CRF completion, data entry, double data entry, laboratory reports and monthly reports and cleaning data. Laboratory work (Microscopy, culture) were processed by James Campbell, Pham Van Minh and Ha Thanh Tuyen. When the study closed, I was involved in cleaning data and developed an analysis plan. I worked with Dr Ruklanthi de Alwis on the analysing and presenting data for this chapter.

Chapter 5: I worked with Le Thi Phuong Tu and Tran Thi Phuong Nhu in optimising the multiplex quantitative real-time PCR assay to detect diarrhoeagenic *Escherichia coli*. Ha Thanh Tuyen and I conducted the assays on 3,000 clinical samples. I analysed the data. The table of logistic models were done with the help of Dr Le Hoang Thanh Nhat (statistic group).

Chapter 6: Voong Vinh Phat and I worked together in validating and calculating Luminex xTAG GPP performance in diagnostic 15 diarrhoeagenic pathogens. Dr Corinne Thompson helped with the co-infection correlation among pathogens.

Chapter 7: Tran Do Hoang Nhu and I worked together to extract the DNA of 478 *Salmonella* isolates. Then we sent the DNA materials to Dr Alison Mather and her research team for whole

genome sequencing (Sanger institute). Dr Chung The Hao and I worked to analyse the raw genome of non-typhoidal *Salmonella* to genoserotype and determine the antimicrobial resistance genes. I analysed and presented the data for the chapter.

This thesis has not been submitted for a degree or other qualification of this or any other university.

## Table of Contents

Abstract ..... i

Acknowledgments ..... iii

Declaration ..... v

Table of Contents ..... vii

List of Tables ..... xiv

List of Figures ..... xvii

Abbreviations ..... xviii

Publications during my DPhil Candidature ..... xxiii

1. Introduction ..... 1

1.1. Diarrhoeal disease ..... 1

1.1.1. The burden of diarrhoeal diseases and aetiology ..... 1

1.1.2. Definition and classification of diarrhoeal diseases ..... 4

1.1.3. The diagnosis of the aetiological agents of diarrhoea ..... 7

1.1.3.1. Microbial culture ..... 7

1.1.3.2. API 20E ..... 8

1.1.3.3. Mass spectrometry-based identification ..... 8

1.1.3.4. Bacterial agglutination ..... 9

1.1.3.5. Real-time Polymerase Chain Reaction (real-time PCR) ..... 10

1.1.3.6. xTAG Gastrointestinal Pathogen Panel assay ..... 10

1.1.3.7. Whole genome sequencing ..... 11

1.1.4. The treatment, management, and prevention or diarrhoea ..... 11

1.1.4.1. Principles of treatment diarrhoea diseases ..... 12

1.1.4.2. Antimicrobial therapy ..... 13

1.1.4.3. Other pharmacological therapies ..... 15

1.1.4.4. Prevention ..... 15

1.2. Bacterial diarrhoea ..... 16
1.2.1. *Campylobacter* ..... 16
1.2.1.1. Microbiology and clinical disease ..... 16
1.2.1.2. Epidemiology and antimicrobial resistance ..... 17
1.2.1.3. Management of campylobacteriosis ..... 18
1.2.2. Non-typhoidal *Salmonella* ..... 19
1.2.2.1. Bacteriology and clinical disease ..... 19
1.2.2.2. Epidemiology and antimicrobial resistance ..... 20
1.2.2.3. Management of NTS infection ..... 21
1.2.3. *Shigella* ..... 22
1.2.3.1. Bacteriology and clinical disease ..... 22
1.2.3.2. Epidemiology and antimicrobial resistance ..... 23
1.2.3.3. Management of shigellosis ..... 24
1.2.4. *Escherichia coli* ..... 25
1.2.4.1. Diarrhoeagenic *Escherichia coli* infection ..... 25
1.2.4.2. Diarrhoeagenic *Escherichia coli* detection ..... 30
1.2.4.3. Treatment ..... 30
1.2.5. Antimicrobial resistance ..... 31
1.2.5.1. Burden of antimicrobial resistance ..... 31
1.2.5.2. Antimicrobial agents: mechanism of action; mechanism of resistance and antimicrobial resistance genes ..... 31
1.2.5.3. Antimicrobial susceptibility testing ..... 38
1.3. Diarrhoeal diseases in Vietnam ..... 38
1.3.1. Diarrhoea is a burden of health in Vietnam ..... 38
1.3.2. The aetiology and antimicrobial susceptibility of diarrhoeal pathogens in Vietnam ..... 39
1.3.3. The management of diarrhoea in Vietnam ..... 40

1.4. Hypothesis, objectives, and aims ..... 41
2. Materials and methods ..... 43
2.1. Methods for chapter 3 (Bacterial infection in children hospitalised with bloody/ mucoid diarrhoea) ..... 43
2.1.1. Study setting ..... 43
2.1.1.1. Ethics ..... 43
2.1.1.2. Study design and enrolment ..... 44
2.1.1.3. Sampling and sample collection ..... 46
2.1.1.4. Case Report Form and data management ..... 47
2.1.2. Microbiological methods (Bacterial identification) ..... 47
2.1.2.1. Microbiological culture ..... 47
2.1.2.2. MALDI-TOF ..... 50
2.1.2.3. API 20E ..... 50
2.1.2.4. Serological agglutination ..... 51
2.1.2.5. Antimicrobial susceptibility testing ..... 52
2.1.3. Statistical analysis ..... 54
2.2. Methods for Chapter 4 (Diarrhoeagenic *Escherichia coli*) ..... 54
2.2.1. Ethics statement and study design ..... 54
2.2.2. Development of multiplex real-time PCR assays for detecting diarrhoeagenic *Escherichia coli* ..... 55
2.2.2.1. Primers and probes design ..... 57
2.2.2.2. Construction of plasmids containing target sequences ..... 61
2.2.2.3. Reproducibility and linearity ..... 61
2.2.3. Specimen procedure and MacConkey sweep storage ..... 62
2.2.4. Data collection and statistical analysis ..... 62
2.3. Methods for Chapter 5 (xTAG GPP, Luminex) ..... 63

2.3.1. Study setting ..... 63
2.3.1.1. Ethics ..... 63
2.3.1.2. Clinical specimens and study procedures ..... 64
2.3.2. Conventional methods (microbial culture and real-time PCR) ..... 64
2.3.2.1. Microbial culture ..... 64
2.3.2.2. Molecular detection of bacterial pathogens ..... 65
2.3.2.3. Molecular detection of rotavirus, norovirus GI/GII, and adenovirus ..... 65
2.3.3. xTAG gastrointestinal pathogen panel assay ..... 66
2.3.3.1. Nucleic acid extraction ..... 66
2.3.3.2. Bead Hybridization and data analysis ..... 67
2.3.4. Statistical analysis ..... 67
2.3.4.1. Sensitivity and specificity calculation ..... 67
2.3.4.2. *Salmonella* detection by xTAG GPP assay ..... 70
2.3.4.3. Spearman's correlation for co-infection among enteric pathogens ..... 70
2.4. Methods for chapter 6 (Whole Genome Sequencing of non-typhoidal *Salmonella*) ..... 70
2.4.1. Ethics and study setting ..... 70
2.4.2. *Salmonella* culture and antimicrobial susceptibility ..... 71
2.4.3. Bacterial genomic DNA extraction ..... 71
2.4.4. Whole genome sequencing and *in-silico Salmonella* serovar typing ..... 71
2.4.5. Identification of antimicrobial resistance genes ..... 72
2.4.6. Correlation of antimicrobial susceptibility phenotypes and genotypes ..... 72
2.4.7. Data analysis ..... 74
3. Assessing the benefit of empirical antimicrobial therapy for paediatric diarrhoea in a high-usage, high-resistance setting, Children's hospitals in Ho Chi Minh City, Vietnam ..... 75
3.1. Abstract ..... 75
3.2. Introduction ..... 76

3.3. Results ..... 77
3.3.1. General characteristics of patients hospitalised with diarrhoea ..... 77
3.3.2. Antimicrobial usage for treatment of hospitalised diarrhoeal diseases ..... 84
3.3.3. Antimicrobial susceptibility ..... 84
3.3.4. Diarrhoeal disease outcome ..... 88
3.4. Discussion ..... 94
3.5. Conclusions ..... 96
4. Validating and using a multiplex real-time PCR assay to detect diarrhoeagenic *Escherichia coli* pathotypes in children with diarrhoea ..... 97
4.1. Abstract ..... 97
4.2. Introduction ..... 99
4.3. Results ..... 100
4.3.1. Multiplex real-time PCR assay for detecting diarrhoeagenic *Escherichia coli* ..... 100
4.3.2. The prevalence of diarrhoeagenic *Escherichia coli* from faecal specimens of diarrhoeal hospitalised children ..... 102
4.3.3. Clinical manifestations of diarrhoeagenic *Escherichia coli* mono-infection ..... 104
4.3.4. Diarrhoeagenic *Escherichia coli* from faecal specimens of diarrhoeal hospitalised children vs. healthy non-diarrhoea children ..... 107
4.4. Discussion ..... 114
4.5. Conclusions ..... 117
5. An evaluation of the Luminex xTAG Gastrointestinal Pathogen Panel assay for the detection of multiple diarrhoeal pathogens in faecal samples ..... 118
5.1. Abstract ..... 118
5.2. Introduction ..... 119
5.3. Results ..... 120
5.3.1. Baseline results ..... 120

5.3.2. Validation ..... 121
5.3.3. Negative specimen evaluation ..... 123
5.3.4. Performance evaluation on clinical specimens ..... 125
5.3.5. Co-infection ..... 127
5.4. Discussion ..... 130
5.5. Conclusion ..... 132
6. Genoserotyping and antimicrobial resistance determinant detection of non-typhoidal *Salmonella* isolated from children hospitalised with gastroenteritis ..... 134
6.1. Abstract ..... 134
6.2. Introduction ..... 136
6.3. Results ..... 137
6.3.1. Study baseline information ..... 137
6.3.2. Clinical manifestations and empirical treatment of NTS infections ..... 137
6.3.3. *Salmonella* genoserotyping ..... 140
6.3.4. Antimicrobial resistance in non-typhoidal *Salmonella* ..... 142
6.3.5. Antimicrobial resistance gene content and genotype-phenotype concordance ..... 147
6.3.5.1. Quinolone resistance ..... 147
6.3.5.2. β-lactam resistance ..... 148
6.3.5.3. Sulphonamide and trimethoprim resistance ..... 148
6.3.5.4. Macrolide resistance ..... 148
6.3.5.5. Aminoglycoside resistance ..... 149
6.4. Discussion ..... 154
6.5. Conclusions ..... 156
7. General discussion ..... 157
8. References ..... 161
9. Appendices ..... 192

9.1. Protocol of 12EN study ..... 192
9.2. Ethic Committee Approval ..... 220
9.3. Patient Information Form ..... 224
9.4. Informed Consent Form ..... 226
9.5. Case Report Form ..... 228
9.6. Microbiology faecal result at OUCRU laboratory ..... 233
9.7. Publications during DPhil candidature ..... 237

## List of Tables

**Table 1.1.** Median number of illnesses, deaths, and Disability Adjusted Life Years (DALYs), 2010, adapted from World Health Organization, sorted by pathogens (virus, bacteria and parasites) and by the number of illnesses, 95% confidence intervals are not included ..... 3

**Table 1.2.** Classification of diarrhoeal diseases and list of pathogens causing diarrhoea ..... 5

**Table 1.3.** Antimicrobial therapy for bacterial gastroenteritis (from clinical guidelines for management of acute Gastroenteritis in children in Europe, update 2014)..... 14

**Table 1.4.** Summary of virulence genes; function and pathogenesis of related genes; and epidemiology and clinical diseases of six major pathotypes of diarrhoeagenic *Escherichia coli*. 27

**Table 1.5.** Summary of antimicrobial drugs, mechanism of action, mechanism of resistance and genes related to antimicrobial resistance ..... 33

**Table 2.1.** Zone diameter interpretive standard according to CLSI guidelines and multidrug resistance determination ..... 53

**Table 2.2.** List of gene code downloaded from BLAST (NCBI) (https://blast.ncbi.nlm.nih.gov)..... 59

**Table 2.3.** Sequences of primers and probes to detect diarrhoeagenic *Escherichia coli* ..... 60

**Table 2.4.** Formulae for calculation of sensitivity, specificity, and 95%CI of xTAG GPP assay with microbiological culture as gold standard..... 68

**Table 2.5.** Formulae for calculation of sensitivity, specificity, and 95%CI of xTAG GPP assay with real-time PCR as gold standard ..... 69

**Table 2.6.** The formulae for the calculation of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and 95%CI ..... 73

**Table 3.1.** Demographic and clinical manifestations of paediatric patients admitted with diarrhoea in Ho Chi Minh City..... 79

**Table 3.2.** Clinical characteristics of those infected with *Campylobacter*, Non-typhoidal *Salmonella,* and *Shigella* ..... 82

**Table 3.3.** Univariate and multivariate analysis of diarrhoeal symptoms and treatment on diarrhoeal disease outcome (i.e. length of stay at hospital) using an accelerated failure time (i.e. Log-Normal) model ..... 93

**Table 4.1.** Reproducibility of the assays on diluted plasmid DNA containing cloned target sequences ..... 101

**Table 4.2.** Diarrhoeagenic *Escherichia coli* detected from bacterial colonies sweeps in children hospitalised with diarrhoea (N=2,815) ..... 103

**Table 4.3.** Demographic and clinical manifestations of diarrhoeagenic *Escherichia coli* mono-infection in children hospitalised with diarrhoea ..... 105

**Table 4.4.** The detection of diarrhoeagenic *Escherichia coli* from children hospitalised with diarrhoea and healthy children ..... 108

**Table 4.5.** Univariate and multivariate analysis of diarrhoeagenic *Escherichia coli* mono-infection and co-infection associated with diarrhoeal disease among children from 24-60 months of age using binary logistic regression model ..... 112

**Table 5.1.** Number of positive Luminex xTAG GPP assay results on samples known to be positive by microbiological culture and real-time PCR ..... 122

**Table 5.2.** Sensitivity and Specificity of Luminex xTAG GPP in comparison to real-time PCR and microbiological culture ..... 126

**Table 5.3.** The value of correlation matrix of co-infections from 479 diarrhoeal stool samples 128

**Table 6.1**. Demographic and clinical manifestations of diarrhoeal paediatric patients infected with non-typhoidal *Salmonella* (N=450) ..... 139

**Table 6.2.** Non-typhoidal *Salmonella* serovars isolated from children with diarrhoeal diseases in three tertiary hospitals in Ho Chi Minh City ..... 141

**Table 6.3.** Prevalence of antimicrobial genes (AMR genes) among non-typhoidal *Salmonella* isolates from children hospitalised with diarrhoeal diseases in Ho Chi Minh City (N=450) ..... 150

**Table 6.4.** The performance of whole genome sequencing in determine the antimicrobial susceptibility of non-typhoidal *Salmonella* ..... 152

**Table 6.5.** The prevalence of mutations in the quinolone resistance determining region (QRDR) in non-typhoidal *Salmonella* isolated from children hospitalised with diarrhoeal diseases and the association with fluoroquinolone resistance..... 153

## List of Figures

**Figure 2.1.** Steps in the study of children hospitalised with bloody and/or mucoid diarrhoea..... 45

**Figure 2.2.** Flow chart for faecal specimen tests of common enteric bacteria identification, confirmation and susceptibility tests ..... 49

**Figure 2.3.** Four multiple real-time PCR assays for detecting target sequences of diarrhoeagenic *Escherichia coli.* ..... 56

**Figure 3.1.** The antimicrobial resistance profiles of *Campylobacter* spp., non-typhoidal *Salmonella* spp., and *Shigella* spp. ..... 86

**Figure 3.2.** Antimicrobial susceptibility and recovery in patients treated with antimicrobials .... 89

**Figure 3.3.** The effect of antimicrobial treatment on clinical outcome ..... 90

**Figure 3.4.** Investigating the effect of antimicrobial resistance on clinical outcome ..... 91

**Figure 4.1.** Mono- and co-infection with diarrhoeagenic *Escherichia coli* in the 24-60 month-old diarrhoeal and healthy groups ..... 110

**Figure 5.1.** Count of pathogens detected by the Luminex xTAG GPP assay in 307 specimens that tested negative by microbiological culture and real-time PCR. ..... 124

**Figure 5.2.** Correlation matrix of co-infections from 479 diarrhoeal stool samples..... 129

**Figure 6.1.** Heat map of antimicrobial resistance profiles of ten most prevalent non-typhoidal *Salmonella* serovars..... 144

**Figure 6.2.** Antimicrobial resistance phenotype and genotype of biphasic variant of *Salmonella* Typhimurium (n=76).....145

**Figure 6.3.** Antimicrobial resistance phenotype and genotype of *Salmonella* Newport (n=29).....146

## Abbreviations

| | |
|---|---|
| AFT | Accelerated failure time |
| AIDS | Acquired immune deficiency syndrome |
| AMC | Amoxicillin-clavulanate |
| AMK | Amikacin |
| AMP | Ampicillin |
| AMR | Antimicrobial resistance |
| AZI | Azithromycin |
| BA | Blood agar |
| BHI media | Brain heart infusion media |
| BHQ | Black Hole Quencher |
| BLAST | Basic Local Alignment Search Tool |
| cAMP | Cyclic adenosine monophosphate |
| CARD | Comprehensive Antibiotic Resistance Database |
| CAZ | Ceftazidime |
| CDC | Centers for Disease Control and Prevention |
| cDNA | Complementary deoxyribonucleic acid |
| cGMP | Cyclic guanosine monophosphate |
| CH1 | Children's Hospital 1 |
| CH2 | Children's Hospital 2 |
| CHCA | Cyano-4-hydroxycinnamic acid |
| CHL | Chloramphenicol |
| CIP | Ciprofloxacin |
| CLI | Clindamycin |
| CLSI | Clinical and Laboratory Standards Institute |

| | |
|---|---|
| CRF | Case report form |
| CRO | Ceftriaxone |
| CRP | C-reactive protein |
| Ct | Threshold cycle |
| DALYs | Disability Adjusted Life Years |
| DEC | Diarrhoeagenic *Escherichia coli* |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxynucleoside triphosphates |
| EAEC | Enteroaggregative *Escherichia coli* |
| EHEC | Enterohemorrhagic *Escherichia coli* |
| EIA | Enzyme immunoassays |
| EIEC | Enteroinvasive *Escherichia coli* |
| EPEC | Enteropathogenic *Escherichia coli* |
| ERY | Erythromycin |
| ESBL | Extended-spectrum β-lactamases |
| ESPGHAN | The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition |
| ESPID | The European Society of Paediatric Infectious Diseases |
| ETEC | Enterotoxigenic *Escherichia coli* |
| EUCAST | European Committee on Antimicrobials Susceptibility Testing |
| FDA | The United States Food & Drug Administration |
| GEMS | The Global Enteric Multicentre Study |
| GEN | Gentamicin |
| HC | Haemorrhagic colitis |
| HCMC | Ho Chi Minh City |
| HIV | Human immunodeficiency virus |

| | |
|---|---|
| HRV | Human rotavirus vaccine |
| HTD | The Hospital for Tropical Diseases |
| HUS | Haemolytic uremic syndrome |
| HVH | Hung Vuong Hospital |
| IBS | Irritable bowel syndrome |
| ICF | The inform consent form |
| IDSA | The Infectious Diseases Society of America |
| IM | Intramuscular |
| IMCI | Integrated Management of Childhood Illness |
| IMP | Imipenem |
| iNTS | Invasive non-typhoidal *Salmonella* |
| *Ipa* | Invasion plasmid antigen |
| IQR | Interquartile range |
| IRB | Institutional Review Board |
| IV | Intravenous |
| IZD | Inhibition zone diameter |
| LMICs | Low-middle income countries |
| LPS | Lipo-polysaccharide |
| MALDI-TOF MS | Matrix-assisted laser desorption ionization - time of light mass spectrometry |
| MAL-ED | Malnutrition and Enteric Disease Study |
| MC | MacConkey agar |
| MDR | Multidrug resistance |
| MFI | Median fluorescence intensity |
| MIC | Minimum inhibitory concentration |
| MLS | Macrolide, Lincosamide and streptogramin |

| | |
|---|---|
| MLST | The Multi-Locus Sequence Typing |
| mRNA | Messenger ribonucleic acid |
| NA | Nutrient agar |
| NAL | Nalidixic acid |
| NCBI | The National Center for Biotechnology Information |
| NPV | Negative predictive value |
| NSP | Non-structural protein |
| NTS | Non-typhoidal *Salmonella* |
| ORF | Open reading frame |
| ORS | Oral rehydration solution |
| OUCRU | Oxford University Clinical Research Unit |
| OxTREC | The University of Oxford Tropical Research Ethics Committee |
| PBPs | Penicillin-binding proteins |
| PMQR | Plasmid-mediated quinolone resistances |
| PO | Per os (orally) |
| PPV | Positive predictive value |
| PRV | Pentavalent rotavirus vaccine |
| qPCR | Quantitative Polymerase Chain Reaction |
| QRDR | Quinolones resistance-determining region |
| real-time PCR | Real-time Polymerase Chain Reaction |
| ROC | Receiver operating characteristic |
| rpm | Revolutions per minute |
| rRNA | Ribosomal ribonucleic acid |
| RT-PCR | Reverse-transcriptase Polymerase Chain Reaction |
| SARGI | *Salmonella* azithromycin resistance genomic island |

| | |
|---|---|
| SISTR | The *Salmonella* In Silico Typing Resource |
| STEC | Shigatoxin-producing *Escherichia coli* |
| SXT | Trimethoprim–sulfamethoxazole |
| TDAS | The xTAG Data Analysis Software |
| TMP | Trimethoprim |
| TR | Time ratio |
| tRNA | Transfer ribonucleic acid |
| UK | United Kingdom |
| UNICEF | United Nations Children's Fund |
| US | United States |
| VIZIONS | Vietnam Initiative on Zoonotic Infections |
| WASH | Water, Sanitation And Hygiene |
| WGS | Whole genome sequencing |
| WHO | World Health Organization |
| XLD | Xylose-lysine-deoxycholate agar |
| xTAG GPP | The Luminex xTAG Gastrointestinal Pathogen Panel assay |

## Publications during my DPhil Candidature


1. Thompson CN, Anders KL, Nhi LTQ, Tuyen HT, Van Minh P, Tu LTP, et al. A cohort study to define the age-specific incidence and risk factors of Shigella diarrhoeal infections in Vietnamese children: a study protocol. BMC Public Health [Internet]. 2014 Dec 17;14:1289. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25514820
2. Thompson CN, Phan MVT, Hoang NVM, Minh P Van, Vinh NT, Thuy CT, et al. A prospective multi-center observational study of children hospitalized with diarrhea in Ho Chi Minh City, Vietnam. Am J Trop Med Hyg [Internet]. 2015 May 23 [cited 2015 Mar 25];92(5):1045–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25802437
3. Thompson CN, Zelner JL, Nhu TDH, Phan MVT, Hoang Le P, Nguyen Thanh H, et al. The impact of environmental and climatic variation on the spatiotemporal trends of hospitalized pediatric diarrhea in Ho Chi Minh City, Vietnam. Heal Place. 2015;35:147–54.
4. Thompson CN, Tu LTP, Anders KL, Hieu NT, Vi LL, Chau NVV, et al. The transfer and decay of maternal antibody against Shigella sonnei in a longitudinal cohort of Vietnamese infants. Vaccine [Internet]. Elsevier Ltd; 2016;34(6):783–90. Available from: http://dx.doi.org/10.1016/j.vaccine.2015.12.047
5. Duong VT, Phat VV, Tuyen HT, Dung TTN, Trung PD, Minh P Van, et al. Evaluation of Luminex xTAG Gastrointestinal Pathogen Panel Assay for Detection of Multiple Diarrheal Pathogens in Fecal Samples in Vietnam. J Clin Microbiol [Internet]. 2016 Apr;54(4):1094–100. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26865681
6. Chung The H, Rabaa M a, Pham Thanh D, De Lappe N, Cormican M, Valcanis M, et al. South Asia as a Reservoir for the Global Spread of Ciprofloxacin-Resistant Shigella sonnei: A Cross-Sectional Study. PLoS Med [Internet]. 2016;13(8):e1002055. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27483136

7. Duong VT, Tuyen HT, Minh P Van, Campbell JI, Phuc H Le, Nhu TDH, et al. No clinical benefit of empirical antimicrobial therapy for pediatric diarrhea in a high usage, high resistance setting. Clin Infect Dis [Internet]. 2017;(November):1–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29029149

8. Thi Quynh Nhi L, de Alwis R, Khanh Lam P, Nhon Hoa N, Minh Nhan N, Thi Tu Oanh L, et al. Quantifying antimicrobial access and usage for paediatric diarrhoeal disease in an urban community setting in Asia. J Antimicrob Chemother [Internet]. 2018 Sep 1;73(9):2546–54. Available from: http://fdslive.oup.com/www.oup.com/pdf/production_in_progress.pdf

# 1. Introduction

## 1.1. Diarrhoeal disease

### 1.1.1. The burden of diarrhoeal diseases and aetiology

Diarrhoea is associated with a major burden of disease worldwide and is the second largest cause of morbidity and mortality globally[1]. This burden is largely associated with children living in low-middle income countries (LMICs)[2,3]. The global incidence of diarrhoeal episodes per year was estimated to be 3 billion cases in 1990, this incidence declined slightly to 2.39 billion cases in 2015[4]. Diarrhoeal diseases is associated with gastroenteritis, gastrointestinal disorders, and other infections[5]. Among those, gastroenteritis and foodborne diseases are the most important issues; resulting in 2 billion cases (95% confidence interval = 1.5-2.9 billion) in 2010[6]. This burden was also associated with >1.31 million deaths[4] and 78.7 million Disability Adjusted Life Years (DALYs)[6]. Diarrhoea is one of the most common reasons for hospital admission globally, with an estimated 1,731 million episodes of diarrhoea in children recorded in 2010[1,7]. This burden is highest in South Asia, Southeast Asia and Africa[1,8].

Infectious diarrhoea, including viral, bacterial, and parasitic infection, is the most significant cause of diarrhoeal disease. Of which, viral diarrhoea is the most common cause[8,9]. Rotavirus infection has been reported to be responsible for 37.3% of all episodes from children hospitalised for diarrhoea worldwide[9]. This disease burden was accompanied by roughly 528,000 and 215,000 deaths in 2000 and 2013, respectively[9]. In Vietnam, rotavirus was identified in the stools of 50% of children <5 years old hospitalised with diarrhoea in 1998-2000 and estimated to be associated with 2,700-5,400 deaths per year[10]. Although rotavirus vaccine has been licensed and is widely available, the uptake and protective immunity in Asia is not as great as in high income regions, this is due, in part, to a reduced immunity in LMICs[11,12]. Hence, rotavirus remains the most common cause of diarrhoea in regions without rotavirus vaccination, with low vaccine coverage, and with poor efficacy. Additionally, burden of norovirus is increasing worldwide[6,13].

The median number of illnesses, deaths, and DALYs associated with diarrhoeal diseases caused by specific enteric pathogens at all ages describes high burdens of diseases caused by enteric bacteria (Table 1.1). These bacteria include Enterotoxigenic *Escherichia coli* (ETEC), *Shigella*, *Campylobacter,* and *Salmonella*. A high number of deaths were associated with norovirus, Enteropathogenic *Escherichia coli* (EPEC), and *Vibrio cholera*. Moreover, the Global Enteric Multicentre Study (GEMS) and Malnutrition and Enteric Disease Study (MAL-ED) were the two largest studies ever designed to measure the burden of diarrhoea caused by various enteric pathogens in developing countries[14,15]. GEMS, a prospective case control study in children <5 years of age, concluded that rotavirus, *Cryptosporidium*, *Shigella*, and ST-ETEC (ST only or LT/ST strains) were the four most common pathogens associated with moderate-to-severe diarrhoea[14]. The pathogens most commonly identified in MAL-ED, a multisite birth cohort study with the follow-up until 24 months of age, were *Campylobacter* and EAEC[15]. These studies show that bacterial infections are important aetiological agents in diarrhoeal disease.

**Table 1.1.** Median number of illnesses, deaths, and Disability Adjusted Life Years (DALYs), 2010, adapted from World Health Organization[8], arranged by pathogens (virus, bacteria and parasites) and by the number of illnesses, 95% confidence intervals are not included

| **Pathogen** | **Illnesses** | **Deaths** | **DALYs** |
|---|---|---|---|
| Diarrhoeal Diseases | 1,912,159,038 | 715,196 | 55,139,959 |
| norovirus | 684,850,131 | 212,489 | 15,105,714 |
| Enterotoxigenic *Escherichia coli* (ETEC) | 240,886,759 | 73,875 | 5,887,541 |
| *Shigella* spp. | 190,849,501 | 65,796 | 5,407,736 |
| *Campylobacter* spp. | 166,175,078 | 37,604 | 3,733,822 |
| Non-typhoidal *Salmonella* | 153,097,991 | 56,969 | 4,377,930 |
| Enteropathogenic *Escherichia coli* (EPEC) | 81,082,327 | 122,760 | 9,717,390 |
| *Vibrio cholera* | 3,183,394 | 105,170 | 7,347,635 |
| Shiga toxin-producing *Escherichia coli* (STEC) | 2,481,511 | 269 | 26,827 |
| *Giardia* spp. | 183,842,615 | 0 | 171,100 |
| *Entamoeba histolytica* | 103,943,952 | 5,450 | 515,904 |
| *Cryptosporidium* spp. | 64,003,709 | 27,553 | 2,159,331 |

### 1.1.2. Definition and classification of diarrhoeal diseases

Diarrhoea is defined as the passage of unusually loose or watery stools at least three times in a 24 hour period[16]. Loose or watery stools mean that the stools take the shape of a container; notably the consistence of stool is more important than the frequency of bowel movements. Diarrhoea is the common symptom leading to health service requirement and hospitalisation[17]. In order to approach diarrhoeal diseases clinically, various types of classification are used to establish the aetiology or underlying pathology. Diarrhoea can be classified into many ways[18], these are briefly described in Table 1.2.

Clinical manifestations are the most practical and common approaches to classify diarrhoea. The clinical manifestation of the various aetiological agents is not specific and diagnostic tests are not used routinely in LMICs. Therefore, the World Health Organization (WHO) recommends that the management of diarrhoeal diseases should follow a clinical approach (i.e. duration of diseases and characteristics of stool)[16]. There are four clinical types of diarrhoea these are 1) acute watery, 2) acute bloody diarrhoea, 3) persistence diarrhoea, and 4) diarrhoea with severe malnutrition. This approach aims to help health workers to manage and treat the main complications associated with each type of diarrhoea, which is independent of aetiology determination[16].

Generally, diarrhoea can be pragmatically classified by its characteristics as being watery or dysenteric. Watery diarrhoea is characterised by liquid or semi-liquid stools, usually of large volume, with or without fever, vomiting, and abdominal pain[16]. This type of diarrhoea is generally associated with any type of viral, bacterial, or parasitic agents[18]. However, dysentery, a syndrome characterised by fever, abdominal cramps, and tenesmus with small, frequent bloody stools, sometimes with mucus (colitis or colonic inflammation)[19], is associated with a narrower list of pathogens, i.e. bacterial infection. This approach is simplistic and impacts both diagnostic evaluation and empirical therapy[20].

**Table 1.2.** Classification of diarrhoeal diseases and list of pathogens causing diarrhoea

| Classification | Main features |
|---|---|
| **Duration** | |
| Acute[16] | < 14 days |
| Persistent[16] | ≥ 14 days. Infection is most common cause of persistent diarrhoea. Persistent diarrhoea associated with mortality due to high rate of malnutrition[21]. |
| Chronic[22] | > 30 days |
| **Stool characteristics** | |
| Watery[16] | Liquid or semi-liquid stools, usually of large volume. The main danger is dehydration. |
| Bloody diarrhoea | The loose or watery stools contain visible red blood[19]. The main dangers are damage of the intestinal mucosa, sepsis and malnutrition[16]. |
| Dysentery[19] | The same meaning as bloody diarrhoea. Although this term may be used to describe a syndrome including fever, abdominal cramps, rectal pain and mucous stools. These symptoms do not always accompany with bloody diarrhoea[19]. |
| **Epidemiology**[18] | |
| Community-acquired | Most common among other types of diarrhoea. Most common cause is foodborne diseases. |
| Hospital-acquired | The onset of diarrhoea 3 days after hospitalisation and not incubating at the time of admission to the hospital[21]. |
| Traveller's diarrhoea | The frequency of traveller's diarrhoea varies between regions with the highest rates seen in Latin America, Africa, Middle East, and India. The intermediate rates are seen for travellers to China, Russia, eastern Europe, and South Africa[23]. |
| **Pathophysiology (more than one pathophysiology mechanism may consist in one clinical diarrhoeal disease)** | |
| Secretory diarrhoea | When pathogens infected small intestine causing an increase of secretion of ions (chloride or bicarbonate) or decrease sodium absorption resulting in water transported to the lumen[24]. |
| Osmotic diarrhoea | Osmotic diarrhoea occurs either when non-absorbable (mannitol, sorbitol) or poorly absorbable solutes (magnesium, sulphate, phosphate) or are ingested or enterocytes cannot absorb them (malabsorption, lactase deficiency[25]). |

| | | | |
|---|---|---|---|
| Inflammatory diarrhoea /invasive diarrhoea | Infectious pathogens causing inflammatory diarrhoea primarily affect the distal small bowel or the colon. This refers to diarrhoea caused by bacterial pathogens that invade the bowel mucosa, causing inflammation and tissue damage[19]. | | |
| Iatrogenic or drug-induced diarrhoea | Antibiotic use may alter the bacterial flora in the colon resulting in impaired colonic salvage of malabsorbed carbohydrates resulting in diarrhoea. Erythromycin interacts with the motilin receptors increasing the motility to cause diarrhoea. | | |
| Functional/ motility diarrhoea | Alteration in colonic transit/motility and hypersensitivity of the rectum play a role in diarrhoea. This rectal hypersensitivity is more likely seen with diarrhoea-predominant irritable bowel syndrome (IBS)[25]. | | |
| **Aetiology**[6,18] | | | |
| Gastrointestinal infection | **Virus** | **Bacteria** | **Protozoa and fungi** |
| | rotavirus | *Campylobacter spp.* | *Entamoeba histolytica* |
| | norovirus | Non-typhoidal *Salmonella* | *Giardia spp.* |
| | adenovirus | *Shigella spp.* | *Cryptosporidium spp.* |
| | astrovirus | *Escherichia coli* (ETEC, EAEC, EIEC, | *Microsporidia* |
| | torovirus | EPEC, EHEC, STEC) | *Cyclospora cayetanensis* |
| | | *Vibrio cholera* | *Candida* |
| | | *Yersinia spp.* | *Aspergillus* |
| | | *Clostridium difficile* | |
| | | *Mycobacterium tuberculosis* | |
| | | *Aeromonas plesoides* | |
| Other infections | Other gastrointestinal tract infection including appendicitis, peritonitis, and intra-abdominal abscesses, meningitis, lower lobe pneumonia, sepsis, or urinary tract infection. | | |
| Non-infectious gastrointestinal disorders | Anatomic or malabsorptive issues such as bowel obstruction or inflammatory bowel disease, metabolic disorders, toxin ingestion, heart failure, and trauma. | | |

enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), Shigatoxin-producing *E. coli* (STEC) and enterohemorrhagic *E. coli* (EHEC)

The list was adapted from a textbook "Diarrhoea-diagnostic and therapeutic advances"[18] and the estimation of global burden of foodborne diseases of World Health Organization[6].

### 1.1.3. The diagnosis of the aetiological agents of diarrhoea

Laboratory studies can be performed to identify the cause of diarrhoea, to guide therapy, and for epidemiology surveillance, but often a laboratory analysis of a diarrhoeal sample is not routinely required because most episodes of diarrhoea are self-limiting. The Infectious Diseases Society of America (IDSA) recommend that stool cultures should be performed in cases presenting with diarrhoea for >1 day, fever, blood in stools, other complications such as dehydration, systemic illness, or a suspicion of a bacterial infection[22]. Moreover, establishing an aetiological diagnosis for a specific episode is often complex because of the wide array and complexity of potential infectious agents[26]. The use of specialised laboratory tests is also dependant on the skill of local laboratory staff and available resources.

In this section, I briefly described the principle and application of diagnostic methods for enteric pathogens used in my research, i.e. bacterial culture, API 20E, matrix-assisted laser desorption ionization-time of light (MALDI-TOF), serological agglutination tests, real-time Polymerase Chain Reaction (real-time PCR), xTAG GPP (Luminex), and whole genome sequencing (WGS).

#### 1.1.3.1. Microbial culture

Bacterial gastroenteritis is most accurately diagnosed by performing microbiological culture on stool specimens. Selective media that inhibit the growth of the normal microbiota, such as MacConkey (MC), or xylose-lysine-deoxycholate (XLD) agar, should be used for culture followed by standard biochemical identification[27]. This conventional method is relatively simple, inexpensive and available in most microbiological laboratories. However, when large numbers of samples are being processed it is also laborious and time consuming due to the time during which microorganisms take to grow in different culture media under specific laboratory conditions. In LMICs, the consumption of antimicrobials in the community can limit the sensitivity[28]. Despite many disadvantages, bacterial culture is an essential primary step for important further

investigations, including antimicrobial susceptibility profiling, molecular characterisation, and bacterial genomics.

#### 1.1.3.2. API 20E

The API strip is a collection of standardised biochemical tests for the identification of bacteria[29]. Different API kits are available for various group of bacteria, the API 20E is a set of 20 biochemical tests in 20 microtubes for identifying organisms within the Gram-negative bacteria family the *Enterobacteriaceae*. A bacterial suspension (pure culture of a single organism) is added into each tube; during incubation, metabolism produces tests various chemical reactions. The results of these reactions are read according to the package insert for a +/- reaction and an identification is generated by using the Analytical Profile Index or software[29]. If specific organisms are isolated, such as *Salmonella* or *Shigella*, identification can be followed by serological identification to confirm the biochemical result and to identify the infection species or serotype.

#### 1.1.3.3. Mass spectrometry-based identification

Mass spectrometry has been used in chemistry for several decades, but in 1975 Anhalt and Fenselau proposed that bacterial detection could be performed using this method[30]. In the late 1980s, a matrix-assisted laser desorption ionization (MALDI) was developed leading to the analysis of large bio-molecules such as proteins[31]. In 1996, Holland reported using spectral fingerprints from MALDI to identify whole bacterial cells without pretreatment[32]. This technique was then developed to identify bacteria at the genus and species levels[33,34]. In short, MALDI is a method which utilises a UV laser strike in matrix of organic material on stain steel plate to form gaseous ions[35]. These ions are put into a high voltage electric field to impart a constant amount of kinetic energy[35]. The ions then are capable to travel from the stain steel plate to the detector. Specific mass spectra are created by measuring the time of flight (TOF) of these ions[32]. The mass

spectra are compared with a database containing multiple fingerprints to characterise and identify the microorganism[36].

MALDI-TOF is a rapid and accurate method for bacterial confirmation. However, unlike blood culture, where detection can be performed directly on a positive blood culture broths[37], identification of a bacteria in a stool sample requires the successful isolation of a probable pathogen[38]. MALDI-TOF can identify *Salmonella* at genus level, but not to the serotype level and can identify *Campylobacter* to the species level[39,40]. Serotyping *Salmonella* by MALDI-TOF to circumvent conventional serotyping by slide agglutination has been established but is limited to only specific serovars[41]. However, current databases cannot differentiate *E. coli* from *Shigella* or *E. coli* from STEC or other *E. coli* pathotypes[38].

#### 1.1.3.4. Bacterial agglutination

*Enterobacteriaceae* have complex antigenic structures. They are identified by more than 150 different heat-stable somatic O (lipopolysaccharide) antigens, more than 100 heat-labile K (capsule) antigens, and more than 50 H (flagella) antigens[42]. O antigens are the external most fraction of the cell lipopolysaccharide[42]. K antigens, which generally form polysaccharide capsules, can interfere with O antisera agglutination, and are often associated with a more virulent phenotype[42]. H antigens are associated with various monophasic and biphasic forms of flagella. Within a single serotype, flagella antigens may be present in either or both of two forms, called phase 1 (conventionally designated by lower-case letters) and phase 2 (conventionally designated by Arabic numerals)[42]. Antigen-antibody complexes (usually IgG) are formed (agglutination) when a bacterial culture is mixed with a specific antiserum directed against bacterial surface components[43]. The complexes are usually visible to the naked eye which allows for the easy determination of O and H antigens by slide agglutination. The application of this method is advantage in bacterial serotyping and detecting pathogens that can difficult to determine by other

conventional methods, i.e. pathogenic *E. coli*, rotavirus, norovirus and parasites (*Cryptosporidium* and *Giardia* spp.)[27,44].

#### 1.1.3.5. Real-time Polymerase Chain Reaction (real-time PCR)

Molecular diagnostics have come to play an important role in diagnosis of infectious diseases[27,45]. The sensitivity of PCR over more traditional methods has allowed for a better understanding of epidemiology of the infectious agents associated with diarrhoeal diseases[27]. The primary difference between real-time and conventional PCR is that amplified DNA in a real-time reaction are measured in "real time", as the PCR reaction is being performed[46]. In real-time PCR, a fluorescent detector is added to the PCR reagents, and the fluorescence of each sample is measured during each cycle of amplification.

Quantitative real-time PCR (qPCR) can often detect pathogens with higher sensitivity and better reproducibility in comparison to microbiological methods, including culture, enzyme immunoassays (EIA), and reverse-transcriptase PCR (RT-PCR)[47]. More specifically, quantitative real-time PCR increased detection of adenovirus 40/41 (fivefold), *Shigella* or EIEC and *Campylobacter jejuni* or *C. coli* (two fold), and ST-ETEC (1.5 times) in stool samples in comparison to conventional methods[47].

#### 1.1.3.6. xTAG Gastrointestinal Pathogen Panel assay

Given the limitations of traditional diagnostics, a multi-panel pathogen identification system that is sensitive and specific and easy to operate represents a significant improvement on classical techniques. The Luminex xTAG Gastrointestinal Pathogen Panel assay (GPP) (Luminex Molecular Diagnostics, Austin, TX, USA) – is a qualitative multiplex test cleared by the US Food & Drug Administration (FDA) that is able to identify 19 enteric pathogens in one reaction in 6 hours[48–50]. However, the assay has a prohibitive cost for routine diagnostic use in Vietnam

(approximately 75USD per sample, as of December 2015). The Luminex platform has been shown to be highly sensitive and specific for a variety of pathogens in several clinical setting globally[51–53]. Nucleic acid from the specimen is extracted and amplified in a single multiplex PCR reaction. Next, tagging primers that have been specific designed to extend the target cDNA are added. Once the specific tag primers match with the target cDNA, a label extension product is generated. Then colour coded beads contained an anti-tag sequence that only bind to the complementary tag sequence on the primer are added. During the hybridisation, the colour beads attached to specific tag primer and specific for the present pathogen. Finally, the complexes are read and analysed by lasers to identify the colour of the bead and the label primer at the same time to call the positive of the pathogen[54].

#### 1.1.3.7. Whole genome sequencing

WGS can have various clinical applications in the diagnosis and epidemiology of infectious diseases. These applications can include identifying the probable pathogen in a culture-independent method in samples from patients with various infectious syndromes[55]. In addition to pathogen identification, WGS can be used for strain typing, and to identify genotypic markers of antimicrobial resistance (AMR) and virulence[55]. Drug resistance mechanisms can be characterised using BLAST (or other sequence comparison methods) to query a sequence against available databases of bacterial AMR genes such as ResFinder[56] or Comprehensive Antibiotic Research Database (CARD; http://arpcard.mcmaster.ca)[57]. Furthermore, genetic information from the organisms when combined with phenotypic, spatial and temporal data allows for more comprehensive insight into the epidemiology of pathogens and their evolution[58].

### 1.1.4. The treatment, management, and prevention or diarrhoea

The management of diarrhoeal disease(s) includes treatment and consultant from the health workers in hospitals, and prevention throughout the public health system (i.e. Water, Sanitation

and hygiene (WASH) program[59] and vaccination). Other social issues such as education within the general public, the food industry and living conditions are important contributors to the management of diarrhoeal diseases[59]. In this section, I summarised the key points associated with managing diarrhoea through the healthcare service, including WASH programs and vaccination.

#### 1.1.4.1. Principles of treatment diarrhoea diseases

As a paediatrician, I have trained and practiced according to the Integrated Management of Childhood Illness (IMCI) developed by WHO and UNICEF[60,61]. These guidelines are augmented by European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) and the European Society of Paediatric Infectious Diseases (ESPID)[62]. The principle of treatment of all types of diarrhoea includes rehydration, continued feeding, and supplementary minerals[60]. The extent of the rehydration therapy is dependent on the degree of dehydration graded according to symptoms and signs (i.e. general condition, sunken eyes, skin pinch, and reaction of the child to fluid), which reflect the amount of fluid lost[61]. After assessment, the hydration status can be classified as severe dehydration, some dehydration, or no dehydration. In some unclear cases, other symptoms (i.e. capillary refill, skin turgor, and urinary output) can and should also be considered. The dehydration status should be noted, not only as a symptom, but also to assess the severity of the diarrhoea[63].

Zinc is an important micronutrient for the general health and development of a child but is lost in large quantities during diarrhoea[61]. Zinc replacement aids the child's recovery, reduces the duration and severity of the disease episode, and lowers the incidence of diarrhoea in the following 2-3 months[61]. Recent studies show that supplementary zinc may also benefit children aged >6 months in developing countries; however, providing zinc supplements in regions where zinc deficiency is rare is not beneficial[62].

During diarrhoea, decreased food intake and nutrient absorption and increased nutrient requirements often combine to cause weight loss and failure to grow[61]. Malnutrition can make diarrhoea more severe, more prolonged and more frequent than in well-nourished children[61]. Diet according to age group including breastfeeding should be continued during rehydration therapy or at 4-6 hours after initial rehydration)[62]. Growth status is a compulsory index needed to be assessed in all paediatric hospitals.

#### 1.1.4.2. Antimicrobial therapy

The recommendations for antimicrobial therapy in a manual for physicians and other senior health workers from the WHO in 2005 stated that "antimicrobials should not be used except for children with bloody diarrhoea (probable shigellosis); suspected cholera with severe dehydration and other serious non-intestinal infections such as pneumonia and urinary tract infection"[16]. ESPGHAN emphasised the unnecessary routine use of antimicrobials for bacterial gastroenteritis; but only for specific pathogens and in defined clinical settings. In Table 1.3, I presented the antimicrobial therapy used to treat the most common enteric bacteria according to a consensus of ESPGHAN and ESPID[62]. However, the application of these guidelines should depend on the antimicrobial susceptibility patterns of local pathogens and the clinical settings. A detailed indication for each enteric bacterium was discussed in section 1.2.

**Table 1.3.** Antimicrobial therapy for bacterial gastroenteritis (from clinical guidelines for management of acute Gastroenteritis in children in Europe, update 2014)[62]

| Pathogen | Indication for antibiotic therapy | Drug of choice* | Alternative agents |
|---|---|---|---|
| *Shigella* spp. | Proven or suspected shigellosis | Oral: azithromycin (12mg/kg on day 1, followed by 6mg/kg for 4 days);<br>Parenteral, IV, IM: ceftriaxone (50mg/kg for 2-5 days)** | Cefixime (8mg/kg/day);<br>Ciprofloxacin PO (20-30mg/kg/day)<br>For a known susceptible strain: TMP/SMX** (8mg/kg/day of TMP) or ampicillin (100mg/kg/day) or nalidixic acid (55mg/kg/day) |
| *Salmonella* spp. (non-typhoidal) | Indicated only in high risk children to reduce the risk of bacteraemia and extra intestinal focal infection | Ceftriaxone (50-100mg/kg/day) | Azithromycin (10mg/kg/day);<br>Ciprofloxacin*** PO (20-30mg/kg/day);<br>For a known susceptible strain, TMP/SMX (8mg/kg/day of TMP) |
| *Campylobacter* spp. | for the dysenteric *Campylobacter* gastroenteritis and most efficacious when stated within 3 days after the onset of the diseases | Azithromycin (10mg/kg/day for 3 days; or a single dose of 30mg/kg) | Doxycycline (>8years) or ciprofloxacin (>17 years) when susceptible |
| STEC | not recommended | - | - |
| ETEC | recommended mainly for traveller's diarrhoea | Azithromycin (10mg/kg/day for 3 days) | Cefixime (8mg/kg/day for 5 days);<br>TMP/SMX (8mg/kg/day of TMP);<br>Ciprofloxacin PO (20-30mg/kg/day)<br>Rifaximin (>12years, 600mg/day, for 3days) |
| *Vibrio cholera* | Antibiotic therapy is recommended for confirmed or suspected case by travel history | Azithromycin (10mg/kg/day for 3 days, or a single 20mg/kg dose) | Doxycycline (>8years);<br>Ciprofloxacin (>17 years), or<br>TMP/SMX (when susceptible) |
| *Clostridium difficile* | Antibiotic therapy is recommended for moderate and severe cases | Metronidazole (30mg/kg/day for 10 days) | Vancomycin PO (40mg/kg/day) |

PO = per os.
*Depends on local antibiotic susceptibility profile, which should be monitored; **TMP/SMX, trimethoprim–sulfamethoxazole.
***Ciprofloxacin is usually not recommended in the paediatric age group, but it can be used in children <17 years when an alternative is not feasible.

#### 1.1.4.3. Other pharmacological therapies

Other drugs, such as probiotics, prebiotics, micronutrients, antiemetic (Ondansetron), antimotility or antiperistalsis drugs (Loperamide), adsorbents (Diosmectite), antisecretory drugs (Racecadotril) were described in the updated evidence-based guidelines from ESPGHAN and ESPID[62]. In summary, probiotics, racecadotril, and diosmectite can be considered in acute gastroenteritis management. Others were not recommended and required more supportive evidence.

#### 1.1.4.4. Prevention

Appropriate treatment of diarrhoeal diseases is effective in preventing fatal cases; however, appropriate treatment has no impact on the incidence of diarrhoea[16]. Health workers try to prevent onward cases of diarrhoeal disease by practicing hygienic principles in healthcare facilities and consulting the patients or the caregivers regarding the care of children at home. The WHO lists seven points of information in preventing diarrhoea, health workers should emphasise one or two appropriate points for the particular mother and child[16]. Exclusive breastfeeding during the first 6 months of age is significant to protect most children from diarrhoea[16]. Introducing nutritious foods, hygienic food preparation and continued breastfeeding are the next stage in maintaining a normal growth status and avoiding diarrhoea[16]. Water and food safety, hand washing and the appropriate disposal of faecal materials are also recommended by the medical practitioner. These various control measures are highlighted in the WASH interventions document developed by WHO and UNICEF[64].

Lastly, immunisation is one of the most effective interventions to control infectious diseases. However, only limited vaccines are available to protect against diarrhoea. Two live attenuated oral rotavirus vaccines are licensed and used globally[11]. Pentavalent human-bovine rotavirus reassortant vaccine (RV5, PRV, RotaTeq- Merck) is administered in three oral doses at two, four,

and six months of age. Attenuated human rotavirus vaccine (RV1, HRV, Rotarix-GCK) is administered in two oral doses at two and four months[65]. The first dose of rotavirus vaccine is administered from 6 weeks of age and the last dose is administered before 6-8 months of age. Rotavirus vaccination has been recommended by WHO since 2009, particularly in countries with high mortality rate associated with rotavirus gastroenteritis, including Southeast Asia and sub-Saharan Africa[66]. Currently, rotavirus vaccine is recommended and used by over 40% of the WHO member countries[67]. Both of these vaccines are effective against rotavirus disease and prevent mortality from the severe diseases caused by this virus. However, it has been well noted that the effectiveness is low in the second year after immunisation[68], and the efficacy of rotavirus vaccine is low (50-70%) in LMICs[11]. In Vietnam, it is estimated that rotavirus vaccination would effectively reduce severe cases of rotavirus by roughly 55% during the first 5 years of life[69].

## 1.2. Bacterial diarrhoea

In this section, I summarised the spectrum of bacterial pathogens causing diarrhoea. I additionally highlighted the issue of AMR and methods used to determine antimicrobial susceptibility for enteric bacteria. For the key bacterial enteric pathogens, i.e. *Campylobacter*, non-typhoidal *Salmonella* (NTS), *Shigella,* and diarrhoeagenic *Escherichia coli* (DEC), I described their bacteriology, clinical manifestations, AMR trends, and disease management.

### 1.2.1. *Campylobacter*

#### 1.2.1.1. Microbiology and clinical disease

*Campylobacter* are gram-negative, slim, curved rods bacteria with a polar flagellum located at one or both ends of the cell, making the bacteria highly motile[26]. *Campylobacter* spp. are part of the normal intestinal microbiota of wild and domesticated animals and birds. *Campylobacter* grow at an optimum temperature of 42°C and in microaerophilic conditions requiring oxygen concentrations of 3-15% and carbon dioxide concentrations of 3-5%. The thermophilic

characteristics of the microorganism is thought to be an adaptation to living in the intestines of birds and the environment of animal[70]. Due to a difficulty in the culture environment, new culture-independent diagnostic tests may provide a better surveillance of campylobacteriosis (the disease caused by *Campylobacter*)[44,71].

*Campylobacter* can be isolated and identified in 2-5 days, or up to 10 days on occasions[72]. *C. jejuni* and *C. coli* cause similar clinical manifestation during human infection. Diarrhoea in campylobacteriosis is described as watery stools, with or without blood. Abdominal pain may be accelerated with severity as suspicious for surgical abdomen[73]. Usually, the diarrhoea is self-limiting, resolving within a week and requiring no antimicrobial therapy. Extra intestinal *Campylobacter* infections, such as bacteraemia, urinary tract infections, cholecystitis, hepatitis, pancreatitis, nephritis, meningitis, abortion, and neonatal sepsis have also been reported[73]. *Campylobacter* bacteraemia is generally uncommon, but it occurs more frequently in patients with human immunodeficiency virus (HIV) infection, malignancy, and liver disease[74]. Sequelae of campylobacteriosis are Guillain-Barré syndrome, reactive arthritis, and irritable bowel syndrome[75,76].

#### 1.2.1.2. Epidemiology and antimicrobial resistance

*Campylobacter* is in the top five most common causes of diarrhoea diseases in children (GEMS and MAL-ED). During 2004-2012, >300,000 culture-confirmed *Campylobacter* spp. cases were reported in the US, leading to the average annual incidence of 11.4 cases/100,000 persons[77]. *Campylobacter* outbreaks have been reported and are commonly associated with raw milk consumption or water contaminated by runoff from livestock butchers[78,79]. Member of the population who are <4 years of age or 15-44 years of age demonstrate a higher risk of *Campylobacter* infection that other members of the population[76]. *C. jejuni* and *C. coli* are the

most common *Campylobacter* spp. associated with human disease; *C. jejuni* is the most common, accounting for >90% of campylobacteriosis cases[80].

Macrolides and fluoroquinolones are commonly used for the treatment of *Campylobacter* infections; however, resistance against these antimicrobials has been widely reported[77,81–83]. In the US, 25% of *Campylobacter* isolates exhibit resistance to ciprofloxacin and 2.5% exhibit resistance to erythromycin; however, these proportions were higher among the *Campylobacter* isolates associated with a history of international travel, i.e. 67.5% resistant to ciprofloxacin and 6.8% resistant to erythromycin[77]. The AMR rates of *C. jejuni* in LMICs is alarming, with >80% isolates reported to be resistant to ciprofloxacin, >90% isolates exhibiting resistance to tetracycline, and 4-20% isolates resistant to macrolides[81–84].

#### 1.2.1.3. Management of campylobacteriosis

Typically, *Campylobacter* infections are mild and self-limiting, lasting only a few days, treatment with antimicrobials is commonly not required[85]. However, antimicrobial therapy is necessary under specific clinical situations such as severe cases, infection in immune-suppressed patients, and prolonged infections[85]. Macrolides, such as erythromycin and azithromycin, are now generally considered to be the optimal drug for treating campylobacteriosis. Fluoroquinolones and tetracycline have been used as an alternative therapy[22,62]. Aminoglycosides or carbapenems are also indicated in very severe cases of *Campylobacter* bacteraemia[73]. A meta-analysis of 11 double-blind, placebo-controlled trials showed that antimicrobial treatment of gastroenteritis caused by *Campylobacter* spp. reduces the duration of intestinal symptoms by 1.3 days[86]. This effect was more pronounced if treatment was started within 3 days of illness onset and in children with *Campylobacter* induced dysentery[86].

Controlling *Campylobacter* infections is complex and associated with multiple steps in food industry. There is a need for better practices in the poultry (meat) production chain, and monitoring in other transmission vehicles, such as raw milk and drinking-water, e.g. pasteurisation of milk and chlorination of water[76]. Various classes of antimicrobials are commonly used in farming in LMICs, these may contribute to increasing AMR in *Campylobacter*[76]. For example, the use of fluoroquinolones, such as enrofloxacin, in food-producing animals has likely resulted in the development of ciprofloxacin-resistant *Campylobacter*; such bacteria have spread throughout the world through travel and food trade[76].

### 1.2.2. Non-typhoidal *Salmonella*

#### 1.2.2.1. Bacteriology and clinical disease

*Salmonella* was named after the veterinary pathologist, Daniel Salmon, in 1900[87]. *Salmonella* are a genus of rod-shaped, Gram-negative facultatively anaerobic bacteria that belong to the family *Enterobacteriaceae*[87]. The culture and confirmation of *Salmonella* infections are relatively straightforward within a standard microbiology laboratory. However, serotyping with the *Salmonella* genus is more complex and requires multiple antisera; there are >2,600 serotypes classified by the standard Kauffman-White scheme[88]. The nomenclature of *Salmonella* is controversial and still evolving. Currently, the Centres for Disease Control and Prevention (CDC) apply the nomenclatural system of *Salmonella* recommended by the WHO Collaborating Centre[89]. Based on this system, the genus *Salmonella* is classified into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is then classified into six subspecies based on their genomic relatedness and biochemical properties[90]. Among all the subspecies of *Salmonella, Salmonella enterica* subsp. *Enterica* (I) is commonly found in mammals and contributes approximately 99% of *Salmonella* infections in humans and warm-blooded animals[26].

The clinical manifestation of NTS infection can range from asymptomatic, to mild self-limiting diarrhoea, to severe diarrhoea, or even systemic infection. Gastroenteritis is the most common manifestation of *Salmonella* infection worldwide, followed by bacteraemia and enteric fever[91]. After a short incubation period of 6 to 12 hours, the disease starts with a period of nausea, vomiting, and cramping abdominal pain; followed by watery or dysenteric diarrhoea (containing blood and mucus)[88]. Fever (38.5°C to 39°C) is present in approximately 70% of cases. Faeces from those with a symptomatic *Salmonella* infection may contain neutrophils and occult blood; and a mild peripheral blood leukocytosis with neutrophil being predominant[72]. Up to 5% of *Salmonella* infection cases can be associated with extra intestinal manifestations such as bacteraemia, arthritis, urinary tract infections, endocarditis, and meningitis[92].

#### 1.2.2.2. Epidemiology and antimicrobial resistance

The prevalence of *Salmonella* globally varies according to the standards of the water supply, waste disposal, food preparation practices, and climate[43,93–95]. The incidence of NTS has been estimated as 93.8 million cases of gastroenteritis worldwide each year, with 155,000 deaths[91]. The highest incidence rates arises in children aged <5 years; especially infants younger than 1 year[96]. In developed countries, NTS has been shown to also cause considerable hospitalisation and death[97]. NTS bloodstream infections (invasive NTS or iNTS ) is a severe clinical manifestation with a correspondingly high mortality rate[98,99].

Globally, *S.* Typhimurium has been identified as most frequent serovar associated with diarrhoea, followed by *S.* Enteritidis. The prevalence of *S.* Typhimurium in the total burden of *Salmonella* associated diarrhoea varies from 20 to 75%[96,100]. In some Asian regions, *S.* Enteritidis has been reported to be the most commonly isolated *Salmonella* serovar, with a prevalence of 19% to 50%[101–104]. Other common serovars include *S.* Kentucky, *S.* Heidelberg[101], *S.* Newport[96], *S.* Haifa, *S.* Braenderup, *S.* Choleraesuis, *S.* Dublin, *S.* Saintpaul, and *S.* Indiana[100,102].

In the 1980s, most NTS were commonly susceptible to a range of clinically relevant antimicrobials. However, in the 1990s, the CDC reported increasing resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole[105]. The resistant rates to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole in NTS isolates during this period were found to be 46.8%, 21.6% and 13.3%, respectively[106]. In Africa, up to 60% of NTS isolates were determined as resistance to ampicillin, chloramphenicol and tetracycline[100]. More problematic is that now >10% of NTS isolates are resistant to important broad spectrum antimicrobials such as ciprofloxacin and the third generation cephalosporins[103,104,107].

#### 1.2.2.3. Management of NTS infection

As with all forms of gastroenteritis, fluid and electrolyte replacement and maintenance are the first concern in the management of NTS. For most patients, oral rehydration is all that is necessary to treat NTS gastroenteritis. The disease is typically limited to the lamina propria of the intestine, and antimicrobial therapy is not indicated[7]. Furthermore, antimicrobial therapy in Salmonellosis does not shorten the course of illness, but prolongs excretion of NTS[108].

Generally, antimicrobial therapy should not be indicated for NTS gastroenteritis; except for children at high risk for developing complications, including children with underlying diseases or receiving therapies that impair host defences[108]. Examples of children who should be given antimicrobials for NTS infections are infants aged 6 months or younger; children infected with HIV, or those with acquired immune deficiency syndrome (AIDS) or malignant disease[108,109]. Those that have been splenectomised, with primary immune-deficiencies, chronic hepatopathy and haemolytic anaemia (particularly sickle-cell anaemia) and infected with NTS should also be administered appropriate antimicrobials[110].

In a case-control study conducted in the US, infants with NTS infection were less likely to have been breastfed and more likely to have consumed liquid infant formula[96]. In LMICs, breastfeeding is key in disease prevention because human milk contains secretory IgA that can protect infants from gastrointestinal infection[111]. Hence, healthcare providers and community education programs should encourage sustained breastfeeding.

Three vaccines for *S*. Typhi are available: an oral live attenuated Ty21a vaccine (Vivotif Berna), a parenteral purified Vi capsular polysaccharide vaccine (Typhim Vi), and a parenteral conjugate Vi capsular polysaccharide vaccine[112,113]. Vaccination of children is indicated when the risk for development of typhoid fever is high (e.g. living with a chronic carrier or in an endemic area)[112].

NTS vaccines have been developed for livestock animals to prevent the diseases in animals and prevent the transmission from animals to humans[93]. In some European countries, the use of *Salmonella* animal vaccines has resulted in a decrease of NTS outbreaks[93]. However, despite the need there are currently no NTS vaccines available for use in humans.

### 1.2.3. *Shigella*

#### 1.2.3.1. Bacteriology and clinical disease

In 1897, Dr. Kiyoshi Shiga discovered a pathogen causing a diarrhoea epidemic that affected >91,000 people in Japan[114]. The bacterium was named *Shigella* and was a non-motile, non-encapsulated, Gram-negative bacillus in the family *Enterobacteriaceae*[72]. The species *Shigella* is now subdivided into serotypes based on the lipopolysaccharide O antigen. Nearly fifty serotypes, belonging to four serogroups have been identified: A (*S. dysenteriae*); B (*S. flexneri*); C (*S. boydii*) and D (*S. sonnei*)[115].

*Shigella* infection may be asymptomatic, mild, watery diarrhoea or stimulate overt dysentery[116]. Dysentery is a syndrome characterised by frequent, but small volume, loose stools, consisting largely of blood and mucus. Fever, pain, and tenesmus are frequently present. Unlike secretory diarrhoea, this syndrome is a result of invasion of the distal small bowel and/or colon by bacteria[117]. Complications with *Shigella* infection are not common in healthy children and mostly associated with *S. dysenteriae* type 1[118]. Intestinal complications of shigellosis included rectal prolapse, intestinal obstruction, toxic megacolon, and perforation[118]. Altered consciousness are more common during shigellosis than with other childhood infections and often associated with febrile convulsions, hypoglycaemia, hypernatremia or meningitis[119,120]. *Shigella* sepsis is often severe and associated with a poor prognosis with risk factors including being very young, being wasted[121] and any immunosuppression status i.e. HIV[122]. Infection with *Shigella* may lead to leukocytosis (>50,000 neutrophil /μl) and haemolytic uremic syndrome[118]. In LMICs, shigellosis can cause long-term complications including persistent diarrhoea and malnutrition due to intestinal protein loss[115]. Functional gastrointestinal disorders after *Shigella* infection have also been reported[123].

#### 1.2.3.2. Epidemiology and antimicrobial resistance

In 2010, 188 million cases of shigellosis occurred globally, 62.3 million of these cases were in children aged <5 years[115]. In LMIC settings, the incidence of *Shigella* infection requiring medical care was 2.0-7.0 cases of *Shigella* per 100 child-years, most commonly in children aged 12-23 months[14]. Globally, *Shigella* is the second-leading cause of diarrhoeal mortality after rotavirus, responsible for roughly 164,300 annual deaths worldwide (12.5% of all diarrhoeal deaths)[124]. This burden is the highest in South Asia and sub-Saharan Africa[124].

The prevalence of *Shigella* serotypes varies over time and geography[125]. *S. sonnei* accounts for approximately 80% of *Shigella* isolates in developed countries, with an increasing relative

prevalence in the last decades[125]. By contrast, in LMICs, *S. flexneri* is most common, with occasional outbreaks caused by *S. dysenteriae* serotype 1[126,127]. However, due to environmental pressures, an epidemiological shift of *S. sonnei* has occurred resulting in *S. sonnei* becoming more predominant across industrialising regions[128–130].

High frequencies of resistance to ampicillin, trimethoprim-sulfamethoxazole, tetracycline, and chloramphenicol have been reported in *Shigella* since the 1990s[131–133]. During this period, quinolones emerged as an alternative antimicrobial agent to treat shigellosis and became the first line treatment by the WHO[19]. However, after a comparatively short period of use, the rate of resistance to ciprofloxacin was reported as 2-6% among six Asian countries in 2003[126]. The ciprofloxacin resistance rate has increased to 30% within 5 years[127,132]. Furthermore, multidrug resistance (MDR) and resistance to third generation cephalosporin have been rapidly increasing globally[134,135]. In the situation of narrowing the choice of antimicrobial drug for shigellosis treatment, azithromycin with the intracellular penetration has become the first choice. A recent study from Vietnam and Laos determined MIC breakpoints inhibition zones for azithromycin and confirmed the azithromycin mechanism resistance rate (4.6%) by PCR[136].

#### 1.2.3.3. Management of shigellosis

Children who have dysentery should always be treated empirically with antimicrobial agents. Appropriate antimicrobial therapy for shigellosis shortens the duration of fever and diarrhoea and reduces the risk for developing complications[19]. In the absence of local antimicrobial susceptibility data, WHO recommendations should be followed[19]. The first-line therapy is ciprofloxacin (in all age groups, including children), and the second-line therapy is pivmecillinam (where available), ceftriaxone, or azithromycin.

No effective, licensed vaccine against shigellosis is available. Candidate *Shigella* vaccines are either against *Shigella* lipopolysaccharide (O antigen) or the invasion plasmid antigens (*Ipa*s). The O antigen is a multivalent antigen; whereas the *Ipa*s antigens are less immunogenic[137]. Recently, the live-attenuated whole cell candidates of *S. sonnei* (known as WRSS1) has entered phase clinical 2a studies in infants and young children in Bangladesh[137] and in adult challenge clinical trial in Thailand[138].

### 1.2.4. *Escherichia coli*

*E. coli* is an important member of human intestinal microbiota. When *E. coli* acquires virulence gene and cause diarrhoea, these are named as pathotypes of diarrhoeagenic *E. coli*.

#### 1.2.4.1. Diarrhoeagenic *Escherichia coli* infection

*E. coli* is a Gram-negative, oxidase-negative, rod-shaped bacterium from the family *Enterobacteriaceae*[139]. *E. coli* can grow both aerobically and anaerobically, preferably at 37°C, and can either be non-motile or motile, with peritrichous flagella. *E. coli* is readily isolated from faecal samples by plating on selective media[139]. The change in pH due to lactose fermentation can be used to differentiate between lactose-fermenting and non-lactose-fermenting strains, as lactose-positive *E. coli* colonies appear red or pink on media such as MC agar[139]. DEC cannot be diagnosed by microbiology culture due to their similarity in morphology to commensal *E. coli*. Classification of the various *E. coli* pathotypes is based upon the various virulence genes that each DEC carries. These virulence genes can, to some extent, be used to explain their pathogenesis and the clinical disease they cause; and act as markers for DEC detection[139,140]. One pathotype of *E. coli* may contain several virulence factors; however, in this section I focused on the virulence factors that may be used to identify DEC. In my work in chapter 5, I aimed to identify on six major DEC pathotypes: enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), Shigatoxin-producing

*E. coli* (STEC) and enterohemorrhagic *E. coli* (EHEC). The various characteristics of these organisms are described in Table 1.4.

**Table 1.4.** Summary of virulence genes; function and pathogenesis of related genes; and epidemiology and clinical diseases of six major pathotypes of diarrhoeagenic *Escherichia coli*

| Patho-types | Virulence genes | Pathogenesis | Epidemiology and Clinical diseases |
|---|---|---|---|
| **ETEC** | *elt* – labile heat enterotoxin | There were two types of LT, LT-I and LT-II, which do not cross-react immunologically. LT-I was expressed by *E. coli* strains that were pathogenic for both humans and animals. LT-II was found primarily in animal *E. coli* isolates. LT and cholera enterotoxin shared many characteristics in structure and function.<br><br>The toxins increased intracellular cyclic adenosine monophosphate (cAMP) leading to hyper secreting of water and electrolytes into the bowel lumen[141]. | ETEC was an important diarrhoeal pathogen in developing countries; and the major cause of "traveller's diarrhoea"[142]. The burden of disease was estimated at 840 million cases per year in the developing world, with an additional 50 million asymptomatic carriers in children <5 years of age[143]. ST-ETEC (or ST-LT-ETEC) were among the most important pathogens associated with diarrhoea in children at this age group in developing countries[144–146].<br><br>The diarrhoea caused by ETEC was the secretory type: the disease began with a sudden onset of watery stool (without blood or inflammatory cells) and often vomiting, low-grade fever, leading to dehydration from the loss of fluids and electrolytes (sodium, potassium, chloride, and bicarbonate) in the stool[147]. Usually the diarrhoea lasted only 3-4 days and then self-limited; if hydration maintained, the patients recovered without any sequelae[142]. |
| | *est* – stable heat enterotoxin | Characteristically, ST-ETEC strains caused the majority of endemic cases[14,141]. ST was a non-antigenic low-molecular-weight peptide[142].<br><br>The toxins activated directly the guanylate cyclase C at membrane of the intestinal cells to produce intracellular cyclic guanosine monophosphate (cGMP) and consequently to generate secretion of $Cl^-$ ions and water from the intestinal epithelium[140]. | |
| **EAEC** | *aggR* (a transcriptional regulator) | The gene encoding transcriptional activator AggR (*aggR*), which was needed for expression of fimbriae[148].<br><br>EAEC caused the diseases by forming of a stacked-brick biofilm lesion resulting in a thick layer of auto-aggregating bacteria adhering loosely to the mucosal surface. EAEC induced mild, but significant, mucosal damage[149]. | EAEC was associated with acute and persistent diarrhoea, diarrhoea in HIV-infected patients, and traveller's diarrhoea in developing countries. This pathogen also caused several outbreaks in Europe, Japan, Mexico and India. EAEC infection including asymptomatic infection was strongly associated with malnutrition and growth deficit[140].<br><br>Diarrhoea caused by EAEC was often watery, but it could be accompanied by mucus or blood. The clinical diseases described with no or low-grade fever and little or no vomiting[141]. |

| Patho-types | Virulence genes | Pathogenesis | Epidemiology and Clinical diseases |
|---|---|---|---|
| **EIEC** | *ipaH* (invasion plasmid antigen H gene) | The invasion plasmid antigen H gene (*ipa*H) encoded for the protein secreted by EIEC/*Shigella*. This protein involved in the entry into the host epithelial cells. This was an important step in pathogenesis of EIEC/*Shigella*[150]. | Epidemiological data may be underestimated due to the difficulty in differentiating between *Shigella* and EIEC. EIEC was responsible for several outbreaks. Water and milk and cheese, beef contaminated were described as potential sources. The direct transmission through person-to-person contact was also reported[140,151].<br><br>EIEC infection described most commonly as watery diarrhoea, which could be indistinguishable from the secretory diarrhoea seen with ETEC. Patients might have the dysentery syndrome, manifested as blood, mucus, and leukocytes in the stool; tenesmus; and fever. Asymptomatic infection due to EIEC was probably unusual[141]. |
| **EPEC** | *eae* (structural gene for intimin of EHEC and EPEC);<br><br>*bfp* (structural gene for the bundle-forming pilus) | Adherence of EPEC to the epithelial cells was mediated by intimin (outer membrane protein) encoded by *eae* (for attaching and effacing lesion). The *eae* gene was present in EPEC and EHEC but was absent from non-pathogenic *E. coli* strains and other bacteria[141].<br><br>Bundle-forming pili (*bfp*) were rope-like fimbriae that help EPEC attach to the surface of the host intestinal epithelium in micro colonies form[152]. | Typical EPEC possessed both *eae* and *bfp* virulence gene; whereas atypical EPEC did not carry *bfp*[153]. Atypical EPEC strains might be less virulent then typical one; hence typical EPEC serotypes were strongly associated with diarrhoea in children in developing regions[14,153]<br><br>EPEC caused acute profuse watery diarrhoea, vomiting and low-grade fever; however, severe diseases due to infection and high mortality rates were reported[141] |

| Patho-types | Virulence genes | Pathogenesis | Epidemiology and Clinical diseases |
|---|---|---|---|
| **EHEC** | *eae* (structural gene for intimin of EHEC and EPEC);<br><br>*rfpE* (encode for lipo-polysaccharide of O157) | The non-eae EHEC strains were reported with the ability of causing severe diseases if these strains carried other virulence genes[154]. | EHEC was a subset of STEC and originally described to be associated with haemorrhagic colitis (HC), which was clinically distinct from shigellosis, and had genotypic and phenotypic features that differed from those of EPEC.<br><br>*E. coli* O157:H7 serotype was the first to be linked to HC and haemolytic uremic syndrome (HUS) cases in the early 1980s. This serotype has been since then responsible for numerous outbreaks and sporadic cases of severe diseases all over the world[140,155].<br><br>Infected with EHEC may be asymptomatic; some may have watery diarrhoea, HC and HUS. HC consists of profuse diarrhoea with visible blood and abdominal pain. Nausea, vomiting, dehydration and low-grade fever may also be seen[156]. |
| **STEC** | *vtx1* and/or *vtx2* (Shiga toxin also known as verocytotoxin) | There were two subgroups of *vtx*, *vtx1* and *vtx2*, with *vtx2* being more prevalent in HC and HUS than *vtx1*[141].<br><br>The *vtx* receptors were the globotriaosylceramides (Gb3s) found on Paneth cells in the human intestinal mucosa and the surface of kidney epithelial cells[141]. The *vtx* was responsible for damage to intestinal and renal tissue by causing inhibition of protein synthesis and apotosis[157]. | Most cases of post-diarrhoeal HUS were caused by STEC. STEC O157:H7 were the most common strains causing many endemics in US, Europe. The incidence of STEC O157 in US was 0.91/100,000 people in 2014. However this incidence significant decreased compared with previous years[155]. Whereas non-O157 STEC incidence significant increased in US[155] and caused sporadic cases and outbreaks in Australia, and Europe[139]. The large STEC O104:H4 outbreak in Germany in 2011 caused by EAEC strain carried *vtx*[158].<br><br>Infection with STEC caused variety diseases at different severity from mild to bloody diarrhoea or HUS (the triad of acute renal failure, thrombocytopenia, and microangiopathic haemolytic anaemia)[159]. |

#### 1.2.4.2. Diarrhoeagenic *Escherichia coli* detection

Traditionally, the diagnosis of DEC is performed by microbiological culture methods followed by serotyping using antisera against the O and H antigens; this approach is feasible in most standard microbiological laboratories. Commercial kits for O and H serotyping of DEC (mostly for O157:H7 and EPEC) are available; however, serotyping has some limitations due to the large number of serotypes, cross-reactivity of antigens, the untypeable nature of some strains, and low sensitivity and specificity[160]. For toxin identification, i.e. Shiga toxin (*Stx1*/*Stx2*) of STEC or *lt*/*st* of ETEC, EIA, ELISA or PCR of toxin genes can be used[139]. For EPEC and EAEC, due to the specific lesions on intestinal epithelium cells, cell culture-base methods can be used if available[139]. However, cell culture methods required substantial labour and time consuming. Currently, STEC/ O157:H7 is the major focus public health and routinely surveillance in many regions[80]. Recently, a rapid RIDA® GENE real-time multiplex PCR assay that can detect most of the gene markers of DEC has been evaluated in Germany and provides reliable and rapid results[161]. However, in a comparable fashion to other molecular approaches, this assay cannot distinguish between STEC carrying *eae*-gene or ST-EPEC or mixed cultures. Yet, despite these limitations multiplex molecular methods are a good option for diagnosing many pathotypes of DEC with high sensitivity and high throughput.

#### 1.2.4.3. Treatment

The treatment of diarrhoea caused by DEC includes rehydration, supportive treatment and WASH for prevention[139]. Among the various pathotypes of DEC, antimicrobial therapy is indicated for ETEC (see section 1.1.4), for EIEC (similar to *Shigella* treatment); and for persistent diarrhoeal cases[139].

### 1.2.5. Antimicrobial resistance

#### 1.2.5.1. Burden of antimicrobial resistance

In 1928, Alexander Fleming initiated a medical revolution by discovering the first antimicrobial agent, penicillin. Soon after his discovery he himself realised the risk of AMR[162]. Between 1940 and 1962, more than 20 classes of antimicrobial agents were developed and widely used[163,164]. In 2011, only two new classes of antimicrobial reached the market, 27 compounds were clinical development and two new classes in phase I clinical trial[165]. As of May 2017, 51 antimicrobials (including combinations) are currently in phases 1-3 of clinical development[166]. Infections caused by AMR organism have become one of the leading threats to human health, with a conservative estimate of 700,000 directly attributed deaths every year worldwide[167]. This figure has been estimated to rise to 10 million by 2050[167]. For foodborne pathogens management, nearly 5,000 million courses of antimicrobial therapy are used to treat diarrhoea each year[167]. AMR of foodborne pathogens have been discussed in previous sections, noting the high rates of resistance to ciprofloxacin and the third-generation cephalosporin and MDR among foodborne pathogens, especially in LMICs. The WHO has specifically noted the lack of data regarding of AMR in Southeast Asia[168]. However, AMR in this region has been recently found to be extremely high, and associated with considerable mortality and economic cost[169]. The reasons for AMR situation in Southeast Asia are complex and multifaceted but include the availability and consumption of antimicrobial drugs in humans and in agriculture i.e. overuse, underuse of diagnostics, self-medication, easy access to antimicrobials, and medicated animal feed[169].

#### 1.2.5.2. Antimicrobial agents: mechanism of action; mechanism of resistance and antimicrobial resistance genes

Broadly, the mechanism of action of antimicrobial drugs can be classified into 4 groups: inhibition of cell wall synthesis and cell membrane function, inhibition of protein biosynthesis, inhibition of DNA replication, and folic acid metabolism inhibitors[170]. Bacteria also have variety

mechanisms for AMR. The main three categories are: prevent access to the target by reducing permeability, efflux pumps, changing the target by mutation, and protection of the target by modifying target or enzymatic the drug[171]. Table 1.5 summarises how antimicrobial agents to kill or inhibit bacteria and how bacteria develop the resistance against specific antimicrobial agents; and the genes encoding AMR.

**Table 1.5.** Summary of antimicrobial drugs, mechanism of action, mechanism of resistance and genes related to antimicrobial resistance

| Antimicrobials | Mechanism of action | Mechanism of resistance | Antimicrobial resistance genes |
|---|---|---|---|
| Penicillins<br>Cephalosporins<br><br>Beta-lactam ring | **Inhibition of cell wall synthesis**<br><br>Penicillins and cephalosporins act by binding to penicillin-binding proteins (PBPs) which are bacterial enzymes that catalyse the last steps of peptidoglycan synthesis cell wall. | Bacteria secrete β-lactamase which open the β-lactam ring of penicillins and cephalosporins (hydrolysis the drug)<br><br>Hundreds of β-lactamases have been described and have been classified on the basic of their structure into 4 molecular classes (A, B, C and D)[172]<br><br>Modification of PBP (mutation of drug targets)<br><br>Cellular permeability (reduce uptake and/or active efflux)[170] | Class A β-lactamases: *bla*TEM, *bla*SHV<br><br>Class A ESBLs (extended-spectrum β-lactamases): *bla*TEM, *bla*SHV, *bla*CTX-M<br><br>Class A carbapenemases: *bla*CARB, *bla*IMI, *bla*SME<br><br>Class B metallo-β-lactamases: *bla*NDM, *bla*MBL<br><br>Class C cephalosporinases: *bla*CMY, *bla*ACT, *bla*DHA<br><br>Class D oxacillinases: *bla*OXA[172–174] |
| β-lactamase inhibitors | β-lactamase inhibitors i.e. clavulanic acid, sulbactam, tazobactam have a high affinity for and irreversibly bind some β-lactamases but are not hydrolysed by the β-lactamases, resulting in enzyme inactivation[174].<br><br>Clavulanic acid acts synergistically with various β-lactam agents to treat β-lactamase-producing bacteria[174]. | Gram negative pathogens gradually adopted ability of producing multiple β-lactamases, clavulanic acid combinations may not be effective for serious infections[175]. | |
| Carbapenems | A unique class of β-lactam agents with the widest spectrum of antibacterial activity Carbapenems bind with high affinity to PBP of Gram-negative bacteria, causing spherical cell formation and lysis. | Two main mechanisms lead to decrease the susceptibility to carbapenems in *Enterobacteriaceae* isolates: production of carbapenemase or production of other β-lactamase in combination with decreased permeability[176]. | *bla*NDM<br>New Delhi metallo-beta-lactamase (NDM) has become one of the most widespread carbapenemases and is found in Gram-negative pathogens[171]. |

| Antimicrobials | Mechanism of action | Mechanism of resistance | Antimicrobial resistance genes |
|---|---|---|---|
| Aminoglycoside<br><br>Presence of aminocyclitol ring links to amino sugars | **Inhibition of protein synthesis**<br><br>Drug acts on 30S ribosomal subunit of the bacteria. Therefore, the bacterial ribosome unable to translate mRNA for protein synthesis. Cell death is final consequence[177]. | The plasmid mediated resistance to aminoglycoside by secreting enzymes that modify the drug so that the drug cannot bind to the bacterial ribosome[178].<br><br>Another plasmid mediated resistance mechanism is that an outer membrane permeability change that reduces active transport of the aminoglycoside into the cell so the drug cannot reach the ribosome[177].<br><br>Chromosomal resistance of microbes to aminoglycosides principally depends on the lack of a specific protein receptor (modification of the target site caused by mutations) on the 30S subunit of the ribosome[179]. | There are more than 50 aminoglycoside-modifying enzymes classified into three main classes: aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferases (ANT) and aminoglycoside phosphotransferases (APH)[178]. The encode genes are known as *aac*, *ant* and *aph*, respectively. |
| Macrolide, Lincosamide and streptogramin (MLS) resistance | **Inhibition of protein synthesis**<br><br>Macrolides are bacteriostatic agents. They bind reversibly to the 23S rRNA of the 50S subunits of ribosome, thereby blocking the elongation of polypeptide chain[180].<br><br>Macrolides, lincosamides, and streptogramins B have a similar mechanism of action. | Resistance mechanism[181]<br>rRNA methylases that modify the ribosomal target sites prevent the drugs from binding to 50S ribosome unit.<br><br>efflux pumps that pump the drug out of the bacterial cell.<br><br>Inactivating enzyme subdivided into esterases, lyases, phosphorylases, and transferases.<br><br>Mutation in 23S rRNA | *erm, cfr* : rRNA methylase<br>These enzymes methylate the adenine residue. The methylated adenine prevents the drugs from binding to the 50S ribosomal subunit[182].<br><br>inactivating enzyme: *mphA*, *mphB*, *mphD* (phosphorylase), *ere* (esterases)<br><br>*mefA*, *msrA*, *lmr*, *vga*: efflux genes[183]<br><br>*mphA-mrx-mphr(A)*: a macrolide inactivation gene cluster within a novel *Salmonella* azithromycin resistance genomic island (SARGI)[184]. |

| Antimicrobials | Mechanism of action | Mechanism of resistance | Antimicrobial resistance genes |
|---|---|---|---|
| Quinolones | **Inhibition of DNA synthesis**<br><br>Quinolones inhibit the bacteria enzymes (topoisomerase II (DNA gyrase) and IV) causing relaxation and decatenating of the supercoiled DNA, leading to termination of chromosomal replication and interference with cell division and gene expression[185].<br><br>DNA gyrase is an enzyme essential for DNA negative supercoiling, which is a vital step for chromosome condensation, relieving torsional strain during replication, and promoting transcription[186].<br><br>The A subunit is encoded by the *gyrA* gene and is involved mainly in DNA breakage and reunion, while the B subunit is encoded by the *gyrB* gene and exhibits ATPase activity. The DNA gyrase A subunit is the main target of quinolones in Gram-negative bacteria.<br><br>Topoisomerase IV has two functions: a decatenating enzyme that resolves interlinked daughter chromosomes after DNA replication; and relaxing positive supercoils[185].<br><br>Topoisomerase IV is also a heterotetramer made of two A subunits (p*ar*C) and two B subunits (*par*E)[187]. | Quinolones resistance due to<br><br>(1) Amino acid substitutions within the quinolones resistance-determining region (QRDR) on chromosome[188]<br>In *Enterobacteriaceae*<br>a single mutation in the *gyrA* gene (at Ser-83/ Asp-87) → low-level of quinolone resistance<br>a single mutation (at Ser-80/ Glu-84) of the *parC* gene →moderate level of ciprofloxacin resistance<br>a third mutation, the second in *gyrA*, is associated with a high level of ciprofloxacin resistance<br>a fourth mutation, the second in *parC*, is associated with the highest level of resistance[185,189].<br><br>(2) Mutations on chromosomes causing reduced drug accumulation, either by mutations in the regulatory genes governing bacterial outer membrane permeability to the drug or by an increased efflux[190].<br><br>(3) Plasmid-mediated quinolone resistances (PMQR)<br><br>efflux pump encoded by the *qepA* and *oqx*AB gene that decreases intracellular drug levels[191]. | Chromosome mutations<br>the genes encoding the modified A and B subunits of topoisomerase (*gyrA*, *gyrB*, *parC* and *parE*)<br><br>Plasmid-mediated quinolone resistances (PMQR)<br>the *qnr* gene (*qnr*A, *qnr*B, and *qnr*S) that encodes a pentapeptide, which bind to and protect the DNA gyrase and topoisomerase IV[192].<br><br>the *aac(6′)-Ib-cr* gene that encodes an acetylase that modifies the amino group of the piperazine ring of the fluoroquinolones[193]. |

| Antimicrobials | Mechanism of action | Mechanism of resistance | Antimicrobial resistance genes |
|---|---|---|---|
| Sulphonamides and Trimethoprim | **Inhibition of folic acid synthesis**<br>Trimethoprim interacts with dihydrofolate reductase (DHFR), whereas sulphonamides interact with dihydropteroate synthase (DHPS). These two enzymes are necessary for the folate biosynthetic pathway that is required for thymine production and bacterial cell growth[194] .<br><br>If used together, they produce sequential blocking, resulting in a marked enhancement (synergism) of activity. | Resistance to TMP by[195]<br>-overproduction of the DHFR in bacteria<br>-mutations in the structural gene for DHFR<br>-acquired *drf* gene encoding resistant DHFR enzyme<br><br>Resistance to sulphonamides by<br>*sul* encoding alternative forms of DHPS that are not inhibited by the drug[196] | *dfr* encoding a DHFR enzyme which is the most resistant mechanism in clinical isolates<br><br>*sul* encoding different forms of DHPS that are not inhibit by the drug. |
| Chloramphenicol | **Inhibition of protein synthesis**<br><br>The drug is a bacteriostatic agent that inhibits protein synthesis by binding reversibly to the peptidyl transferase of the 50S ribosomal subunit, then preventing the transpeptidation process of peptide chain elongation[197]. | Inactivation the drug by enzyme (chloramphenicol acetyltransferase) encode by *cat*-gene<br><br>Inactivation the drug by phosphotransferases<br>Mutations of the target site<br>Decrease outer membrane permeability<br>Active efflux systems[197] | *flo*R, *cml*, *cmr*, *pex*A encode for the bump which efflux the drug from cell.<br><br>*cat*-genes encode for chloramphenicol acetyltransferase which inactivate the drug[183] |
| .Lincosamide | **Inhibition of protein synthesis**<br><br>Lincosamides bind to the 50S ribosomal subunits of susceptible bacteria and prevent elongation of peptide chains by interfering with peptidyl transfer, thereby suppressing protein synthesis. The ribosomal binding sites are the same as, or closely related to, those that bind macrolides, streptogramins, and chloramphenicol; hence there are cross resistance among these drugs[198]. | Target site modification by methylation or mutation that prevents the drug from binding to ribosomal target<br><br>Efflux pump<br><br>Drug inactivation | *lin*, *lnu* encode inactivating enzyme (lincosamide nucletidyltransferases)[198] |

| Antimicrobials | Mechanism of action | Mechanism of resistance | Antimicrobial resistance genes |
|---|---|---|---|
| Tetracyclines | **Inhibition of protein synthesis**<br><br>Tetracyclines bind reversibly to the 30S ribosomal subunits, preventing aminoacyl-tRNA to bind to the ribosomal acceptor A site[199]. | Efflux of drug<br><br>Ribosome protection proteins that bind to ribosome causing the drug unable to bind to.<br><br>Modification of the drug: enzymatic alteration of the drug[183]. | *tet*, *otr*(B/C), *tcr* encode efflux pumps<br><br>*otr*(A), *tet*B(P), *tet*(M/O/Q/S/T/W) encode ribosome protection |
| Colistin | **Inhibition of cell membrane function**<br><br>The polymyxin E (also known as colistin) is cyclic antimicrobial peptides that target gram negative bacteria by binding to lipolysaccharide (LPS) leading to disrupt cell membrane[171] | changing in the expression of regulators affecting LPS production, which result in alterations in the target and reduce binding of the drug[171]. | *mcr-1* from the GenBank database (accession number LC114018.1) |
| Rifampicin | **Inhibition of RNA transcription**<br><br>Rifampin is an bactericidal agent by binding to bacterial RNA polymerase, preventing DNA transcription[200]. | Alteration in RNA polymerase enzyme<br><br>Inactivation of the drug<br><br>Decrease in bacterial cell membrane permeability[200] | *arr*[201] |

#### 1.2.5.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing methods measure the phenotypes of susceptibility based on minimum inhibitory concentration (MIC) or inhibition zone diameter (IZD). MIC is defined as the lowest concentration of an antimicrobial agent that inhibit visible growth of a microorganism after overnight incubation[202]. Determining an MIC can be performed using different methods i.e. E tests, or dilution methods using broth or agar[203]. IZD is the measurement in millimetres of the zone around an antimicrobial disc in which the bacteria colonies do not grow[204]. Determining IZD is usually performed using the disk diffusion method (Kirby-Bauer test) on Mueller Hilton agar[204]. The results of MIC or IZD can be interpreted using international standards such as the Clinical and Laboratory Standards Institute (CLSI)[205] or the European Committee on Antimicrobials Susceptibility Testing (EUCAST)[206](www.eucast.org).

Antimicrobial susceptibility of bacteria can be determined by susceptibility testing and now by WGS. The recent survey regarding foodborne pathogens and AMR pathogens using genomic surveillance in Europe showed that WGS provides a good correlation with phenotype for many antimicrobials and has a high throughput and rapid turnaround time[207,208].

## 1.3. Diarrhoeal diseases in Vietnam

### 1.3.1. Diarrhoea is a burden of health in Vietnam

Vietnam is a rapidly developing LMIC in Southeast Asia with a population of >96 million people living an area of 331,210 square kilometres[209]. Although the morbidity and mortality of diarrhoeal disease has decreased in Vietnam in recent years, the incidence of diarrhoea in children in Vietnam is relatively high in comparison to developed countries; 3.23 episodes per child year in infants[210] vs. 1.4 episodes per child year in US[97]. Diarrhoea remains the second largest cause of illness and hospitalisation in Vietnamese children after respiratory tract infections[211]. The prevalence of all-cause diarrhoea in children aged <5 years in Vietnam was estimated to be 7-

11% (Multiple Indicator Cluster Survey in 2000, 2006, and 2011)[211] and accounted up to 12% of all-cause deaths in this age group[212].

### 1.3.2. The aetiology and antimicrobial susceptibility of diarrhoeal pathogens in Vietnam

Diarrhoeal diseases remains common in Vietnam and during a recent study at least one enteric pathogen was identified in > 70% faecal samples of children with diarrhoeal diseases[213,214]. Epidemic diarrhoea was common in Vietnam in the 1960s and 1970s; *Shigella flexneri* was the most common pathogen; followed by DEC and *Vibrio cholera*[215]. After 2000, viral pathogens emerged and became more frequently detected than bacterial pathogens in diarrhoeal cases, potentially reflecting the effect of the recent economic transition in Vietnam on the epidemiology of enteric pathogens[216].

The epidemiology of enteric bacterial pathogens in Vietnam appear to change with the time, this is exemplified by *Shigella* species. With the application of genomic analysis, Holt *et al.* found that the current dominant genotype of *S. sonnei* was introduced into Vietnam in the 1980s and underwent a national clonal expansion[217]. Data collected from provinces across Vietnam over a 10 year (1991-2001) period showed that shigellosis was the most common cause of dysentery with ~39,500 cases annually[218]. Vinh *et al.* identified a change in dominant *Shigella* species over 14 years (1995-2008) and reported an epidemiological shift in dominant infecting species (*S. flexneri* to *S. sonnei*) and their accompanying resistance profile in southern Vietnam[128]. Since this period, *S. sonnei* has become the predominant *Shigella* serotype in diarrhoeal cases in Vietnam[213]. This trend has been reflected internationally[129,130,219–221].

In contrast to *Shigella* spp., which infect only humans, NTS and *Campylobacter* can infect a variety of animals, including poultry, livestock, reptiles, and rodents[42]. Hence, in this setting with economic development and an increase in industrial food products (meat and dairy), the

prevalence of foodborne diseases causing by NTS and *Campylobacter* has increased in Vietnam over the last 20 years. NTS accounted for 0.8% diarrhoeal cases in 1999[210] versus 4% in 2010[213] and was found to be more common than *Campylobacter* and *Shigella* infections. Although the prevalence of enteric bacterial infection in children with acute diarrhoea is less than viral infection[213,222], these bacteria may be more problematic due to increasing rates of AMR. However, AMR data from bacteria associated with enteric bacteria in Vietnam is limited. A recent publication from 2010 identified high rates of resistance and MDR in *Campylobacter*, NTS and *Shigella*, i.e. >80% of *Campylobacter* exhibited resistance to ciprofloxacin; >75% *Shigella* isolates were resistance to third generation cephalosporins[213]. Notably, resistant bacteria were more associated with diarrhoeal disease than susceptible bacteria. Generally, the extensive use of antimicrobials in farming and improper prescription of antimicrobials in humans has led to a major AMR crisis in Asia, with increased resistance against first-line antimicrobials, including fluoroquinolones, and third generation cephalosporins in many Gram-negative pathogens across the region[168].

### 1.3.3. The management of diarrhoea in Vietnam

A large burden of diarrhoeal disease, combined with the lack of financial and diagnostic resources in Vietnam, means that the causative agent of most diarrhoeal episodes goes undiagnosed. As a consequence, antimicrobials are empirically administered to children with diarrhoea based on clinical manifestations and without adequate causative diagnostic information. Prior studies found that antimicrobials were prescribed in 38% of acute watery diarrhoea in Vietnamese children caused by a viral pathogen and in 60% of cases of unknown aetiology[213]. The Vietnamese healthcare system currently follows the WHO guidelines for treatment of paediatric diarrhoea. These guidelines recommend the use of low osmolarity oral rehydration solution (ORS), zinc, and ciprofloxacin or one of the three second-line antimicrobials: pivmecillinam, azithromycin, or ceftriaxone, for all patients with bloody diarrhoea, irrespective of

age[16,19]. In this limited resource setting without good diagnostics and antimicrobial susceptibility data empirical antimicrobial therapy may be excessive and require further assessment.

### 1.4. Hypothesis, objectives, and aims

Diarrhoeal disease places a major burden on the healthcare system in Vietnam. Diarrhoea caused by pathogenic bacteria in children is a major concern due to an epidemiology shift and increasing rates of AMR. The management of bacterial diarrhoea infection, i.e. antimicrobial therapy is mostly empirical due to lack of current data regarding the prevalence of major enteric pathogens and their AMR profiles. Therefore, my thesis aimed to provide an overview of children hospitalised with diarrhoeal diseases in Vietnam. This included the prevalence of common diarrhoeagenic bacteria, the clinical manifestations of infectious diarrhoea, the utility of diagnostic tests, AMR profiles of infecting pathogens, and the usage of antimicrobial drugs.

My thesis was conducted from the main research protocol (12EN) (Appendices 9.1) which was initially designed focusing on *Shigella* infection. After two year of enrolment, I had collected enough data to describe the epidemiology of bacterial infection in diarrhoea in children. Moreover, the prevalence of *Shigella* infection was low. Hence, I proposed the alternative research aims to describe the prevalence, clinical manifestations and AMR patterns of most common enteric bacteria in this setting.

The aims of my thesis were:

1. To define the key bacterial aetiologies of diarrhoea and their AMR profile in hospitalised children, specifically to assess their treatment and outcome (Chapter 3).
2. DEC are common diarrhoeal pathogens. However, DEC have never been studied in Vietnam and distinguished between commensal *E. coli* and DEC cannot be performed by conventional microbial culture. Therefore, a series of multiplex real-time PCR assays

were designed and I aimed to measure the prevalence of DEC in Vietnamese children with and without diarrhoea (Chapter 4).

3. Novel diagnostic assays are available for diarrhoeal disease. I aimed to evaluate the Luminex xTAG GPP to ask if this assay should be used for detecting enteric pathogens in a clinical setting in Vietnam (Chapter 5).
4. NTS are the most prevalence bacteria associated with diarrhoea in Vietnamese children. However, NTS research is complex due to difficulty in serotyping and the multi-host nature of the organisms. WGS is the latest method that can provide information for NTS genoserotyping, mechanisms of AMR, virulence genes, and phylogeny. Here, I focused to genoserotype a collection of NTS and evaluation the performance of WGS in determining antimicrobial susceptibility profile (Chapter 6).

Four result chapters were conducted from the analysis of samples in the 12EN project. The DEC diagnostic tests were performed on both faecal samples of 12EN project (diarrhoeal diseases) and anal swabs of cohort project (healthy without diarrhoeal diseases). Lastly, the Luminex xTAG GPP was performed on a collection of faecal samples from three studies including 12EN study, acute diarrhoea in children (09AV), and the Vietnam Initative on Zoonotic Infection (VIZIONS) study (see section 2.3.1).

# 2. Materials and methods

For the purposes of the work in this thesis I designed a prospective, observational study to describe the role of bacterial infections in diarrhoeal diseases in children living in Ho Chi Minh City (HCMC), Vietnam. The aims of this study (named 12EN) were to diagnose bacterial diarrhoeal infection, describe the clinical manifestations of the disease in the population, and measure empirical antimicrobial therapy. As the attending physician at Children's Hospital 1 in HCMC, I recruited children hospitalised with bloody and/or mucoid diarrhoea which were likely more associated with bacterial infection clinically. By collecting faecal samples of enrolled participants and processing the samples using microbiological culture, the bacterial isolates were identified and then stored for further investigation.

The main study (Chapter 3) of this work was a hospital-based study and applying conventional diagnostic methods (microbiological culture, API 20E, MALDI TOF). Samples from the study in Chapter 3 were screened for the presence of DEC using a novel combination of multiplex real-time PCRs (Chapter 4). Aiming to assess newer diagnostic models samples from Chapter 3 were used to evaluate a multi-panel diagnostic approach (Luminex xTAG GPP assay) (Chapter 5). Lastly, the NTS organisms isolated in Chapter 3 were subjected to WGS, these data were subjected to genoserotyping and the detection of genes associated with AMR (Chapter 6).

## 2.1. Methods for chapter 3 (Bacterial infection in children hospitalised with bloody/ mucoid diarrhoea)

### 2.1.1. Study setting

#### 2.1.1.1. Ethics

Ethical approval for this study was provided by the ethics committees of all three participating local hospitals and the University of Oxford Tropical Research Ethics Committee (OxTREC No. 1045-13). Written consent from parents or legal guardians of all participants was obtained prior to

enrolment. Protocol of the study named 12EN and ethical approval were presented in Appendix 9.1 and 9.2 respectively.

#### 2.1.1.2. Study design and enrolment

A prospective, observational, multicentre cross-sectional study was performed to evaluate the aetiology, epidemiology, and outcomes in children (aged <16 years) hospitalised for diarrhoea. Study participants were recruited from 3 tertiary hospitals, i.e. Children's Hospital 1(CH1), Children's Hospital 2 (CH2), and the Hospital for Tropical Diseases (HTD) in HCMC, Vietnam from May 2014 to April 2016.

Children hospitalised with diarrhoea defined as ≥3 passages of loose stools within 24 hours[16] along with at least one loose stool containing blood and/or mucus were recruited into the study. Based on faecal characteristics and duration of illness, participants were classified into three groups: acute non-bloody diarrhoea (diarrhoea with mucus, <14 days), acute bloody diarrhoea (diarrhoea with blood, <14 days), and persistent diarrhoea (diarrhoea with mucus and/or blood, ≥14days). Children were not eligible if they had suspected or confirmed intussusception at the time of enrolment. Following enrolment, a short questionnaire (requesting clinical and demographic information) was completed, and a faecal sample was collected and processed within 24 hours (Figure 2.1). All enrolled patients were provided with the routine standard of care practices at each hospital. Treatment and proxy outcomes, including patient recovery status at three days post enrolment and duration of hospitalisation, were recorded by clinical staff at study sites (Figure 2.1). Patient status were recorded as "recovered" if they had <3 passages of loose stools in the past 24 hours or "improved" if the patients had less episodes of diarrhoea and less mucus and/or bloody in comparison to the condition of the patients at enrolment.

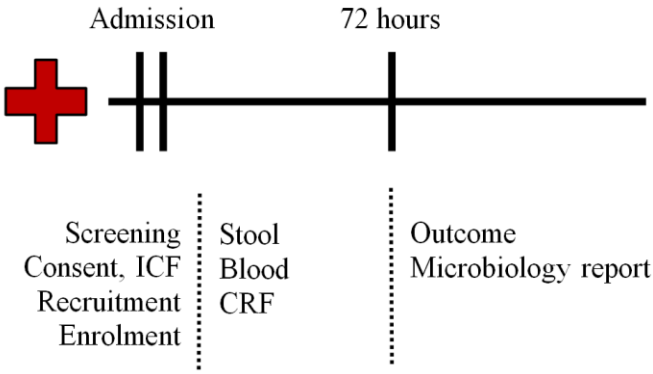



**Figure 2.1.** Steps in the study of children hospitalised with bloody and/or mucoid diarrhoea

The study was performed in three paediatric wards in tertiary hospitals. Firstly, the study nurses/ doctors screened the paediatric patients to assess whether these patients were eligible to participate in the study. Next, the parents or guardians of the child were informed of the content of the study, risk and benefit of being in the study, confidentiality, costs, and voluntary participation; and the parents or guardians of the child had given time to read the Patient Information Form (Appendix 9.3) and sign the informed consent form (ICF) (Appendix 9.4) to agree entry into the study. After recruitment and enrolment, a fresh faecal sample and a small volume of blood were collected and transferred to the laboratory for microbiological testing. After 72 hours, a preliminary outcome was assessed and noted in case report form (CRF) (Appendix 9.5) with other demographic and clinical information. At this point, a microbiology report (Appendix 9.6) had sent to clinical wards, usually after 3 days of sampling.

#### 2.1.1.3. Sampling and sample collection

Stool specimens were collected in sterile containers on the day of hospital admission. Where possible, samples were collected before the hospital prescribed antimicrobial treatment. However, a stool sample was also collected after treatment was started. A total of 2-5 ml of stool was collected by the study nurse/clinician in a sterile container using a rectal catheter. A collection form was completed for each sample, and the patient was given a unique personal identification number, which was written on the sample collection container, the collection form, and the CRF. All participants were anonymous and identified only by a unique ID number. All collected samples from each hospital were stored at room temperature and transported on the same day (apart from Sundays) to the Oxford University Clinical Research Unit (OUCRU) Microbiology laboratory by a member of OUCRU staff for analysis.

#### 2.1.1.4. Case Report Form and data management

To understand the symptoms and disease duration, study clinicians completed a CRF for each participant. All of the metadata in the form of a paper CRF were entered on CliRes (an operational data management system) and saved immediately to the server at OUCRU via internet connection. Only the named investigators or their designee(s) had access to this information. Investigators from CH1, CH2 and HTD were updated regularly and granted access to data when requested.

In order to guarantee the match between paper and electronic data, double data entry was performed manually for every CRF. All of the corrections and reasons for the correction were recorded. All the paper CRFs were stored according the regulations of OUCRU. The paper CRFs were schedule scanned into *pdf* files and double checked to guarantee completeness.

### 2.1.2. Microbiological methods (Bacterial identification)

#### 2.1.2.1. Microbiological culture

Culturing and bacteria identification were performed for all collected fresh stool samples on the day of sampling following the standard procedure in clinical microbiology[27]. Briefly, stool specimens were cultured on different selective media to isolate common diarrhoeagenic bacteria including *Campylobacter* spp., NTS, *Shigella* spp., and *E. coli*. The media used were MacConkey agar (MC, Oxoid), xylose-lysine-deoxycholate agar (XLD, Oxoid), selenite broth (Oxoid), blood agar (BA), *Campylobacter* selective agar (Oxoid), and nutrient agar (NA, Oxoid).

Faecal specimens were inoculated onto MC, XLD and into selenite broth and incubated at 37°C for 18-24 hours (Figure 2.2). *Salmonella* and *Shigella* were detected by their characteristic appearance on XLD and MC agar (non-ferment colonies on MC; *Shigella* appeared pink, small colonies on XLD; *Salmonella* appeared black on XLD); then confirmed using API 20E

(bioMerieux) and MALDI TOF (Bruker) following the manufacturer's guidelines. The identification and speciation were confirmed by serological agglutination for *Shigella* and NTS. *Campylobacter* were isolated using *Campylobacter* selective agar under microaerophilic conditions (in an anaerobic jar supplying a gas mixture of 10% $CO_2$, 5% $O_2$ and 85% $N_2$) at 42$^{o}$C for 48 hours, and then confirmed at sub-species level using MALDI TOF.

Bacterial sweeps on MC were taken using a sterile loop and then inoculated into 20% glycerol, mixed well and added into a cryovial tube. These sweeps were stored at -80$^{o}$C for DEC identification (Chapter 4).

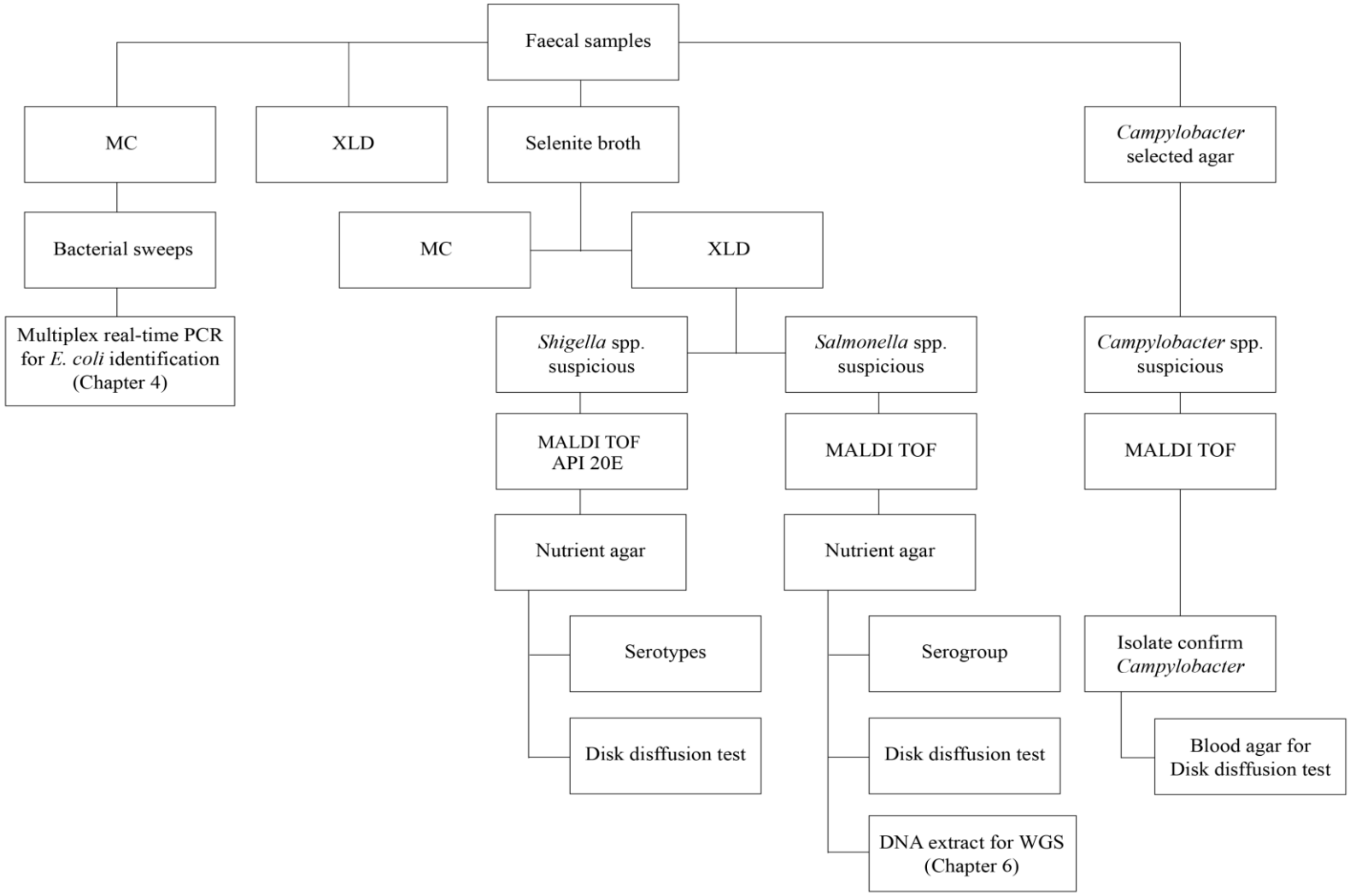



**Figure 2.2.** Flow chart for faecal specimen tests of common enteric bacteria identification, confirmation and susceptibility tests

#### 2.1.2.2. MALDI-TOF

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has recently been introduced into diagnostic microbiology laboratories for the identification of bacterial and yeast strains isolated from clinical samples. A MALDI-TOF MS was purchased by OUCRU in 2014. Since then, the MALDI-TOF has become part of the routine operating procedures to identify *Enterobacteriaceae* at the species or genus level.

For MALDI-TOF identification the samples were prepared using a sterile tip to pick a pure bacterial colony (single colony) from an appropriate agar medium. This was then inoculated in a thin film onto a ground steel MALDI target plate. The samples were overlaid with 1.0µL α-cyano-4-hydroxycinnamic acid (MALDI-TOF CHCA matrix). The sample-matrix mixture was dried at room temperature and subsequently inserted into the MALDI-TOF mass spectrometer[223]. *Staphylococcus aureus* 29213 was used as the control and placed in the first spot of the steel plate. The data were processed automatically by the instrument software and the resulting spectra were compared with a bacterial identification reference library. Using a biostatistical algorithm, this peak list is compared to the pattern of organisms in the reference library database and a log (score) value between 0.00 and 3.00 is calculated. Interpretation was performed using a 'traffic light' colour scheme: <1.70 (colour red) "no organism identification possible"; 1.70-1.99 (colour yellow) "low confidence identification/ to genus level"; and 2.00-3.00 (colour green) "high confidence identification/ to species level"[223].

#### 2.1.2.3. API 20E

Bacterial confirmation was also performed using the API 20E test strip of biochemical reactions for the *Enterobacteriaceae*[29]. The test strip included 20 microtubes of dehydrated substrates. For the purpose of this study, API 20E was mainly used in *Shigella* spp. confirmation. Briefly, the strip was prepared by adding 5ml of sterile distilled water into the base of each tray. Single

colonies of bacteria were suspended in 5ml sterile saline, mixed thoroughly to create homogenous bacterial suspension. Next, ~140µl of the suspension was added into each well to fill the tube part of the well. For the wells trisodium citrate (CIT), sodium pyruvate (VP) and gelatine (GEL), both the tube and the cupule of the well were filled with suspension. For the wells L-arginine (ADH), L-lysine (LDC), L-ornithine (ODC), sodium thiosulfate (H2S) and urea (URE), the tube was filled with suspension and the cupule was filled with API mineral oil. The trays were covered and incubated overnight (18-24hr) in air at 36±2$^{o}$C. After the incubation, the strip was checked to read the result i.e. ≥ 3 positive reactions, not including D-glucose (GLU). Three reagents needed to add to test TDA, INA and VP to read the result. The results then converted to number and calculated in groups. The profile for this combination of the reaction was generated in 7-digit code. Finally, this code was used to identify tested bacteria via *apiweb* online (https://apiweb.biomerieux.com).

#### 2.1.2.4. Serological agglutination

For all isolates confirmed as *Shigella*/*E. coli* and *Salmonella* by MALDI-TOF, serologic identification was performed by slide agglutination with antigen grouping sera and monovalent antisera for specific serotype identification (Denka Seiken, Japan). Pure colonies were picked from the nutrient agar plate and mixed with a drop of the appropriate testing agglutinating sera on a sterile glass slide. The slide was gently rocked following thorough mixing for 10-15 seconds and checked for agglutination. *Shigella* spp. isolates were serotyped with polyvalent group A (*S. dysenteriae*), B (*S. flexneri*), C (*S. boydii*) and D (*S. sonnei*) according to the manufacturer's instructions (Denka Seiken, Japan). *Salmonella* isolates were serotyped with polyvalent O and Vi in to group B, C, D, and other. As MALDI-TOF provided confirmation of *Campylobacter* spp. to genus level (*C. jejuni* and *C. coli*), no serological test was performed for *Campylobacter* spp.

#### 2.1.2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid) for *Salmonella* and *Shigella*, and on blood agar containing 5% sheep blood for *Campylobacter* and interpreted using the CLSI Guidelines[224]. Single colonies from the overnight plate cultures were suspended in 1ml of sterile distilled water and mixed thoroughly to obtain a homogenous suspension of 0.5 McFarland standard. The mixed inoculum was swabbed evenly over the surface of the agar plate in three different directions. Plates were set to dry on room temperature before placing antimicrobial discs. After incubating for 16 hours at 42$^{o}$C (for *Campylobacter* spp.) or 37$^{o}$C (for all other bacteria), the diameter of the clear zones of inhibition around the antimicrobial discs on the growth was measured and interpreted according to CLSI guidelines. The breakpoints of antimicrobials used in this study are shown in Table 2.1. MDR was defined as non-susceptibility to ≥1 agent in ≥3 antimicrobial categories listed in Table 2.1. The microbiology results were reported to the collaborating hospitals within three days of sampling.

The production of extended-spectrum β-lactamases (ESBL) was investigated using the double-disc synergy test by comparing zone sizes between amoxicillin (20µg) discs against amoxicillin-clavulanic acid (20/10µg) discs and ceftriaxone or ceftazidime discs (30µg) against ceftriaxone/ ceftazidime-clavulanic acid discs. Isolates with an increase ≥5mm in diameter of inhibitory zone by the synergy of clavulanate were considered to be ESBL positive.

**Table 2.1.** Zone diameter interpretive standard according to CLSI guidelines and multidrug resistance determination

| Test/ Report group | Antimicrobial Agent | | Disk Content | Zone Diameter Interpretive Criteria (nearest whole mm) [a] | | | Note |
|---|---|---|---|---|---|---|---|
| | | | | **R** | **I** | **S** | |
| **PENICILLINS** | | | | | | | |
| A | ampicillin | AMP | 10µg | ≤13 | 14-16 | ≥17 | |
| **β-LACTAM/ β-LACTAMASE INHIBITOR COMBINATIONS** | | | | | | | |
| B | amoxicillin-clavulanate | AMC | 20/10µg | ≤13 | 14-17 | ≥18 | |
| **CEPHEMS (cephalosporins III)** | | | | | | | |
| B | ceftriaxone | CRO | 30µg | ≤19 | 20-22 | ≥23 | |
| C | ceftazidime | CAZ | 30µg | ≤17 | 18-20 | ≥21 | |
| **CARBAPENEMS** | | | | | | | |
| B | imipenem | IMP | 10µg | ≤19 | 20-22 | ≥23 | |
| **AMINOGLYCOSIDES [b]** | | | | | | | |
| A | gentamicin | GEN | 10µg | ≤12 | 13-14 | ≥15 | |
| B | amikacin | AMK | 30µg | ≤14 | 15-16 | ≥17 | |
| **MACROLIDES [c]** | | | | | | | |
| A | azithromycin | AZI | 15µg | ≤12 | - | ≥13 | except *Shigella flexneri* |
| A | azithromycin | AZI | 15µg | ≤15 | - | ≥16 | for *Shigella flexneri* |
| A | erythromycin [d] | ERY | 15µg | ≤13 | 14-22 | ≥23 | |
| **QUINOLONES AND FLUOROQUINOLONES** | | | | | | | |
| B | ciprofloxacin | CIP | 5µg | ≤15 | 16-20 | ≥21 | except *Salmonella* spp. |
| B | ciprofloxacin | CIP | 5µg | ≤20 | 21-30 | ≥31 | for *Salmonella* spp. |
| O | nalidixic acid [c] | NAL | 30µg | ≤13 | 14-18 | ≥19 | |
| **FOLATE PATHWAY INHIBITORS** | | | | | | | |
| B | trimethoprim-sulfamethoxazole | SXT | 1.25/ 23.75µg | ≤10 | 11-15 | ≥16 | |
| **PHENICOLS** | | | | | | | |
| C | chloramphenicol | CHL | 30µg | ≤12 | 13-17 | ≥18 | |
| **LINCOSAMIDES** | | | | | | | |
| A | clindamycin [d] | CLI | 2µg | ≤14 | 15-20 | ≥21 | |

There are no standard guidelines for *Campylobacter* spp., hence we used *Enterobacteriaceae* guidelines for tested antimicrobials apart from erythromycin and clindamycin. Multidrug resistance (MDR) was defined as non-susceptibility to ≥1 agent in ≥3 antimicrobial categories described above.

[a] S = Sensitive; I = Intermediate; R = Resistance. Sensitive was classified as susceptible; Intermediate and Resistance were classified as non-susceptible to the tested antimicrobials

[b] For *Salmonella* spp. and *Shigella* spp., aminoglycosides may appear active in vitro but are known to not be effective clinically[224]. Therefore, susceptibility profiles of *Salmonella* spp. and *Shigella* spp. to aminoglycosides were not reported

[c] Not included in MDR estimation.

[d] Tested for *Campylobacter* isolates only and used zone diameter interpretive standard from *Staphylococcus* spp.

### 2.1.3. Statistical analysis

Data were analysed in Stata v11 (StataCorp, College Station, TX, USA) and R v3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). Figures were constructed in R v3.2.2. using the ggplot2[225] and prodlim packages. Descriptive comparisons between groups were conducted using non-parametric tests including Fisher's exact test for categorical variables and Mann-Whitney U test for continuous data. Statistical comparisons between more than two groups were conducted using the $\chi^2$ test and the Kruskal-Wallis test for categorical and continuous variables, respectively. Kaplan-Meier curves of length of hospital stay were compared between groups using log-rank test. Growth status of participating patients were assessed using the WHO global database on growth and nutrition and Prevention and Management of Obesity for Children and Adolescents-Healthcare guidelines[226,227] using the macro package of Stata v11 developed by WHO.

An accelerated failure time (AFT) regression model (incorporating all study patients hospitalised for ≥1 day) was constructed in three steps. Firstly, the best AFT distribution fit was identified for the dependent variable, i.e. length of hospital stay. Secondly, eleven demographic variables (e.g. sex and age), clinical symptoms of disease severity, treatment types, and MDR were tested by univariate analysis using a log-normal distribution. The eleven variables were chosen due to their potential to affect duration of hospital stay. Thirdly, a multivariate log-normal model was constructed using a stepwise backward elimination method, where variables were removed based on the likelihood ratio test ($p<0.05$).

## 2.2. Methods for Chapter 4 (Diarrhoeagenic *Escherichia coli*)

### 2.2.1. Ethics statement and study design

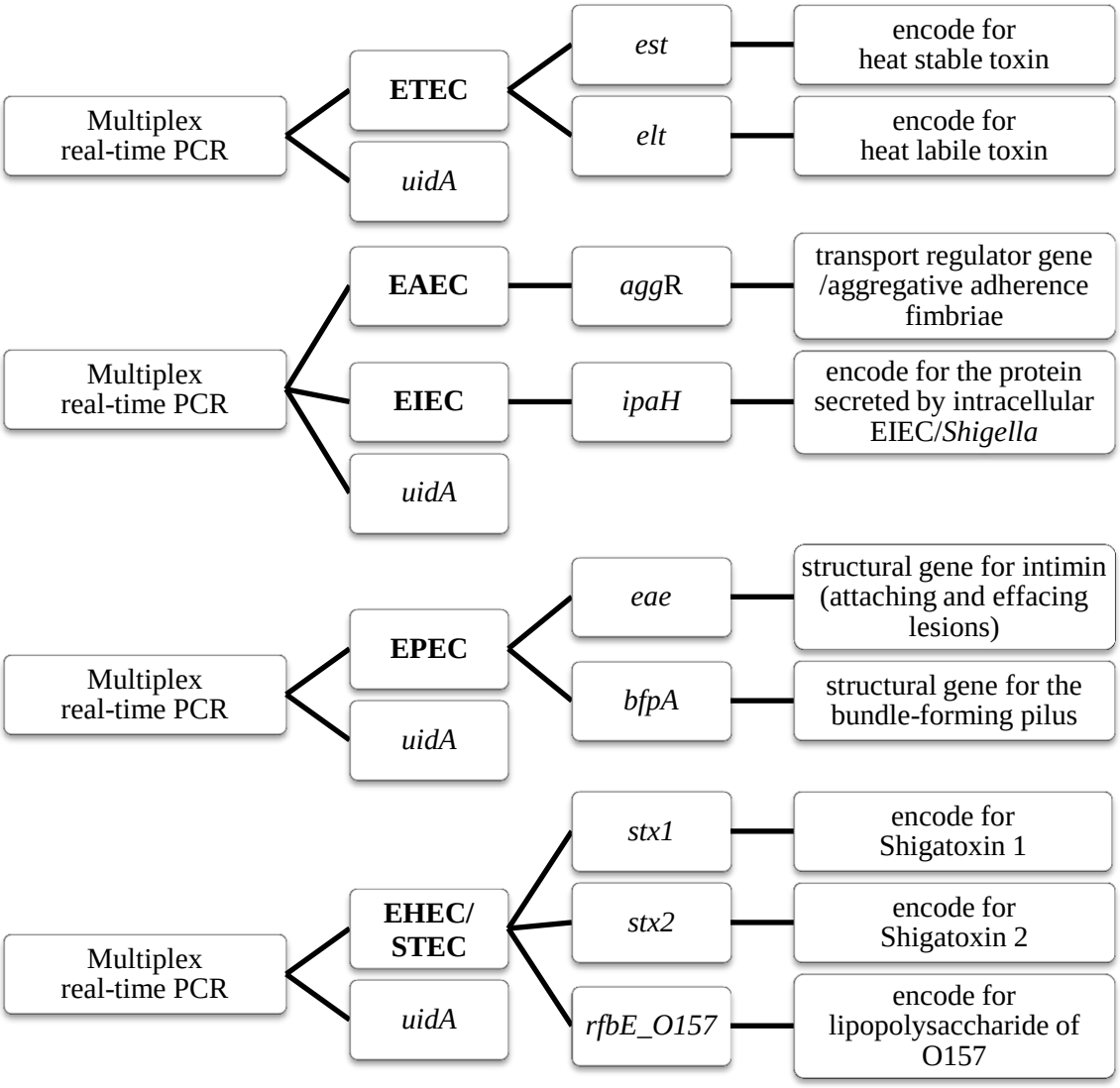
Ethical approval for this study was obtained from the OxTREC (OxTREC No 1058-13 for controls and OxTREC No. 1045-13 for cases) as well as from local partners including the

Institutional Review Board (IRB) at the HTD, CH1, CH2 and the IRB at Hung Vuong Hospital (HVH). An informed consent form signed by parent or guardian was required for enrolment.

Children with diarrhoea were enrolled as described in section 2.1.1.2. Controls were healthy children between the age of 12-36 months enrolled in diarrhoeal disease cohort in District 8 in HCMC from 2014 to 2016[228]. The enrolled children attended HVH for routine health check every 6 months. An anal swab of non-diarrhoeal child was collected by study nurses at these routine visits.

### 2.2.2. Development of multiplex real-time PCR assays for detecting diarrhoeagenic *Escherichia coli*

Control DEC strains were provided by Public Health England (UK) (Many thanks to Dr Claire Jenkins), with the exception of EIEC strain ATCC 43893 which was purchased from ATCC collection (USA). DNA templates of control strains were prepared from pure colonies culture using the Wizard Genomic DNA Purification Kit (Promega). I employed simultaneously four multiplex real-time PCR to detect virulence factors of six different *E. coli* pathotypes (ETEC, EAEC, EIEC, EPEC, EHEC, and STEC as followed: *elt* (heat-labile) and/or *est* (heat-stable) (encoding enterotoxins of ETEC); *aggR* (a transport regulator gene of EAEC[148]); *ipaH* (located on plasmid pINV, encoding a protein secreted by intracellular EIEC/*Shigella*[150]); *eae* (gene encoded for intimin, outer membrane protein mediated adherence to intestine epithelial cells of EHEC and EPEC[141]); *bfpA* (structural gene for the bundle-forming pilus on plasmid responsible for bacterial interaction of EPEC[152]), *stx1* and/or *stx2* (Shiga toxins 1 and 2 of STEC[141]); *rfbE_O157* (encode for lipopolysaccharide which is O antigen of O157) and *uidA* (encoding beta-glucuronidase present in all *E. coli*, an internal control to monitor both DNA extraction and PCR amplification process) (Figure 2.3).




*uidA* (gene encoded for β-glucuronidase and presented in all *E. coli*) was chosen as an internal control

**Figure 2.3.** Four multiple real-time PCR assays for detecting target sequences of diarrhoeagenic *Escherichia coli*.

The criteria for classifying strains as DEC pathotypes were as followed. ETEC included LT-ETEC (the presence of *elt* only); ST-ETEC (the presence of *est* only); and LT-ST-ETEC required to carry both *elt* and *est*. The presence of *aggR* demonstrated for EAEC; whereas the presence of *ipaH* concluded for EIEC/*Shigella* infection. EPEC is classified into typical EPEC (carry both *eae* and *bfpA)* and atypical EPEC (the presence of only *eae*). As gene *eae* is also virulence mechanism for EHEC, I was unable to distinguish atypical EPEC and EHEC. Hence I aimed to identify the EHEC O157 which is the most virulence serotype of EHEC causing several outbreaks worldwide[140,155]. Gene *rfbE_O157* was used to identify EHEC O157 (the presence of *eae* and *rfbE_O157*) and non-*eae* EHEC O157 strains[157] (the presence of only *rfbE_O157*). Although Shiga toxin also known as *vero*-toxin causing haemorrhagic colitis and haemolytic uremic syndrome was usually reported associated with EHEC[140,141,155], emerging other pathotypes of DEC harboured these virulence genes (s*tx1*/s*tx2*) and caused severe sequela[229]. Hence, I defined Shigatoxigenic *E. coli* (STEC) separately from EHEC for analysis. The presence of s*tx1* and/or s*tx2* demonstrated for STEC infection.

#### 2.2.2.1. Primers and probes design

Among the ten gene targets, I adapted primer and probe sequences of four genes (*aggR*, *ipaH*, *eae*, *uidA*) from previous studies and designed the remained 6 genes (*etl*, *est*, *bfpA*, *rfbE_O157*, *stx1* and *stx2*). Briefly, reference sequences were downloaded from GenBank (Accession number in Table 2.2) and aligned using AlignX (Vector NTI, Invitrogen) to find conserved region of each gene. Primer Quest (IDT, USA) was employed to generate primers and probes of amplicon size 100 to 150bp. To find optimal pairs, these candidate primers and probes were re-analysed for Tm, %GC, hairpin, self-dimer and hetero-dimer using Oligo Analyzer 3.1 (http://sg.idtdna.com/calc/analyzer). Final candidates were then blast to NCBI database using PrimerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm *in-silico* specificity of each pairs. I then tested the designed primers and probes on control DEC strains and a range of

other enteric bacterial pathogens (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella spp*., *Campylobacter coli*, *Campylobacter jejuni*, *Shigella sonnei*, *Shigella flexneri*, *Enterobacter*, and *Proteus*) and viruses (norovirus, rotavirus) to confirm specificity of primers and probes. Sequences of primers and probes to detect DEC are shown in Table 2.3.

**Table 2.2.** List of gene code downloaded from BLAST (NCBI) (https://blast.ncbi.nlm.nih.gov)

| Gene | Accession number | | | | |
|---|---|---|---|---|---|
| *elt* | FN649417.1 | AP010910.1 | EU113252.1 | S60731.1 | AB011677.1 |
| | J01646.1 | FN822745.1 | FJ407053.1 | NC_009786.1 | NC_014232.1 |
| | NC_017640.1 | | | | |
| *est* | AJ868113.1 | FN822745.1 | J03311.1 | M18346.1 | M18345.1 |
| | AB702969.1 | M29255.1 | M34916.1 | NC_014232.1 | |
| *bfpA* | KJ641929.1 | KJ020707.1 | AB364244.1 | FN391181.1 | DQ388534.1 |
| | KJ020711.1 | KJ020697.1 | AB364243.1 | FM180569.1 | |
| *rfbE_O157* | CP007592.1 | CP008957.1 | JQ907520.1 | CP001368.1 | AF163334.1 |
| | CP010304.1 | CP008805.1 | JN578668.1 | AF163335.1 | |
| *stx1* | NC_002695.1 | NC_011356.1 | JX206444.1 | EU754740.1 | KM406321.1 |
| | NC_013364.1 | AP010953.1 | JX161807.1 | LM997224.1 | JQ327854.1 |
| | CP001925.1 | GQ429158.1 | | | |
| *stx2* | HF572917.2 | KF932362.1 | CP008805.1 | CP006027.1 | HF558443.1 |
| | KM516099.1 | LM997391.1 | KJ158456.1 | HF558445.1 | HF558442.1 |
| | AB854290.1 | | | | |

**Table 2.3.** Sequences of primers and probes to detect diarrhoeagenic *Escherichia coli*

| Diarrhoeagenic *E. coli* target | Primer/probe name | Sequence (5'-3') | Amplicon size (bp) |
|---|---|---|---|
| ETEC | *elt*_ETEC_F<br>*elt*_ETEC_R<br>*elt*_ETEC_ probe | CTCGGTCAGATATGYGATTCTT<br>AACATTTCAGGTCGAAGTCC<br>**FAM**-TGTGTCCTTCATCCTTTC AATGGCTT-**BHQ1** | 100 |
| | *est*_ETEC_F<br>*est*_ETEC_R<br>*est*_ETEC_ probe | GCTAAACCAGYAGRGTCTTCAA<br>GCAGGATTACAACACAATTCAC<br>**LCCyan500**-AGTRGTCCTGAAA GCATGAATAGTAGCA-**BHQ1** | 137 |
| EAEC | *aggR*_F<br>*aggR*_R<br>*aggR*_probe | CCATTTATCGCAATCAGATTAA<br>CAAGCATCTACTTTTGATATTCC<br>**FAM**-CAGCGATACATTAAGA CGCCTAAAGGA-**BHQ1** | 92[230] |
| EIEC/ *Shigella* | *ipaH*_F<br>*ipaH*_R<br>*ipaH*_probe | AGGTCGCTGCATGGCTGGAA<br>CACGGTCCTCACAGCTCTCA<br>**LCCyan500**-AACTCAGTGCCTCT GCGGAGCTTCGACA-**BHQ1** | 99[231] |
| EPEC/ EHEC | *eae*_F<br>*eae*_R<br>*eae*_probe | CATTGATCAGGATTTTTCTGGTGA TA<br>CTCATGCGGAAATAGCCGTTA<br>**FAM**-ATAGTCTCGCCAGTA TTCGCCACCAATACC-**BHQ1** | 102[232] |
| EPEC | *bfpA*_EPEC_F<br>*bfpA*_EPEC_R<br>*bfpA*_EPEC_probe | GTCTRTCTTTGATTGAATCKGC<br>CATTCTGYGMCTTATTGGAATC<br>**LCCyan500**-ACCGTTACYGCM GGTGTGATGTTT-**BHQ1** | 108 |
| STEC | *stx1*_EHEC_F<br>*stx1*_EHEC_R<br>*stx1*_EHEC_probe | GCATCTGATGAGTTTCCTTCTA<br>GTTCTGCGCATCAGAATTG<br>**FAM**-AAGAGKCCGTGGGA TTACGCACAAT-**BHQ1** | 113 |
| | *stx2*_EHEC_F<br>*stx2*_EHEC_R<br>*stx2*_EHEC_probe | ACRACGGACAGCAGYTATWC<br>GAACTCCATTAAMKCCAGATA<br>**LC Red 610**-ATGCAAATCAGTCGTCA CTCACTGGT-**BHQ1** | 111 |
| EHEC O157 | *rfbE*_O157_F<br>*rfbE*_O157_R<br>*rfbE*_O157_probe | CAAGTCCACAAGGAAAGTAAAG<br>GAGTTTATCTGCAAGGTGATTC<br>**LCCyan500**-AACTCAGTGCCTCT GCGGAGCTTCGACA-**BHQ1** | 111 |
| Internal control | *uidA*_F<br>*uidA*_R<br>*uidA*_probe | GTGTGATATCTACCCGCTTCGC<br>AGAACGGTTTGTGGTTAATCAGGA<br>**CY5**-TCGGCATCCGGTCAGTGGCAGT-**BHQ2** | 82[233] |

R (A/G), Y(C/T), S (G/C), W (A/T), M (A/C), K (G/T) according to International Union of Pure and Applied Chemistry (IUPAC)
Probe detection format (Roche Light Cycler II 480) as followed **FAM**: 498-580; LC**Cyan500**: 440-488; **CY5**: 618-660; **LC Red 610**: 533-610; BlackBerry® Quencher: **BHQ1**, **BHQ2**
ETEC: Enterotoxigenic *Escherichia coli*; EIEC: Enteroinvasive *Escherichia coli*;
EPEC: Enteropathogenic *Escherichia coli*; EHEC: Enterohemorrhagic *Escherichia coli*;
STEC: Shiga-toxin producing *Escherichia coli*

#### 2.2.2.2. Construction of plasmids containing target sequences

Each PCR amplicon generated from the six *E. coli* pathotypes was purified and ligated to vector pCR™ 2.1-TOPO® (Invitrogen, Applied Biosystem, UK) and subsequently cloned into competent *E. coli* cells to generate positive control templates. These templates were used to optimise PCR reaction and calculate reproducibility of PCR reaction. Plasmid DNA contained target amplicon were purified and concentration was quantified using a Nanodrop spectrophotometer (Thermo- Scientific, UK). Plasmid concentrations (ng/µl) were converted to copy number using via online tool (http://cels.uri.edu/gsc/cndna.html).

Multiplex real-time PCR was set up in a 25µl reaction mixture contained a final concentration of 1X buffer, 0.2mM deoxynucleoside triphosphates (dNTPs), 3.5mM of $MgCl_2$, 0.2µM of forward and reverse primers, 0.08µM of each probe and 1U of Hotstart Taq polymerase (QIAGEN, Germany). Five µl of DNA templates were subjected to each PCR reaction. Real-time PCR cycling condition was as follows: 95°C for 15 min, followed by 45 cycles of 95$^{o}$C for 15s, then 60$^{o}$C for 60s on Light Cycler 480 II system (Roche, Germany). The threshold cycle (Ct) value for a positive result was 38 or less.

#### 2.2.2.3. Reproducibility and linearity

The precision and reproducibility of real-time PCR assay were assessed using co-efficient of variance (CV%), measured by dividing Cp value deviation to the mean Cp of selected concentration. The Cp values of three replicates in the same day over each plasmid concentration were compared in order to measure intra-assay reproducibility. The inter-assay reproducibility indicates precision of Cp values generated by three replicates of each plasmid concentration each day over a period of three days. Linearity of the plasmid DNA containing cloned target sequences (concentration $5x10^0$ to $5x10^7$) was calculated by linear regression through Cp value of a ten-fold serial dilution.

### 2.2.3. Specimen procedure and MacConkey sweep storage

The multiplex real-time PCR was applied for the detection of DEC in diarrhoeal and non-diarrhoeal faecal samples. All anal swabs from non-diarrhoeal children were transported to the laboratory at OUCRU for processing. Briefly, 1.5mL of BHI media and glycerol 20% was added into the swab tube before the swab tubes were shaken in shaker (Thermomixer comfort, Eppendorf) at 300 rpm for 20 minutes. The well-mixed suspensions from the anal swab (control) and fresh diarrhoeal faecal samples (cases) were cultured. The microbiological culture procedure was as described in section 2.1.2.1. A sweep of colonies from MC agar was stored at -80$^{o}$C by sweeping into a tube contained BHI media and glycerol 20% until investigation.

Eighty µl of colony sweep (BHI+glycerol) suspension was centrifuged at 4,000rpm for 10mins, and the pellet was resuspended in 80µl of molecular grade water (Sigma). The resulting suspension was mixed well by gently by pipetting up and down, boiled at 96°C for 10 minutes and cooled to room temperature. The lysate was centrifuged at 4,000 rpm for 10 minutes and 5µl of DNA from the supernatant was analysed by real-time PCR.

### 2.2.4. Data collection and statistical analysis

Data were exported into Microsoft Excel (Microsoft, USA), and analysed using Stata v11 (StataCorp, College Station TX, USA). Descriptive comparisons between groups were conducted using non-parametric tests including $\chi^2$ test or Fisher's exact test for categorical variables and the Kruskal-Wallis test for continuous data. Growth status of participating patients were assessed using the WHO global database on growth and nutrition and Prevention and Management of Obesity for Children and Adolescents-Healthcare guidelines[226,227] using the macro package of Stata v11 developed by WHO.

Due to the age difference between diarrhoeal and non-diarrhoeal groups, the comparison analyses were performed between all the children in healthy group and the subset children in the diarrhoea group that were aged 24-60 months.

Logistic regression was performed to determine the associations with diarrhoea using each type of infection considered as an independent variable. Infections were classified as mono-infection with each pathotype of DEC or co-infections with each specific pathotype and other pathotypes. Types of co-infection were repeated due to multi pathotype co-infection; hence *P*-value for univariate model was considered significant when $p<0.01$. Next, multivariable logistic regression models were performed including mono-infection, each specific type of co-infection, gender and growth status with the binary outcomes as diarrhoea and non-diarrhoea (performed on Stata v11, StataCorp, College Station TX, USA). For the latter, a *p*-value of <0.05 was considered significant. The figure for mixed-infections (Figure 4.1) was generated using the UpSetR package[234] and then restructured manually to generate the side by side bar graphs for comparing two groups.

## 2.3. Methods for Chapter 5 (xTAG GPP, Luminex)

### 2.3.1. Study setting

#### 2.3.1.1. Ethics

Stool samples were collected from three studies, all of which were approved by the local ethical committees and OxTREC (OxTREC no. 0109, 15-12, and 1045-13). Written informed consent was required for all patients or from parents/legal guardians if the patient was a child prior to enrolment into all three studies

#### 2.3.1.2. Clinical specimens and study procedures

Faecal specimens were collected from 479 patients hospitalised with diarrhoeal disease from three different studies across Vietnam during 2009 to 2014. The hospitals included in the study were CH1, CH2, and HTD in HCMC. Provincial hospitals in Dong Thap, Dak Lak, Khanh Hoa, and Thua Thien Hue also participated as part of the Vietnam Initiative on Zoonotic Infections (VIZIONS) initiative[235].

Patients were admitted at the discretion of the treating clinician and were excluded from each of the studies if they had suspected or confirmed intussusception, multiple complications unrelated to diarrhoeal disease, or suspected antimicrobial-induced diarrhoea. Diarrhoea was defined as three watery or loose stools within 24h or one episode of bloody and/or mucoid diarrhoea[16]. After collection in a sterile container, fresh stool samples were stored at 4°C at the sites and transported to the central study microbiology laboratory within 24h. The specimens were tested using microbiological culture and real-time PCR and then stored in 20% glycerol solution at -80°C.

This study consisted of three components. First, I selected 172 samples with known aetiologies from microbiological culture for validation of the xTAG GPP system. Second, I selected an additional 307 specimens that were negative by conventional culture methods, microscopy, and real-time PCR to test with the xTAG GPP to detect pathogens that I may not have identified through conventional methodologies. Finally, I used all of the 479 stool tested samples to evaluate the sensitivity and specificity of the xTAG GPP for seven pathogens by using culture and real-time PCR as the gold standards.

### 2.3.2. Conventional methods (microbial culture and real-time PCR)

#### 2.3.2.1. Microbial culture

As in section 2.1.2

#### 2.3.2.2. Molecular detection of bacterial pathogens

The presence of *Shigella* spp. (target, *ipaH*), *Salmonella* spp., and *Campylobacter* spp. (*C. jejuni* target, *hipO*; *C. coli* target, *glyA*) by real-time PCR using the following cycling conditions: 95°C for 15min, 40 cycles of 95°C for 5s, 60°C for 30s, and 72°C for 30s, as described previously[236,237]. *Salmonella* screening was performed using an in-house assay targeting the *invA* gene (forward primer, 5'-TCATCGCACCGTCAAARGA-3'; reverse primer, 5'-CGATTTGAARGCCGGTATTATT-3'; probe, 5'-**FAM**-ACGCTTCGCCGTTCRCGYGC-**BHQ1**-3' ; BHQ1, black hole quencher 1), under the following conditions: 95°C for 15min, 45 cycles of 95°C for 5s, and 60°C for 60s[238].

#### 2.3.2.3. Molecular detection of rotavirus, norovirus GI/GII, and adenovirus

For rotavirus and norovirus molecular testing, total RNA was extracted from fresh stool samples, reverse transcribed into cDNA, and used as the template to detect viruses by real-time PCR, as previously described[239]. Rotavirus detection was performed by targeting the non-structural protein 3 (NSP3). The norovirus primers and probes targeted the open reading frame 1 (ORF1)-ORF2 junction of norovirus genotype 1/2 (GI/GII)[240]. PCR amplifications for rotavirus and norovirus GI/GII were performed using RNA Master hydrolysis probes (Roche Applied Sciences, UK) and optimised with 1.4µl of activator on a LightCycler 480II (Roche Applied Sciences). Five microliters of RNA was mixed with a concentration of 20µM for each primer and 10µM probe, and thermal cycling was initiated at 61°C for 5min for reverse transcription, 5min at 95°C for amplification, and then by 45 cycles at 95°C for 5s and 60°C for 4s.

Adenovirus real-time PCR amplification was performed using primers AdV-F (TCTTACAAAGTGCGC TTTACGC) and AdV-R (TTAAAG CTGGGRCCACGATC) and probe AdV-probe (**Cy5**-GACAACCGGGTKTTGGACATGGCCAG-**BHQ3**). Adenovirus DNA was mixed with a concentration of 20µM for each primer and 10µM probe and LightCycler 480

probes master (Roche Applied Sciences) and subjected a PCR cycle of 95°C for 5min, followed by 45 cycles of 95°C for 5s and 60°C for 1min. The assay was validated with positive controls using a previously described procedure[237].

### 2.3.3. xTAG gastrointestinal pathogen panel assay

The xTAG GPP assay is a multiplex assay that simultaneously detects *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *C. difficile* (toxins A and B), enterotoxigenic *E. coli* (ETEC) heat labile (LT) and heat stable (ST) enterotoxins, *E. coli* O157, Shiga toxin-producing *E. coli* (STEC) spp., *Vibrio cholerae*, *Yersinia enterocolitica*, faecal adenovirus 40/41, rotavirus A, norovirus GI/GII, Giardia spp., *E. histolytica*, and *Cryptosporidium* species.

#### 2.3.3.1. Nucleic acid extraction

For Nucleic acid extraction, 200µl of each stool specimen was automatically extracted with 10µl xTAG MS2 (internal control) by MagNA Pure-96 machine (Roche) according to the manufacturer's instruction.

The xTAG GPP assay was performed according to the manufacturer's recommendations (Luminex Molecular Diagnostics, Austin, TX, USA). Briefly, each 15µl-master mixture contained 7.5µl of 5X xTAG OneStep Buffer, 2.5µl of xTAG GPP Primer Mix, 0.5µl of xTAG BSA (10mg/ml), 2µl of xTAG OneStep Enzyme Mix, and water is up to 15µl. A 10µl of the appropriate extracted nucleic acid sample was used as template for PCR step. The PCR reaction was performed at 53°C for 20min 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 2min; and holding at 4°C.

#### 2.3.3.2. Bead Hybridization and data analysis

The xTAG GPP Bead mix and xTAG Reporter Buffer (containing 0.15M NaCl) kits were thawed and brought to ambient temperature. The 0.22 SAPE was diluted 75-fold with the xTAG Reporter Buffer and stored in the dark until required. The xTAG Bead Mix was mixed and sonicated. Each reaction contained 20µl xTAG Bead Mix, 5µl PCR product and 75µl of reporter solution. The hybridization was performed at 60$^{o}$C for 3 min, 45$^{o}$C for 45 min, and holding at 45$^{o}$C.

When the hybridization was complete, the plate was placed on Luminex plate heater block and samples read by Luminex 200 machine. Each Luminex bead population detects a specific pathogen target through a specific anti-tag/tag hybridization. A signal or median fluorescence intensity (MFI) was generated for each bead population. These fluorescence values are analysed to establish the presence or absence of bacterial, viral or parasitic targets and/or controls in each sample. An output file is created under Luminex batch run folder using the xTAG Data Analysis Software (TDAS).

### 2.3.4. Statistical analysis

The Luminex data were read by the TDAS and imported and analysed in Stata version 11 (College Station, TX, USA). Plots were made in R version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria) using the ggplot2 package[225].

#### 2.3.4.1. Sensitivity and specificity calculation

The sensitivity and specificity were calculated separately using two gold standards: (i) microbiological culture and (ii) real-time PCR. Formulae were presented in Table 2.4 and Table 2.5.

**Table 2.4.** Formulae for calculation of sensitivity, specificity, and 95%CI of xTAG GPP assay with microbiological culture as gold standard

| | | Microbiological culture | | Total |
|---|---|---|---|---|
| | | Positive | Negative | |
| Detected by xTAG GPP, Luminex | Positive | a<br>true positive | b<br>false positive | a + b |
| | Negative | c<br>false negative | d<br>true negative | c + d |
| Total | | a + c | b + d | |
| | | Sensitivity = $\frac{a}{a+c}$ | Specificity = $\frac{d}{b+d}$ | |
| | | 95%CI = $p \pm 1.96 \times \sqrt{\frac{p(1-p)}{n}}$ | | |

p: sensitivity (or specificity) as proportion (not percentage)

n: number of culture positive (a+c) and culture negative (b+d), respectively for sensitivity or specificity

**Table 2.5.** Formulae for calculation of sensitivity, specificity, and 95%CI of xTAG GPP assay with real-time PCR as gold standard

| | | Real-time PCR | | Total |
|---|---|---|---|---|
| | | Positive | Negative | |
| Detected by xTAG GPP, Luminex | Positive | a<br>true positive | b<br>false positive | a + b |
| | Negative | c<br>false negative | d<br>true negative | c + d |
| Total | | a + c | b + d | |
| | | Sensitivity = $\frac{a}{a+c}$ | Specificity = $\frac{d}{b+d}$ | |
| | | 95%CI = $p \pm 1.96 \times \sqrt{\frac{p(1-p)}{n}}$ | | |

p: sensitivity (or specificity) as proportion (not percentage)

n: number of real-time PCR positive (a+c) and real-time PCR negative (b+d), respectively for sensitivity or specificity

#### 2.3.4.2. *Salmonella* detection by xTAG GPP assay

As the specificity of the original Luminex cut-offs for *Salmonella* spp. was low (60%), I attempted to redefine the cut-offs to improve the sensitivity and specificity for the *Salmonella* assay. *Salmonella* detection occurs via 2 probes, a positive result was recorded when probe 1 has a signal ≥1400 MFI; or when both probe 1 and 2 have a signal ≥200 MFI. If probe 1 has a signal <200 MFI, the result is negative regardless the signal of probe 2. To evaluate the capacity of probes 1 and 2 in discriminating between positive and negative *Salmonella* infection, an area under the receiver operating characteristic (ROC) curve was computed for univariate logistic regression models (containing either probe 1 or 2 alone as a predictor) and a multivariate logistic regression model (containing both probes 1 and 2 as predictors). The optimal cut points for probe 1, probe 2, or for probes 1 and 2 combined were estimated using the Youden index method (in which the sum of sensitivity and specificity of the logistic models were maximised)[241].

#### 2.3.4.3. Spearman's correlation for co-infection among enteric pathogens

I calculated the value of Spearman's correlation to describe the relationship of two specific pathogens in the same clinical faecal sample. By using this correlation, one enteric pathogen demonstrated infected with another pathogen with correspondent value of the correlation coefficient. The interpretation of value of the correlation coefficient was as followed: -0.50 to -0.25 "very unlikely"; -0.25 to 0.00 "unlikely"; 0.00 to 0.25 "likely"; 0.25 to 0.50 "very likely". Finally, I illustrated the value by colour to create a heat-map of co-infection among enteric pathogens.

## 2.4. Methods for chapter 6 (Whole Genome Sequencing of non-typhoidal *Salmonella*)

### 2.4.1. Ethics and study setting

I investigated the NTS organisms isolated from the study described in Chapter 3. The ethics and study design were as described in section 2.1.1.

### 2.4.2. *Salmonella* culture and antimicrobial susceptibility

As described in section 2.1.2

### 2.4.3. Bacterial genomic DNA extraction

Total DNA was extracted from each confirmed *Salmonella* specimen using the Wizard genomic DNA extraction Kit (Promega, USA). Briefly, DNA extraction of Gram negative bacteria i.e. *Salmonella* spp. is comprised of three main steps: lysis, RNA and protein removal, and DNA precipitation and rehydration[242]. The surface of culture plates were scraped and added into 600µl Nuclei Lysis Solution in 1.5ml micro-centrifuge tube. The mixture was gently pipetted until the cells were fully re-suspended. Then the tubes were incubated at 80$^{o}$C for 5min and cooled to room temperature. To digest RNA, 3µl of RNase Solution was added and incubated at 37$^{o}$C for 5min before cooling the sample to room temperature. Next 200µl of protein precipitation solution was added to each tube and vortex vigorously at high speed for 20s. These tubes were incubated on ice for 5-15min, then centrifuged at 13,200rpm for 15min. Lastly, supernatants containing the DNA were transferred to labelled micro-centrifuge tube containing 600µl of isopropanol. The tubes were mixed gently by inversion until the thread like strands of DNA formed; then tubes were centrifuged at 13,200rpm for 2 min. The DNA pellet was washed in 600µl of 70% ethanol and rehydrated with 100µl of DNA Rehydration solution. DNA was stored at 4$^{o}$C until required.

### 2.4.4. Whole genome sequencing and *in-silico Salmonella* serovar typing

All DNA samples were shipped to the Wellcome Trust Sanger Institute (Hinxton, UK) for WGS using the Illumina Hiseq 2500 platform. Raw sequences in FASTQ format were input into Kraken (v0.10.6) for taxonomic identification by comparison to a pre-set database[243]. A *de novo* sequence assembly was performed using Velvet v1.2.03 and VelvetOptimizer for each isolate, and each read sets were mapped back to the corresponding assembly[244].

*In silico* molecular serotyping for *Salmonella* was performed for individual genome assembly using the *Salmonella* In Silico Typing Resource (SISTR)[245]. The analysis was based on the Multi-Locus Sequence Typing (MLST) scheme for *Salmonella*, and serovar prediction was based on identification of genetic elements coding for the O (somatic) and H (flagellar) antigens.

### 2.4.5. Identification of antimicrobial resistance genes

AMR genetic determinants were predicted from each sample's raw sequencing reads using ARIBA (version 0.4.1), which performs target gene identification by assembly and alignment[246]. The Comprehensive Antibiotic Resistance Database (CARD – McMaster University; https://card.mcmaster.ca/home) was used as the input database[57]. Resistance determinants were identified if they fit the criteria of ≥95% nucleotide identity and ≥50% sequence length identity. ARIBA results were then manually curated and summarised.

### 2.4.6. Correlation of antimicrobial susceptibility phenotypes and genotypes

All NTS isolates were tested for susceptibility to 14 antimicrobial agents. The presence of a certain resistance gene or mutation was interpreted as non-susceptible phenotype to the corresponding antimicrobial. Intermediate phenotypes were presented as non-susceptible. To determine the correlation between antimicrobial susceptibility phenotypes and genotypes, I calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) using the presence of resistance genes and mutations, with laboratory susceptibility phenotypes as gold standard. Formulae were as followed in Table 2.6.

**Table 2.6.** The formulae for the calculation of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and 95%CI

| | | Laboratory susceptibility phenotypes | | Total | |
|---|---|---|---|---|---|
| | | Non-susceptible | Susceptible | | |
| The presence of antimicrobial resistance genes | Yes | a<br>true positive | b<br>false positive | a + b | PPV = $\frac{a}{a+b}$ |
| | No | c<br>false negative | d<br>true negative | c + d | NPV = $\frac{d}{c+d}$ |
| Total | | a + c | b + d | | |
| Sensitivity = $\frac{a}{a+c}$ Specificity = $\frac{d}{b+d}$ | | | | | |
| 95%CI = $p \pm 1.96 \times \sqrt{\frac{p(1-p)}{n}}$ | | | | | |

p: sensitivity (or specificity) as proportion (not percentage)

n: number of non-susceptible (a+c) and susceptible (b+d) samples, respectively for sensitivity or specificity

### 2.4.7. Data analysis

All clinical information was anonymised and identified only by the ID number in the database. The data was analysed using Stata v11 (StataCorp, College Station, TX, USA). Growth status of the study's participants were assessed using the WHO global database on growth and nutrition and Prevention and Management of Obesity for Children and Adolescents-Healthcare guidelines, using the macro package of Stata v11 developed by WHO[226,227]. Haemoglobin concentration cut-off for anaemia diagnosis was assessed using the recommended WHO guidelines[247].

# 3. Assessing the benefit of empirical antimicrobial therapy for paediatric diarrhoea in a high-usage, high-resistance setting, Children's hospitals in Ho Chi Minh City, Vietnam

## 3.1. Abstract

Paediatric diarrhoeal disease presents a major public health burden in LMICs. The clinical benefits of empirical antimicrobial treatment for diarrhoea are unclear in settings that lack reliable diagnostics and have high AMR. I conducted a prospective multicentre cross-sectional study of paediatric patients hospitalised with diarrhoea containing blood and/or mucus in HCMC, Vietnam. Clinical parameters, including disease outcome and treatment, were measured. *Shigella*, NTS, and *Campylobacter* were isolated from faecal samples and their antimicrobial susceptibility profiles were determined. Statistical analyses, comprising Log-rank tests and accelerated failure time models, were performed to assess the effect of antimicrobials on disease outcome. Among 3,166 recruited participants (median age 10 months, IQR 6.5-16.7 months), one-third (1,096/3,166) had bloody diarrhoea and 25% (793/3,166) were culture-positive for *Shigella*, NTS, or *Campylobacter*. Over 85% (2,697/3,166) of patients were treated with antimicrobials; fluoroquinolones were the most commonly administered antimicrobials. AMR was highly prevalent among the isolated bacteria, including resistance against fluoroquinolones and third generation cephalosporins. Antimicrobial treatment and MDR status of the infecting pathogens were found to have no significant effect on outcome. Antimicrobial treatment was significantly associated with an increase in the duration of hospitalisation in particular groups of diarrhoeal diseases. In a setting with high antimicrobial usage and high AMR, my results imply a lack of clinical benefit for treating diarrhoea with antimicrobials; adequately powered randomised controlled trials are required to assess the role of antimicrobials for diarrhoea.

## 3.2. Introduction

Diarrhoea remains the second most significant cause of morbidity and mortality in children aged <5 years worldwide[1]. In 2010, the global burden of diarrhoea was estimated to be 1.73 billion episodes, of which 36 million were characterised as moderate or severe; 26% (9.3 million) of the severe episodes were estimated to arise in Southeast Asia[1]. Among the bacterial pathogens causing diarrhoea in children *Campylobacter spp.*, NTS, *Shigella spp.*, *E. coli*, and *Yersinia enterocolitica* are the most commonly identified[97,248]. *Campylobacter*, NTS, and *Shigella* are major contributors to the global morbidity of diarrhoea and account for an estimated 7.5, 7.1, and 4.8 million DALYs, respectively; the majority of these occur in LMICs[249].

The prevalence of all-cause diarrhoea in children aged <5 years in Vietnam is estimated to be 7-11% (Multiple Indicator Cluster Survey in 2000, 2006, and 2011)[211] and accounts for as much as 12% of all-cause deaths in this age group[212]. A large burden of diarrhoeal disease, combined with the lack of financial and diagnostic resources in Vietnam, means that the causative agent(s) of most diarrhoeal episodes are never determined. As a consequence, antimicrobials are empirically administered to children with diarrhoea based solely on clinical presentation. A previous study found that antimicrobials were prescribed in 38% of acute watery diarrhoea episodes in Vietnamese children found to be associated with a viral pathogen and in 60% of cases with unknown aetiology[213]. The excessive use of antimicrobials in animals and humans in Southeast Asia has led to the current AMR crisis in the region, with increasing resistance against many first-line antimicrobials, including fluoroquinolones and third generation cephalosporins, in many Gram-negative pathogens across the region[168]. Therefore, a better understanding of the bacterial agents of diarrhoea, their corresponding AMR profile, the impact of antimicrobial treatment on clinical outcome, and the effects of empirical antimicrobials are required.

Little has been reported regarding the disease burden and clinical management of hospitalised paediatric diarrhoeal diseases in Vietnam. Despite NTS, *Campylobacter*, and *Shigella* being previously identified as major bacterial causes of diarrhoea in Vietnamese children[94,128,213,250], the epidemiology, AMR profiles, treatment, and the associated outcome of these bacteria in paediatric diarrhoea was not well described. The Vietnamese healthcare system currently follows the WHO guidelines for treatment of paediatric diarrhoea. These guidelines recommend the use of low osmolarity ORS, zinc, and ciprofloxacin or one of the three alternatives: pivmecillinam, azithromycin, or ceftriaxone, for all patients with bloody diarrhoea, irrespective of age[16,19]. However, as the prevalence of AMR in enteric pathogens increases across the region, it is uncertain how existing guidelines correspond to circulating AMR profiles, antimicrobial treatment practices and patient outcomes in children hospitalised with diarrhoea. Therefore, I conducted a prospective multicentre cross-sectional study in HCMC to improve the understanding of bacteria-associated diarrhoea in Vietnamese children and to assess the duration of hospital stay in diarrhoeal patients infected with bacterial pathogens receiving antimicrobials.

## 3.3. Results

### 3.3.1. General characteristics of patients hospitalised with diarrhoea

Between May 2014 and April 2016, 3,166 hospitalised children meeting the study criteria were recruited at the three study hospitals. The majority of patients were male (1,945/3,166; 61.4%), with ages ranging from one month to 15 years (median age 10 months, IQR 6.5-16.7 months). Patients were hospitalised for a median of five days (IQR 3-7 days) with 88.7% (2,808/3,166) of patients improving or resolving symptoms within three days of enrolment.

Patients were segregated into three diarrhoeal types: acute non-bloody (1,775/3,166; 56.1%), acute bloody (1,096/3,166; 34.6%), and persistent (295/3,166; 9.3%) (Table 3.1). Due to the clinical complexity of persistent diarrhoea, statistical comparisons were conducted between the

two acute diarrhoea groups only. Patients with non-bloody diarrhoea were more likely to experience vomiting than those with bloody diarrhoea (66.8% vs. 38.7%; $p$=0.001), corresponding with a greater proportion of non-bloody diarrhoeal patients experiencing dehydration (15.0% vs. 2.6% in bloody diarrhoea; $p$=0.001). A significantly greater proportion of bloody diarrhoea patients experienced abdominal pain than patients with non-bloody diarrhoea (26.4% vs. 19.3%; $p$=0.001). Systemic C-reactive protein (CRP) concentrations were significantly higher in patients with bloody diarrhoea in comparison to those with non-bloody diarrhoea (21.0 mg/L, IQR 6.7-40.2 vs. 7.0 mg/L, IQR 4.0-24.0; $p$<0.001).

**Table 3.1.** Demographic and clinical manifestations of paediatric patients admitted with diarrhoea in Ho Chi Minh City

| | **Non-bloody diarrhoea** [a] | | **Bloody diarrhoea** | | **Persistent diarrhoea** | | ***p*-value** [b] |
|---|---|---|---|---|---|---|---|
| Total, *N* | 1,775 | | 1,096 | | 295 | | - |
| Socio-demographic | | | | | | | |
| Male, *n* (%) | 1135 | (63.9) | 633 | (57.8) | 177 | (60.0) | **0.001** |
| Age in months, *median* [IQR] | 11.5 | [7.6-18.5] | 9.0 | [5.9-15.5] | 5.5 | [4.0-8.2] | **<0.001** |
| Growth [c] | | | | | | | |
| Obese or overweight, *n* (%) | 184 | (11.0) | 83 | (8.3) | 24 | (8.7) | **0.018** |
| Wasted or severely wasted, *n* (%) | 176 | (10.5) | 107 | (10.6) | 25 | (9.1) | 0.688 |
| Clinical symptoms | | | | | | | |
| Number of episodes per day, *median* [IQR] | 7 | [5-10] | 7 | [5-10] | 7 | [5-10] | - |
| Moderate or severe dehydration, *n* (%) [d] | 267 | (15.0) | 28 | (2.6) | 9 | (3.1) | **0.001** |
| Abdominal pain, *n* (%) | 343 | (19.3) | 289 | (26.4) | 50 | (17.0) | **0.001** |
| Fever (≥37.5°C) at enrolment, *n* (%) | 1137 | (64.1) | 615 | (56.1) | 83 | (28.2) | **0.001** |
| Vomit, *n* (%) | 1185 | (66.8) | 424 | (38.7) | 101 | (34.4) | **0.001** |
| Haematology | | | | | | | |
| Neutrophil count ($10^3$/μL), *median* [IQR] | 4.4 | [2.6-7.0] | 4.1 | [2.4-6.7] | 2.5 | [1.6-3.9] | **0.014** |
| C-reactive protein (mg/L), *median* [IQR] | 7.0 | [4.0-24.0] | 21.0 | [6.7-40.2] | 5.0 | [3.3-7.0] | **<0.001** |
| Stool culture positive | | | | | | | 0.7 |
| *Campylobacter*, *n* (%) | 100 | (5.6) | 155 | (14.1) | 2 | (0.7) | **<0.001** |
| *Salmonella*, *n* (%) | 179 | (10.1) | 289 | (26.4) | 10 | (3.4) | **<0.001** |
| *Shigella*, *n* (%) | 39 | (2.2) | 41 | (3.7) | 1 | (0.3) | **0.019** |
| Treatment | | | | | | | |
| Low-osmolarity oral rehydration solution, *n* (%) | 1740 | (98.0) | 956 | (87.2) | 260 | (88.1) | 0.8 |
| IV Rehydration, *n* (%) | 278 | (15.7) | 48 | (4.4) | 15 | (5.1) | **0.001** |
| Antimicrobials, *n* (%) | 1392 | (78.4) | 1079 | (98.4) | 226 | (76.6) | **0.001** |
| Fluoroquinolones, *n* (% of antimicrobial treated) [e] | 739 | (53.1) | 920 | (85.3) | 140 | (61.9) | **0.001** |
| Zinc, *n* (%) | 1586 | (89.4) | 962 | (87.8) | 276 | (93.6) | 0.200 |
| Probiotics, *n* (%) | 1395 | (78.6) | 627 | (57.2) | 200 | (67.8) | **0.001** |
| Outcome | | | | | | | |
| Hospital stay in days, *median* [IQR] | 5 | [3-6] | 4 | [3-6] | 9 | [4-10] | **0.003** |
| Improved and recovered after 3 days, *n* (%) [f] | 1624 | (91.5) | 961 | (87.7) | 223 | (75.6) | 0.008 |

[a] All the children with non-bloody diarrhoea presented mucus in stools

[b] Comparisons between non-bloody and bloody diarrhoea using Fisher's exact test for categorical data or Mann-Whitney *U* test for continuous data.

[c] Obese: weight for length z score >3SD in children age < 24months; BMI for age z score >3SD in children age ≥24months,

Overweight: weight for length z score >2SD in children age < 24months; BMI for age z score >2SD in children age ≥24months,

Wasted: weight for length z score <-2SD in children age < 24months; BMI for age z score <-2SD in children age≥24months,

Severely wasted: weight for length z score <-3SD in children age < 24months); BMI for age z score <-3SD in children age ≥24months)[251]

[d] Dehydration classified according to Integrated Management of Childhood Illness[60]

[e] Fluoroquinolones included ciprofloxacin and norfloxacin.

[f] Defined as "recovered" if patient had <3 passages of loose stool in the past 24 hours or "improved" if patient had less episodes of diarrhoea and less mucus and/or bloody in comparison to the condition of the patient at enrolment.

This study focussed on culturing the three key bacterial diarrhoeal pathogens in Vietnam (i.e. NTS, *Campylobacter*, and *Shigella*), isolating 816 of these bacteria from 804 cases (12 co-infections) and stratified clinical manifestations and treatment data by these organisms. At least one of these bacteria was isolated from the faecal specimens of 44.3% (485/1,096) of the bloody diarrhoeal cases and 17.9% (318/1,775) of the non-bloody diarrhoeal cases ($p<0.001$) (Table 3.1). Overall, NTS was the most frequently isolated of the three bacterial pathogens from the diarrhoeal children, accounting for 15.1% (478/3,166) of all diarrhoeal cases, followed by *Campylobacter* and *Shigella*. *Shigella* infections were more common in older children (median age 3 years) and associated with more severe symptoms, including abdominal pain (51.9% vs. 25.4% in NTS infection and 22.4% in *Campylobacter* infection; $p<0.001$) and vomiting (64.6% vs. 41.8% in NTS infection and 48.6% in *Campylobacter* infection; $p<0.001$) (Table 3.2). In those with more severe symptoms, patients infected with *Shigella* were more likely to have dehydration (16.5% vs. 5.5% in NTS infection and 5.7% of *Campylobacter* infection; $p=0.004$). These children also demonstrated had a greater inflammatory response with higher neutrophil counts and CRP, $p<0.001$. As a consequence of these observations, patients infected with *Shigella* were more likely to require IV fluid (19.0% vs. 6.8% in NTS infection and 6.5% in *Campylobacter* infection; $p<0.001$) and fluoroquinolones therapy (89.3% vs. 74.1% of NTS infection and 79.1% of *Campylobacter* infection, p=0.010). However, children with *Shigella* infections recovered more rapidly than those infected with NTS and *Campylobacter* with shorter hospital stay and higher percentage of number of patients who recovered or improved after 3 days of follow-up (Table 3.2).

**Table 3.2.** Clinical characteristics of those infected with *Campylobacter*, Non-typhoidal *Salmonella*, and *Shigella*

| | ***Campylobacter*** | | **Non-typhoidal *Salmonella*** | | ***Shigella*** | | ***p*-value** [b] |
|---|---|---|---|---|---|---|---|
| Total, *N* [a] | 245 | | 469 | | 79 | | |
| Socio-demographic | | | | | | | |
| Male, *n* (%) | 153 | (62.4) | 285 | (60.8) | 40 | (50.6) | 0.165 |
| Age in months, *median* [IQR] | 11.0 | [8.0-16.5] | 9.2 | [6.4-15.1] | 36.8 | [25.0-56.3] | **<0.001** |
| Growth [c] | | | | | | | |
| Obese or overweight, *n* (%) | 26 | (11.2) | 49 | (10.9) | 13 | (21.0) | 0.067 |
| Wasted or severely wasted, *n* (%) | 34 | (14.7) | 59 | (13.2) | 1 | (1.6) | 0.055 |
| Type of diarrhoea | | | | | | | 0.209 |
| Non-bloody diarrhoea [d], *n* (%) | 95 | (38.8) | 175 | (37.3) | 38 | (48.1) | |
| Bloody diarrhoea, *n* (%) | 148 | (60.4) | 284 | (60.6) | 40 | (50.6) | |
| Persistent diarrhoea, *n* (%) | 2 | (0.8) | 10 | (2.1) | 1 | (1.3) | |
| Clinical manifestations | | | | | | | |
| Number of episodes per day, *median* [IQR] | 6 | [5-10] | 10 | [6-10] | 8 | [6-10] | **<0.001** |
| Moderate and severe dehydration, *n* (%) [e] | 14 | (5.7) | 26 | (5.5) | 13 | (16.5) | **0.004** |
| Abdominal pain, *n* (%) | 55 | (22.4) | 119 | (25.4) | 41 | (51.9) | **<0.001** |
| Fever (≥37.5°C at enrolment), *n* (%) | 151 | (61.6) | 306 | (65.2) | 57 | (72.2) | 0.666 |
| Vomit, *n* (%) | 119 | (48.6) | 196 | (41.8) | 51 | (64.6) | **<0.001** |
| Haematology | | | | | | | |
| Neutrophil count ($10^3$/μL), *median* [IQR] | 4.5 | [3.0-7.0] | 4.9 | [3.3-7.2] | 8.0 | [5.0-11.4] | **<0.001** |
| C-reactive protein (mg/L), *median* [IQR] | 13.4 | [6.5-31.5] | 29.0 | [10.0-48.0] | 52.0 | [24.0-82.0] | **<0.001** |
| Treatment | | | | | | | |
| Low-osmolarity oral rehydration solution, *n* (%) | 208 | (84.9) | 401 | (85.5) | 59 | (74.7) | |
| IV rehydration, *n* (%) | 16 | (6.5) | 32 | (6.8) | 15 | (19.0) | **<0.001** |
| Antimicrobials, *n* (%) | 230 | (93.9) | 440 | (93.8) | 75 | (94.9) | 0.927 |
| Fluoroquinolones, *n* (% of antibiotic treated) [f] | 182 | (79.1) | 326 | (74.1) | 67 | (89.3) | **0.010** |
| Zinc, *n* (%) | 226 | (89.0) | 426 | (90.8) | 68 | (86.1) | 0.256 |
| Probiotics, *n* (%) | 172 | (70.2) | 315 | (67.2) | 55 | (69.6) | 0.686 |
| Outcomes | | | | | | | |
| Hospital stay in days, *median* [IQR] | 4 | [2-5] | 5 | [3-7] | 3 | [3-5] | **<0.001** |
| Improved and recovered after 3 days, *n* (%) [g] | 222 | (90.6) | 407 | (86.8) | 74 | (93.7) | **<0.001** |

[a] All co-infections (which amounted to 11 co-infections) between *Campylobacter,* NTS and/or *Shigella* were removed from above analysis. Therefore, above table presents data and statistical analysis for mono-infections only.

[b] Comparison of three bacterial pathogens using $\chi^2$ test for categorical data or Kruskal-Wallis test for continuous data

[c] Obese: weight for length z score >3SD in children age < 24months; BMI for age z score >3SD in children age ≥24months,

Overweight: weight for length z score >2SD in children age < 24months; BMI for age z score >2SD in children age ≥24months,

Wasted: weight for length z score <-2SD in children age < 24months; BMI for age z score <-2SD in children age≥24months,

Severely wasted: weight for length z score <-3SD in children age < 24months); BMI for age z score <-3SD in children age ≥24months)[251]

[d] All the children with non-bloody diarrhoea presented mucus in stools

[e] Dehydration classified according to Integrated Management of Childhood Illness[60]

[f] Fluoroquinolones included ciprofloxacin and norfloxacin.

[g] Defined as "recovered" if patient had <3 passages of loose stool in the past 24 hours or "improved" if patient had less episodes of diarrhoea and less mucus and/or bloody in comparison to the condition of the patient at enrolment.

### 3.3.2. Antimicrobial usage for treatment of hospitalised diarrhoeal diseases

I additionally recorded the treatment regimens of the enrolled patients, which included ORS, intravenous (IV) rehydration, zinc, probiotics, and antimicrobials. More than 90% of patients were administered ORS, and >80% were given zinc supplementation. The use of antimicrobials within this population was high, with 85.2% (2,697/3,166) of patients receiving empirical antimicrobial treatment following admission to hospital and prior to obtaining a bacterial culture result. Fluoroquinolones were the most commonly used class of antimicrobials (1,799/2,697; 66.7%). Differences in standard of care treatment were observed between bloody and non-bloody diarrhoea patients (Table 3.1); antimicrobials were more regularly administered to patients with bloody diarrhoea than non-bloody diarrhoea ($p$<0.001). Antimicrobials, specifically fluoroquinolones, were commonly (>70%) prescribed prior to an etiological diagnosis in those eventually found to be infected with C*ampylobacter*, NTS, or *Shigella* (Table 3.2).

### 3.3.3. Antimicrobial susceptibility

C*ampylobacter*, NTS and *Shigella* isolates displayed a high prevalence of non-susceptibility against many of the screened antimicrobials (Figure 3.1). Notably, a high proportion of *Campylobacter*, NTS, and *Shigella* exhibited non-susceptibility against ciprofloxacin: 94.2% (242/257), 58.4% (279/478), and 70.4% (57/81), respectively. Additionally, 54.3% (44/81) of *Shigella* and 13.2% (63/478) of NTS isolates were ESBL positive. As a result of that, 56.8% (46/81) of *Shigella* and 13.8% (66/478) of NTS isolates were non-susceptible to the third generation cephalosporins, ceftriaxone and ceftazidime. A smaller fraction of the *Campylobacter*, NTS, and *Shigella* isolates additionally exhibited resistance against azithromycin: 9.7% (25/257), 17.4% (83/478) and 22.2% (18/81), respectively. The majority (65.4%, 531/816 isolates) of the organisms were categorised as MDR. The prevalence of MDR was highest within the *Campylobacter isolates* (218/257, 84.8%), with three isolates additionally exhibiting non-

susceptibly to imipenem. The MDR prevalence in NTS and *Shigella* was 53.9% (258/478) and 67.9% (55/81), respectively (Figure 3.1).

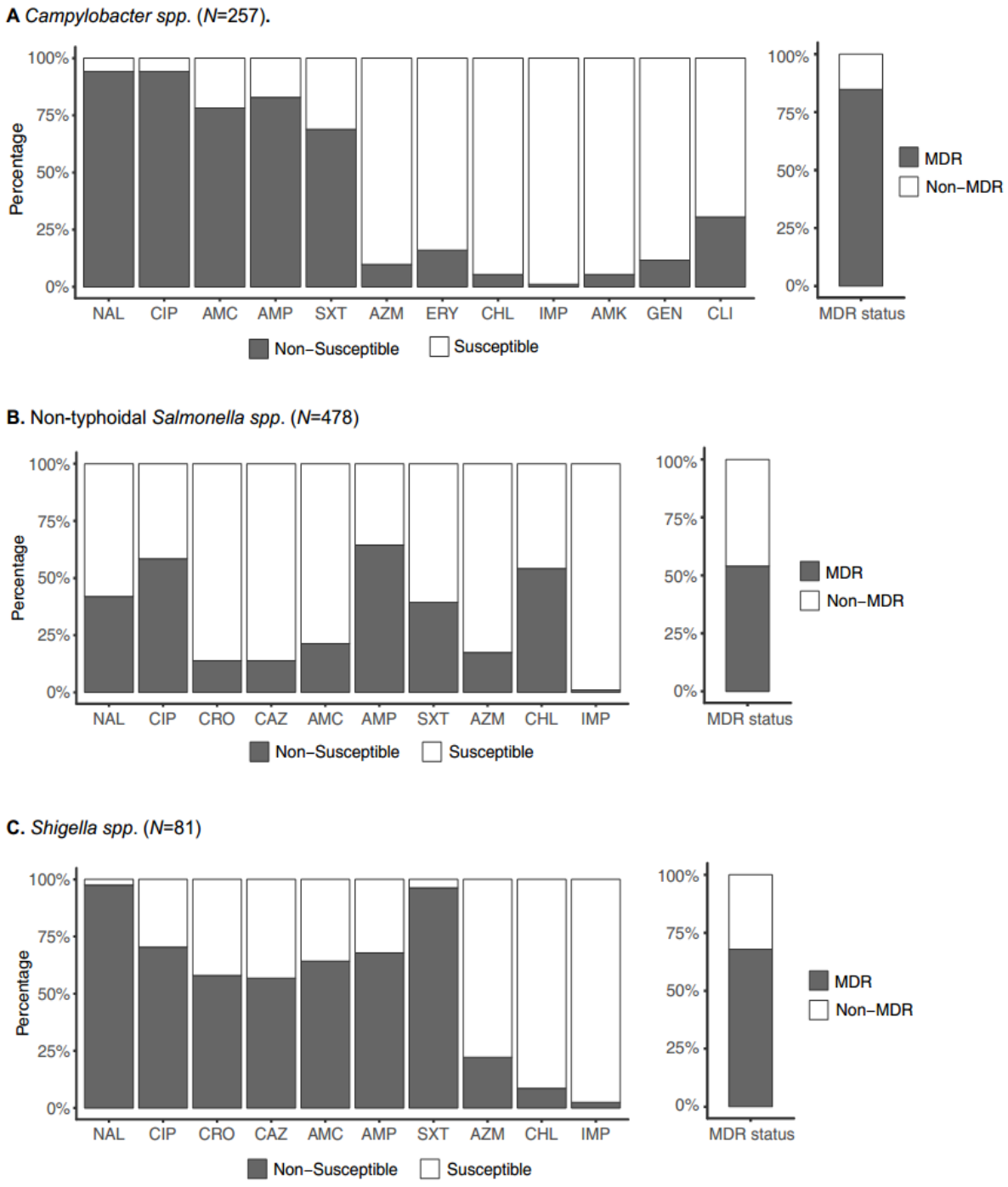



**Figure 3.1.** The antimicrobial resistance profiles of *Campylobacter* spp., non-typhoidal *Salmonella* spp., and *Shigella* spp.

Antimicrobial susceptibility and multidrug resistance (MDR defined as non-susceptibility to ≥1 agent in ≥3 antimicrobial categories) profiles for isolated *Campylobacter* spp. (A), non-typhoidal *Salmonella* spp. (B), and *Shigella* spp. (C). Bar graphs showing the proportion of organisms exhibiting non-susceptibility (dark grey) to nalidixic acid (NAL), ciprofloxacin (CIP), ceftriaxone (CRO), ceftazidime (CAZ), amoxicillin-clavulanic acid (AMC), ampicillin (AMP), trimethoprim-sulfamethoxazole (SXT), azithromycin (AZM), chloramphenicol (CHL), amikacin (AMK), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), imipenem (IMP).

For *Salmonella* spp. and *Shigella* spp., aminoglycosides (i.e. amikacin) may appear active *in vitro* but are known to not be effective clinically[224]. Therefore, susceptibility profiles of *Salmonella* spp. and *Shigella* spp. to aminoglycosides were not reported. Erythromycin and clindamycin were tested for *Campylobacter* isolates only and used zone diameter interpretive standard from *Staphylococcus* spp.

### 3.3.4. Diarrhoeal disease outcome

I next assessed the effect of antimicrobial treatment on two proxy disease outcome measures, clinical outcome (i.e. improved/recovered) at three days post-enrolment and the duration of hospital stay. More than 80% of patients improved or recovered at three days post-enrolment regardless of antimicrobial treatment or susceptibility profiles of infectious bacteria (MDR or ciprofloxacin non-susceptible) (Figure 3.2). However, those given an antimicrobial, specifically a fluoroquinolone, had a longer hospital stay in comparison to those not receiving an antimicrobial ($p<0.0001$ and $p=0.01$, respectively) (Figure 3.3). Notably, the duration of hospital stay was not significantly different between those receiving and not receiving an antimicrobial within those with bloody diarrhoea (Figure 3.3C). However, antimicrobial treatment in those with non-bloody diarrhoea was significantly associated with a longer hospital stay (antimicrobial usage: median hospital stay 5 days, IQR 3-7; no antimicrobial use: median hospital stay 4 days, IQR 3-5, $p<0.001$) (Figure 3.3C). Similarly, antimicrobial treatment in patients with low CRP (≤5mg/L) was significantly associated with an increased hospital stay in comparison to patients with high CRP (>5mg/L), $p<0.001$ (Figure 3.3D).

I then stratified all patients by antimicrobial treatment, and within those treated with antimicrobials I compared disease outcome (i.e. duration of hospitalisation) between those infected with MDR and non-MDR organisms and between those infected with fluoroquinolone-susceptible and non-susceptible organisms (Figure 3.4). In patients empirically treated with any antimicrobial or a fluoroquinolone, more than 90% (526/564) and 70% (361/515) were infected with an MDR or a fluoroquinolone non-susceptible organism, respectively. However, regardless of the MDR status and fluoroquinolone non-susceptibility of the infecting organisms, no differences in the duration of hospitalisation were observed among patients treated with antimicrobials (Figure 3.4).

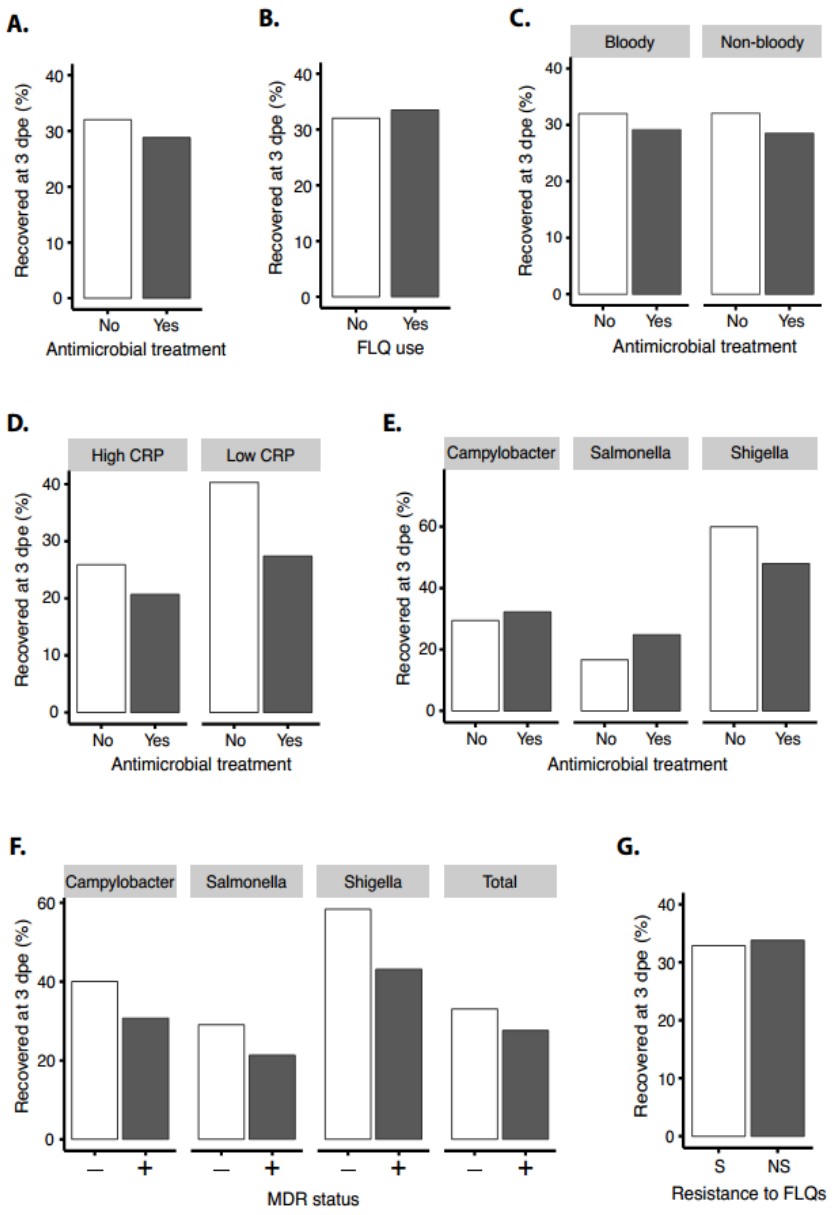



**Figure 3.2.** Antimicrobial susceptibility and recovery in patients treated with antimicrobials

At 3 days post-enrolment (dpe), disease outcome was recorded as "recovered" if the patients had less than 3 passages of loose stool in the last 24 hours. The effect of antimicrobial treatment (A) or fluoroquinolones (FLQs) (B) on recovery status at 3 dpe. Also investigated the effect of antimicrobial treatment on recovery status in different diarrhoea types (C), blood C-reactive protein concentration (CRP) (5mg/L cuff-off) (D), and isolated bacterial pathogens (E). The effect of multidrug resistance (MDR) status (F) or FLQ susceptibility (G) on the recovery from diarrhoea under antimicrobial or FLQ treatment, respectively. FLQ resistance: NS = non-susceptible, S = susceptible. No statistically significant differences in disease outcome were observed between groups.

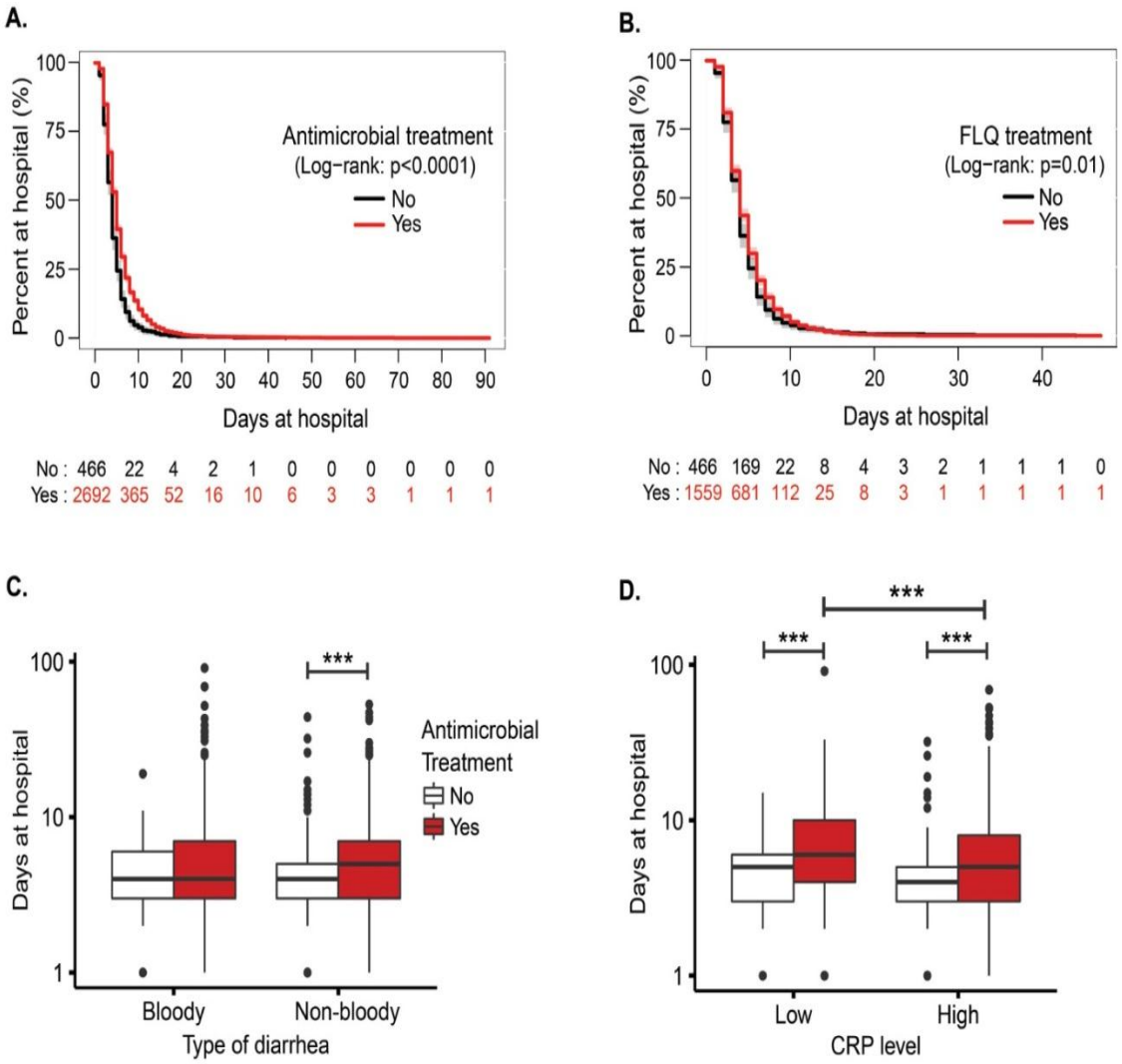



**Figure 3.3.** The effect of antimicrobial treatment on clinical outcome

Kaplan-Meier curves of days at hospital in diarrhoeal children treated with antimicrobials (A) or specifically, fluoroquinolones (FLQs) (B). Effect of antimicrobial usage on the length of hospital stay within different diarrhoeal types (C) and blood C-reactive protein concentration (CRP) (5mg/L cut-off) (D). Statistical comparisons for categorical variables were conducted using the Kruskal-Wallis test, where * for $0.05<p< 0.01$, and *** for $p<0.001$. Log-rank test was used to compare Kaplan-Meier curves of length of hospital stay between groups.

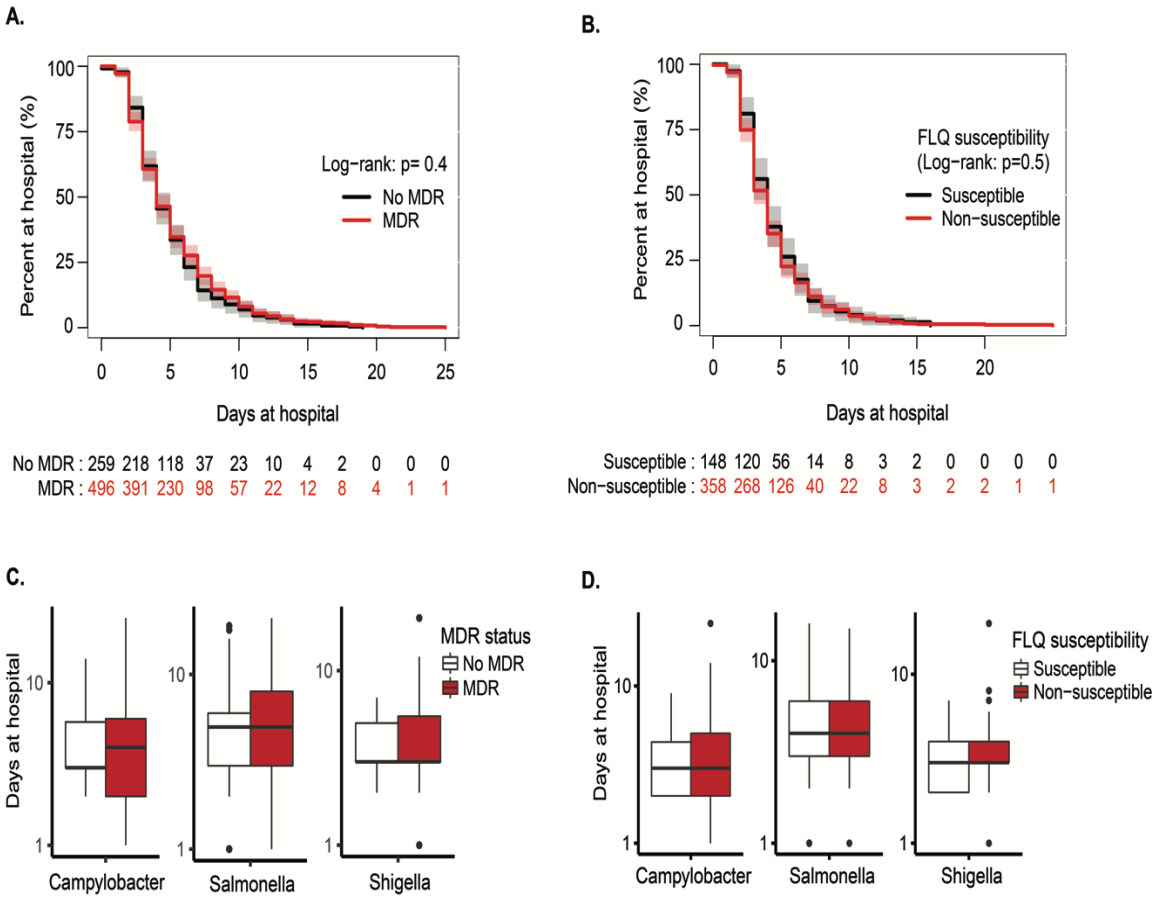



**Figure 3.4.** Investigating the effect of antimicrobial resistance on clinical outcome

Kaplan-Meier curves of length of hospital stay in diarrhoeal children treated with either antimicrobials or fluoroquinolones (FLQs) and stratified by multidrug resistance (MDR) (A) or FLQs resistance (B) profile of the isolated bacteria. Effect of MDR (C) and FLQ resistance (D) on the length of hospital stay in diarrhoeal children infected with *Campylobacter*, non-typhoidal *Salmonella* and *Shigella*, while being treated with antimicrobials or FLQs, respectively. Statistical comparisons for categorical variables were conducted using the Kruskal-Wallis test. Log-rank test was used to compare Kaplan-Meier curves of length of hospital stay between groups.

An AFT multiple regression model was considered to be an appropriate method for describing how each of the adjusted variables multiplicatively alters the duration of hospitalisation. Therefore, an AFT was constructed to investigate the effect of antimicrobials on the duration of hospital stay, adjusting for age, disease severity, and other prescribed treatments. After adjusting for age, diarrhoea presentation, and dehydration, antimicrobials were associated with a significant increase in the duration of hospital stay of diarrhoeal patients by a time ratio (TR) of 1.32 [1.24-1.41]. Lastly, infection with an MDR organism was found to not significantly prolong hospitalisation ($p$=0.552, Table 3.3).

**Table 3.3.** Univariate and multivariate analysis of diarrhoeal symptoms and treatment on diarrhoeal disease outcome (i.e. length of stay at hospital) using an accelerated failure time (i.e. Log-Normal) model

| Variable | Univariate Model | | | Final Multivariate Model | | |
|---|---|---|---|---|---|---|
| | **β** | **TR**[*95% CI*]** | ***P*-value** | **β*** | **TR [*95% CI*]** | ***P*-value** |
| Age | | | | | | |
| 0 to 6 months | 1 | 1 | - | 1 | 1 | - |
| 7 to 12 months | -0.075 | 0.93 [0.88-0.98] | **0.011** | -0.106 | 0.90 [0.85-0.95] | **0.0003** |
| 13 to 60 months | -0.188 | 0.83 [0.78-0.88] | **<0.0001** | -0.231 | 0.79 [0.75-0.84] | **<0.0001** |
| > 60 months | -0.381 | 0.68 [0.59-0.79] | **<0.0001** | -0.445 | 0.64 [0.56-0.74] | **<0.0001** |
| Sex | | | | | | |
| Female | 1 | 1 | - | 1 | 1 | - |
| Male | 0.017 | 1.02 [0.97-1.07] | **0.440** | | | |
| Diarrhoeal types | | | | | | |
| Non-bloody | 1 | 1 | - | 1 | 1 | - |
| Bloody | -0.059 | 0.94 [0.90-0.98] | **0.010** | -0.122 | 0.89 [0.84-0.93] | **<0.0001** |
| Fever | -0.047 | 0.95 [0.88-1.03] | 0.257 | | | |
| Dehydration | 0.193 | 1.21 [1.13-1.31] | **<0.0001** | 0.179 | 1.20 [1.11-1.29] | **<0.0001** |
| Abdominal pain | -0.049 | 0.95 [0.90-1.01] | 0.101 | | | |
| Probiotics | 0.067 | 1.07 [1.02-1.12] | **0.005** | | | |
| Zinc | 0.043 | 1.04 [0.97-1.12] | 0.232 | | | |
| Rehydration | 0.081 | 1.08 [0.99-1.18] | 0.074 | | | |
| Antimicrobial Use | 0.229 | 1.26 [1.18-1.34] | **<0.0001** | 0.279 | 1.32 [1.24-1.41] | **<0.0001** |
| MDR | -0.027 | 0.97 [0.89-1.06] | 0.552 | | | |

*Adjusted β, and TR values from the Multivariable log-normal analysis.

**Time Ratio (TR), TR>1 indicates an extended time in hospital, and TR<1 indicates a decreased time in hospital.

## 3.4. Discussion

Moderate-to-severe diarrhoea has a significant healthcare burden in Vietnamese children[238]. Although previous observational studies of diarrhoea in Vietnam have described some of the epidemiological features, the bacterial aetiologies and their associated AMR profiles, these studies have focused chiefly on children with acute watery diarrhoea[213]. Little is known about the epidemiology and clinical management of bloody and/or mucus diarrhoea in Vietnam. Therefore, I aimed to address this paucity of data by enrolling >3,000 of children hospitalised with diarrhoeal. This large sample size not only enabled the isolation of >800 enteric pathogens, but also provided data regarding antimicrobial usage in medical practice and outcome. Furthermore, this study investigated the clinical role of AMR in a relevant population empirically prescribed antimicrobials on presentation in hospital.

AMR in pathogenic bacteria, including those associated with diarrhoea, is a global public health problem[252,253]. Data from Vietnam highlights the increasing trend of AMR in diarrhoeagenic bacteria to the current first line antimicrobials, such as fluoroquinolones and third generation cephalosporins. Moreover, despite recent increases in fluoroquinolone resistance in Asia and beyond, current guidelines still endorse the use of this class of antimicrobials to treat bloody diarrhoea[254–256]. Many of the organisms isolated during this investigation were additionally non-susceptible to other (non-fluoroquinolone) antimicrobials, including some "last resort" choices such as imipenem. In comparison to estimates from other industrialising countries, I observed a similar or elevated prevalence of NTS exhibiting non-susceptibility to third generation cephalosporins, and increased non-susceptibility to both ciprofloxacin and azithromycin[103,257]. An extraordinarily high prevalence (~90%) of fluoroquinolone-resistant *Campylobacter* have been recently observed in other industrialising countries[84,258]. The situation in Vietnam appears to be exacerbated by the non-susceptibility of *Campylobacter* isolates to macrolides. The *Shigella* isolated here additionally had a high MDR rate; emerging MDR *Shigella* isolates with resistance

to fluoroquinolones and extended-spectrum cephalosporins are now commonly reported across Asia[126,128,259].

The treatment of diarrhoea with antimicrobials is a complex issue. Apart from the limited capability of most LMICs to confirm etiological agents associated with disease and the current complication of increasing AMR, there are conflicting data regarding the clinical efficacy of antimicrobials in reducing symptoms[260]. In line with WHO recommendations, I observed that >85% of hospitalised diarrhoeal cases containing mucus and/or blood were prescribed an antimicrobial, with fluoroquinolones (ciprofloxacin/norfloxacin) being the most common. Our current analysis found that antimicrobial use during hospitalised diarrhoea did not add benefit to supportive therapy only (i.e. rehydration and zinc supplementation). The recovery of patients regardless of antimicrobial treatment or AMR status when treated with a first-line antimicrobial for bloody diarrhoea treatment (such as ciprofloxacin) may be explained by either the differing *in vivo* effects of antimicrobials or the self-limiting nature of the infections. Additionally, in diarrhoea with a less pronounced inflammation (indicated by non-bloody and/or low CRP), the use of antimicrobials was associated with a prolonged hospital stay. These observations are in support of previous findings in studies of NTS and *Campylobacter* where antimicrobial treatment did not provide a clinical advantage and sometimes even caused harm (in comparison to placebo) in decreasing the duration of symptoms[86,108]. The routine antimicrobial treatment may also impact on the transmission of diarrhoea-causing bacteria by increasing faecal carriage and consequently spreading AMR organisms[108].

The main limitation in the present study is that this investigation was observational, with patients receiving standard of care treatment, which made it difficult to assess whether our observations were biased by more severe cases being prescribed antimicrobials. Furthermore, the duration of hospital stay and recovery of patients at three days post-enrolment were proxy measures of

clinical outcome, and I cannot discount that some children may have been discharged prior to the cessation of symptoms.

### 3.5. Conclusions

In conclusion, the bacteria commonly associated with paediatric diarrhoea in southern Vietnam displayed an extensive AMR profile, thereby emphasising the significance of etiological diagnosis of diarrhoea in LMICs. My key finding was that antimicrobial treatment was not associated with a reduction of diarrhoeal symptoms and even prolonged hospital stay in some groups. Therefore, I suggested a need adequately powered randomised controlled trials to be conducted to better assess the potential benefits of antimicrobial therapy for treatment of diarrhoea. These data will become essential for controlling antimicrobial usage during the present AMR crisis.

# 4. Validating and using a multiplex real-time PCR assay to detect diarrhoeagenic *Escherichia coli* pathotypes in children with diarrhoea

## 4.1. Abstract

*E. coli* are the most common bacterial genus associated with diarrhoea in children. These infections are particularly common in LMICs. Detecting the various pathotypes of DEC is complex and they are unable to be differentiated by classical microbiology alone. The distribution of DEC in people with diarrhoea has never been investigated in Vietnam and there is currently not a validated assay for DEC detection in Vietnam. These issues limit our understanding of aetiology of diarrhoea in this location.

Four multiplex real-time PCR assays were developed to detected specific virulence markers of six DEC pathotypes. Firstly, primer and probe sequences of four genes (*aggR, ipaH, eae, uidA*) were adapted from previous studies. Next, primers and probes for the remaining 6 gene targets (*etl*, *est*, *bfpA*, *rfbE_O157, stx1* and *stx2*) were designed and the specificity was tested using control DEC and other enteric pathogens. Additionally, each PCR amplicon generated from the six *E. coli* pathotypes was purified and amplified to be used to optimise PCR reaction and to calculate the reproducibility of PCR reaction. Finally, multiplex real-time PCR assays were validated and applied to clinical samples from diarrhoea and non-diarrhoea children.

Designed primers and probes were specific for each target in monoplex tests. The intra-assay and inter-assay CVs ranged and linear regressions of the standard curves showed the consistent results, demonstrating a well optimised, reproducible, and specific collection of multiplex real-time PCRs. At least one DEC variant was detected in 34.7% (978/2,815) of the faecal samples from diarrhoeal group, with EAEC, EIEC and atypical EPEC most frequent; whereas 41.2%

(205/498) of the samples from non-diarrhoeal children was identified as positive with DEC pathotype. Due to the age difference, the comparison analyses were performed in children aged 24-60 months. Only EIEC, which was detected in 34.3% (99/289) of diarrhoeal samples vs. 0.8% (4/498) non-diarrhoeal samples ($p<0.001$), was significantly associated with the diarrhoeal samples. The mixed-infection among DEC were complex and scattered; however, after controlling for confounders by performing binary univariate and multivariate logistic regression, I conclude that mono or co-infection with EIEC and being wasted were significantly associated with diarrhoea.

Multiplex real-time PCR applied on enteric bacterial sweeps was an efficient approach to detect six major pathotypes of DEC. The prevalence of DEC infection was high among both diarrhoeal and healthy children. EAEC and atypical EPEC were common in both healthy and ill children. EIEC were strongly associated with diarrhoeal diseases.

## 4.2. Introduction

Diarrhoea remains the second-highest cause of mortality and morbidity worldwide[1,8,261], with the main burden of disease occurring in children in South Asia, Southeast Asia, and Africa[8]. Among the bacterial pathogens associated with diarrhoea in children, *E. coli* is repeatedly the most common food borne pathogen identified[8,14,97,248]. However, the DEC landscape is complex as the organisms associated with disease cannot be delineated from commensal *E. coli* solely by microbiological culture. DEC pathotypes can only be differentiated at the time of isolation by molecular methods identifying specific virulence markers that are encoded on plasmids or chromosomal islands. To date, six major pathotypes of DEC have been identified: ETEC, EAEC, EIEC, EPEC, STEC, and EHEC. ETEC, EAEC, and EPEC are all implicated in causing diarrhoea in young children in developing countries[145,146,262]. EIEC and *Shigella* spp. are invasive DEC, they also important in childhood diarrhoea, and share common virulence genes, resulting in disease with similar clinical manifestations. EHEC and STEC are commonly associated with outbreaks in different parts of the world and can also be fatal through haemolytic uremic syndrome[139,140,155]. Notably, the epidemiology of EHEC and STEC in South East Asia areas are not well described.

An earlier study in Ha Noi (the capital of Vietnam) detected DEC in 22% of cases and 12% of controls[263] but the prevalence of DEC in children in Vietnam has been likely underestimated and screening for DEC is resource intensive. Here, I aimed to develop a multiplex real-time PCR to identify pathotypes of *E. coli* efficiently and precisely in a comparatively short turnaround time. In order to set up the multiplex real-time PCR to identify the six pathotypes of DEC, I gathered and also designed specific primers and probes for virulence genes of DEC. Some sequence of primers and probes were adopted from previous studies. Next, I tested the reliability and efficiencies of the assay *in vitro*. I used our method to determine the prevalence of DEC in faecal samples among diarrhoea children (case) and anal swab samples in healthy children (control) to

describe the clinical features of children with DEC and determine the pathogenicity of the presence of DEC in intestinal lumen.

## 4.3. Results

### 4.3.1. Multiplex real-time PCR assay for detecting diarrhoeagenic *Escherichia coli*

I aimed to establish a panel of multiplex real-time PCRs that could be used to detect ETEC, EAEC, EIEC, EPEC, EHEC, and STEC in symptomatic/asymptomatic faecal samples (Table 2.2). The various PCRs were first performed in monoplex using cloned target sequences and then validated and optimised using genomic DNA extracted from the various *E. coli* pathovars.

After thorough optimisation I tested the various amplifications using a panel of DNA extracted from a range of different organisms. There was no cross reactivity with *Staphylococcus aureus, Klebsiella pneumoniae, Salmonella spp., Campylobacter coli, Campylobacter jejuni, Shigella sonnei, Shigella flexneri, Enterobacter, Proteus*, norovirus, or rotavirus. I next generated dilutions of cloned target sequences and produced a series of consecutive standard curves (Table 4.1). The various PCRs were multiplexed and then I measured the intra-assay and inter-assay CVs across the nine target sequences using the four multiplexed PCRs. The intra-assay and inter-assay CVs ranged from 0.01 to 1.54% and from 0.01 to 2.12%, respectively. The target copy numbers ranged from $5x10^0$ - $5x10^7$ copies per reaction. The linear regressions of the standard curves were between 0.992-0.999 for all targets tested.

The resulting efficiency of the amplification ranged from 90.87 to 105.72%, demonstrating that I had generated a well optimised, reproducible, and specific collection of multiplex real-time PCRs.

**Table 4.1.** Reproducibility of the assays on diluted plasmid DNA containing cloned target sequences

| Target sequence | Co-efficient of variance (%) | Target concentration | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $5 \times 10^7$ | $5 \times 10^6$ | $5 \times 10^5$ | $5 \times 10^4$ | $5 \times 10^3$ | $5 \times 10^2$ | $5 \times 10^1$ | $5 \times 10^0$ |
| *elt* | Intra-assay variation [a] | 0.03 | 0.04 | 0.06 | 0.10 | 0.35 | 0.16 | 0.27 | 0.35 |
| | Inter-assay variation [b] | 0.07 | 0.11 | 0.05 | 0.23 | 0.41 | 0.50 | 0.38 | 0.67 |
| *est* | Intra-assay variation | 0.01 | 0.05 | 0.03 | 0.25 | 0.19 | 0.16 | 0.15 | 0.06 |
| | Inter-assay variation | 0.15 | 0.03 | 0.04 | 0.05 | 0.52 | 0.36 | 0.12 | 0.76 |
| *aggR* | Intra-assay variation | 0.11 | 0.08 | 0.08 | 0.15 | 0.20 | 0.37 | 0.29 | - |
| | Inter-assay variation | 0.06 | 0.11 | 0.33 | 0.03 | 0.29 | 0.23 | 1.05 | - |
| *ipaH* | Intra-assay variation | 0.19 | 0.17 | 0.20 | 0.12 | 0.12 | 0.60 | 0.15 | 0.11 |
| | Inter-assay variation | 0.23 | 0.28 | 0.20 | 0.07 | 0.42 | 0.20 | 0.52 | 1.42 |
| *eae* | Intra-assay variation | 0.07 | 0.24 | 0.29 | 0.06 | 0.26 | 0.76 | 0.20 | 0.48 |
| | Inter-assay variation | 0.38 | 0.16 | 0.06 | 0.46 | 0.82 | 0.64 | 0.63 | 0.88 |
| *bfpA* | Intra-assay variation | 0.03 | 0.09 | 0.11 | 0.17 | 0.50 | 0.90 | 0.63 | 0.90 |
| | Inter-assay variation | 0.01 | 0.11 | 0.05 | 0.06 | 0.18 | 0.38 | 0.75 | 1.23 |
| *rfbE* | Intra-assay variation | 0.03 | 0.11 | 0.09 | 0.42 | 0.20 | 0.70 | 0.98 | 0.30 |
| | Inter-assay variation | 0.02 | 0.04 | 0.10 | 0.23 | 0.60 | 1.49 | 1.29 | 1.08 |
| *stx1* | Intra-assay variation | 0.13 | 0.13 | 0.17 | 0.21 | 0.50 | 0.74 | 0.97 | 0.60 |
| | Inter-assay variation | 0.22 | 0.50 | 0.71 | 0.98 | 1.19 | 1.57 | 1.95 | 0.55 |
| *stx2* | Intra-assay variation | 0.11 | 0.14 | 0.18 | 0.17 | 0.25 | 1.54 | 0.81 | 0.24 |
| | Inter-assay variation | 0.12 | 0.22 | 0.43 | 0.47 | 0.87 | 1.54 | 2.12 | 1.97 |

a) Intra-assay variation was calculated by measuring the co-efficient of variance of the Cp value on three concurrently run assays.
b) Inter-assay variation was calculated by comparing variation in Cp value on three independently run assays

### 4.3.2. The prevalence of diarrhoeagenic *Escherichia coli* from faecal specimens of diarrhoeal hospitalised children

Between May 2014 and April 2016, I amassed 2,815 MC sweeps (i.e. faecal samples plated on MC media) cultured from 3,166 children hospitalised with bloody and/or mucoid diarrhoea at three tertiary hospitals in HCMC. A single faecal sample was collected from each child within their first two days of hospital admission for diarrhoea. The majority of patients were male (1,731/2,815; 61.5%), with ages ranging from one month to 15 years (median age 10 months, IQR 6.6-17.1 months).

I employed four multiplex real-time PCRs onto all 2,815 MC sweeps to identify and sub-group the 6 different DEC. At least one DEC variant was detected in 34.7% (978/2,815) of the faecal samples from paediatric patients hospitalised with diarrhoea. Among those, EAEC was the most commonly detected DEC, with an associated amplicon recorded in 15.7% (443/2,815) of the MC extractions (Table 4.2). Other commonly detected DEC included EIEC/*Shigella* and EPEC, which had a respective prevalence of 12.4% (349/2,815) and 12.2% (343/2,815) in the extracted samples.

Notably, atypical EPEC (*eae* positive, *bfpA* negative) amplifications were more prevalent than typical EPEC (*eae* positive, *bfpA* positive); 93.9% (322/343) versus 6.1% (21/343), respectively. ETEC was detected in 6% (182/2,815) of the MC extractions, with a limited number of samples (8.2%; 15/182) producing an amplicon for heat stable toxin (*est*). The *rfbE* gene, encoding the O157 lipopolysaccharide was detected in 46 diarrhoeal samples. Only four diarrhoeal patients harboured samples containing the Shiga toxin-producing genes (*stx*1/*stx*2). Among these STEC cases, one case carried *eae* and one case carried both *eae* and *rfbE_O157*; whereas the two STEC cases without *eae* and *rfbE_O157* carried *elt* and *aggR*.

**Table 4.2.** Diarrhoeagenic *Escherichia coli* detected from bacterial colonies sweeps in children hospitalised with diarrhoea (N=2,815)

| **Pathotypes** | **Target gene** | **N** | **%** |
|---|---|---|---|
| ETEC | | 182 | 6.5 |
| LT-ETEC | *elt* | 167 | 5.9 |
| ST-ETEC | *est* | 7 | 0.2 |
| LT-ST-ETEC | *elt* & *est* | 8 | 0.3 |
| EAEC | *aggR* | 443 | 15.7 |
| EIEC/*Shigella* | *ipa*H | 349 | 12.4 |
| EPEC | | 343 | 12.2 |
| Atypical EPEC | *eae* | 322 | 11.4 |
| Typical EPEC | *eae* & *bfpA* | 21 | 0.7 |
| EHEC | | 46 | 1.6 |
| O157 | *eae* & *rfbE_O157* | 20 | 0.7 |
| non-eae O157 | *rfbE_O157* | 26 | 0.9 |
| STEC | *stx1*/*stx2* | 4 | 0.1 |
| Negative | | 1,837 | 65.3 |

ETEC: Enterotoxigenic *Escherichia coli*; EIEC: Enteroinvasive *Escherichia coli*; EPEC: Enteropathogenic *Escherichia coli*; EHEC: Enterohemorrhagic *Escherichia coli*; STEC: Shiga-toxin producing *Escherichia coli*

### 4.3.3. Clinical manifestations of diarrhoeagenic *Escherichia coli* mono-infection

To investigate clinical syndromes associated with the various DEC, I accessed clinical data associated with the patients and excluded those infected with more than one known bacterial pathogen (i.e. *Shigella* (n=76), NTS (n=448), or *Campylobacter* (n=235)) and those infected more than one DEC (n=205).

I found that patients from whom different pathotypes of DEC were detected in their faecal sample presented with some distinguishing clinical characteristics (Table 4.3). Approximately 70% of those with an ETEC, EAEC, EPEC, or EHEC positive sample were associated with mucoid, non-bloody diarrhoea, whereas EIEC was significantly associated with visible bloody diarrhoea (39.7%, 46/116, $p<0.001$, $\chi^2$ test).

EAEC was the most commonly identified DEC mono-infection, this pathotype was more commonly associated with children that had wasting or severe wasting (13.5%, 23/170; $p=0.013$, Fisher's exact test). Whilst EHEC was identified less frequently amplified than other variants, it was significantly associated with moderate and severe dehydration (40%, 8/20; $p=0.010$, Fisher's exact test), which commonly required intravenous rehydration therapy.

Generally, infections caused by DEC were uncomplicated; >90% of patients had improved or recovered after three days and their median hospital stay was 5 days [IQR 3-7 days]. The use of antimicrobials within this study population was high, with 81.3% (1,513/1,861) of patients receiving empirical antimicrobial treatment. Fluoroquinolones, specifically ciprofloxacin, were the most commonly used class of antimicrobials (957/1,512, 63.3%).

**Table 4.3.** Demographic and clinical manifestations of diarrhoeagenic *Escherichia coli* mono-infection in children hospitalised with diarrhoea

| Characteristics | Number of patients (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ETEC (n=34) | | EAEC (n=170) | | EIEC (n=116) | | EPEC (n=137) | | EHEC (n=20) | | Negative (n=1,385) | | *p* value [a] |
| Socio-demographic | | | | | | | | | | | | | |
| Male | 18 | (52.9) | 117 | (68.8) | 70 | (60.3) | 82 | (59.9) | 16 | (80.0) | 852 | (61.5) | 0.118 |
| Age in months, *median* [IQR] | 11.2 | [7.0-20.4] | 11.5 | [7.4-18.1] | 14.8 | [6.7-24.5] | 12.4 | [8.4-18.7] | 15.6 | [8.3-21.2] | 9.1 | [5.7-14.5] | 0.388 |
| Growth [b] | | | | | | | | | | | | | |
| Obese or overweight | 0 | - | 15 | (8.8) | 11 | (9.5) | 13 | (9.6) | 0 | - | 129 | (9.3) | 0.321 |
| Wasted or severely wasted | 4 | (11.8) | 23 | (13.5) | 7 | (6.0) | 5 | (3.7) | 1 | (5.0) | 126 | (9.1) | **0.013** |
| Type of diarrhoea [c] | | | | | | | | | | | | | **<0.001** |
| Non-bloody diarrhoea | 23 | (67.6) | 142 | (83.5) | 63 | (54.3) | 98 | (71.5) | 15 | (75.0) | 812 | (58.6) | **<0.001** |
| Bloody diarrhoea | 8 | (23.5) | 23 | (13.5) | 46 | (39.7) | 33 | (24.1) | 4 | (20.0) | 368 | (26.6) | **<0.001** |
| Persistent diarrhoea | 3 | (8.8) | 5 | (2.9) | 7 | (6.0) | 6 | (4.4) | 1 | (5.0) | 205 | (14.8) | 0.414 |
| Clinical manifestations | | | | | | | | | | | | | |
| Number of episodes per day, *median* [IQR] | 5 | [4-9] | 6 | [5-10] | 6 | [5-10] | 6 | [4-10] | 5.5 | [4-8] | 7 | [5-10] | 0.284 |
| Moderate and severe dehydration [d] | 3 | (8.8) | 20 | (11.8) | 15 | (12.9) | 23 | (16.8) | 8 | (40.0) | 123 | (8.9) | **0.010** |
| Abdominal pain | 5 | (14.7) | 30 | (17.7) | 25 | (21.6) | 23 | (16.8) | 6 | (30.0) | 287 | (20.7) | 0.363 |
| Fever (≥37.5°C at enrolment) | 19 | (55.9) | 104 | (61.2) | 69 | (59.5) | 83 | (60.6) | 12 | (60.0) | 764 | (55.2) | 0.985 |
| Vomit, *n* (%) | 15 | (44.1) | 108 | (63.5) | 76 | (65.5) | 90 | (65.7) | 15 | (75.0) | 753 | (54.4) | 0.238 |
| Haematology | | | | | | | | | | | | | |
| Neutrophil count ($10^3$/µL), *median* [IQR] | 5.6 | [3.3-9.3] | 4.3 | [2.3-6.6] | 5.4 | [2.9-9.4] | 4.2 | [2.5-6.9] | 5.5 | [3.5-7.8] | 3.5 | [2.2-5.7] | **0.014** |
| C-reactive protein (mg/L), *median* [IQR] | 8.4 | [5.0-31.0] | 5.0 | [1.0-19.3] | 8.0 | [5.0-40.2] | 5.0 | [1.3-12.0] | 8.0 | [6.0-15.9] | 6.0 | [4.0-21.4] | 0.132 |
| Treatment | | | | | | | | | | | | | |
| Low-osmolarity ORS | 29 | (85.3) | 164 | (96.5) | 110 | (94.8) | 131 | (95.6) | 20 | (100) | 1,285 | (92.8) | 0.125 |
| IV rehydration, | 3 | (8.8) | 19 | (11.2) | 15 | (12.9) | 26 | (19.1) | 8 | (40.0) | 141 | (10.2) | **0.005** |
| Antimicrobials, | 30 | (88.2) | 123 | (72.4) | 101 | (87.1) | 109 | (79.6) | 16 | (80.0) | 1134 | (81.9) | **0.027** |
| Fluoroquinolones, *n* (% of antibiotic treated) [e] | 20 | (66.7) | 66 | (53.7) | 65 | (64.4) | 77 | (70.6) | 12 | (75.0) | 717 | (63.2) | 0.077 |
| Zinc | 29 | (85.3) | 148 | (87.1) | 100 | (86.2) | 118 | (86.1) | 20 | (100.0) | 1,243 | (89.7) | 0.488 |
| Probiotics | 22 | (64.7) | 123 | (72.4) | 89 | (76.7) | 99 | (72.3) | 16 | (80.0) | 957 | (69.1) | 0.631 |
| Outcomes | | | | | | | | | | | | | |
| Hospital stay in days, *median* [IQR] | 4 | [3-5] | 4 | [3-6] | 3.5 | [3-6] | 4 | [3-6] | 4 | [3-6.5] | 5 | [3-7] | 0.582 |
| Improved and recovered after 3 days [f] | 32 | (94.1) | 157 | (92.4) | 106 | (91.4) | 129 | (94.2) | 18 | (90.0) | 1,204 | (86.9) | 0.872 |

ETEC: Enterotoxigenic *Escherichia coli*; EIEC: Enteroinvasive *Escherichia coli*; EPEC: Enteropathogenic *Escherichia coli*;

EHEC: Enterohemorrhagic *Escherichia coli*; ORS: Oral Rehydration Solution; IQR: Interquartile range

[a] Comparison among diarrhoeagenic *Escherichia coli* mono-infection using $\chi^2$ test or Fisher's exact test for categorical data or Kruskal-Wallis test for continuous data (negative group was not included)

[b] Obese: weight for length z score >3SD in children age < 24months; BMI for age z score >3SD in children age ≥24months,

Overweight: weight for length z score >2SD in children age < 24months; BMI for age z score >2SD in children age ≥24months,

Wasted: weight for length z score <-2SD in children age < 24months; BMI for age z score <-2SD in children age≥24months,

Severely wasted: weight for length z score <-3SD in children age < 24months); BMI for age z score <-3SD in children age ≥24months)[34]

[c] Type of diarrhoea including non-bloody diarrhoea (diarrhoea with mucus, <14 days), bloody diarrhoea (diarrhoea with blood, <14 days), and persistent diarrhoea (diarrhoea with mucus and/or blood, ≥14days)

[d] Dehydration classified according to Integrated Management of Childhood Illness[35]

[e] Fluoroquinolones included ciprofloxacin and norfloxacin.

[f] Defined as "recovered" if patient had <3 passages of loose stool in the past 24 hours or "improved" if patient had less episodes of diarrhoea and less mucus and/or bloody in comparison to the condition of the patient at enrolment.

### 4.3.4. Diarrhoeagenic *Escherichia coli* from faecal specimens of diarrhoeal hospitalised children vs. healthy non-diarrhoea children

Between March 2016 and August 2016, I additionally collected 498 MC sweeps from faecal samples taken from healthy children residing in HCMC and participating in a cohort study[228]. The majority of healthy children were male (269/498; 54.0%), with their age when sampled ranging from 24 months to 5 years (median age 46.4 months, IQR 35.6-52.5 months). In a comparable manner to the diarrhoeal samples, I tested MC extractions from these healthy children with the multiplex real-time PCRs to detect the various DEC. At least one type of DEC was detected in 41.2% (205/498) of the samples associated with non-diarrhoeal children (Table 4.4). To attribute the various DEC to diarrhoea, I compared data from these non-diarrhoeal children with those from the subset children from the diarrhoeal group in the same age range; 319 children (median age 31.5 months, IQR 26.7-38.9 months) (Table 4.4 and Figure 4.1).

The proportions of ETEC, EAEC, and EHEC were not significantly different between faecal samples from children with or without diarrhoea. However, EPEC was detected significantly more frequently in the non-diarrhoeal samples (18.7%, 93/498) than the diarrhoeal samples (11.4%, 33/289) ($p$=0.019, $\chi^2$ test) (Table 4.4). The only organism that was significantly associated with the diarrhoeal samples was EIEC, which was detected in 34.3% (99/289) of diarrhoeal samples vs. 0.8% (4/498) non-diarrhoeal samples ($p$<0.001, Fisher's exact test).

**Table 4.4.** The detection of diarrhoeagenic *Escherichia coli* from children hospitalised with diarrhoea and healthy children

| **Diarrhoeagenic *Escherichia coli*** | **Target gene** | **Diarrhoea N (%)** | | **Non-diarrhoea N (%)** | | ***p* value*** |
|---|---|---|---|---|---|---|
| Number | | 319 | | 498 | | |
| ETEC | | 29 | (9.1) | 42 | (8.4) | 0.745 |
| LT-ETEC | *elt* | 25 | (7.8) | 39 | (7.8) | 0.998 |
| ST-ETEC | *est* | 1 | (0.3) | 3 | (0.6) | 1.000 |
| LT-ST-ETEC | *elt* & *est* | 3 | (0.9) | 0 | (0.0) | 0.059 |
| EAEC | *aggR* | 50 | (15.7) | 89 | (17.9) | 0.415 |
| EIEC/*Shigella* | *ipa*H | 93 | (29.2) | 4 | (0.8) | **<0.001** |
| EPEC | | 39 | (12.2) | 93 | (18.7) | **0.015** |
| Atypical EPEC | *eae* | 38 | (11.9) | 90 | (18.1) | **0.018** |
| Typical EPEC | *eae* & *bfpA* | 1 | (0.3) | 3 | (0.6) | 1.000 |
| EHEC | | 13 | (4.1) | 20 | (4.0) | 0.712 |
| O157 | *eae* & *rfbE_O157* | 5 | (1.6) | 5 | (0.6) | 0.524 |
| non-eae O157 | *rfbE_O157* | 8 | (2.5) | 18 | (3.4) | 0.379 |
| STEC | *stx1*/*stx2* | 0 | - | 7 | (1.4) | **0.033** |
| Negative | | 163 | (51.1) | 293 | (58.8) | **0.030** |

*$p$ value from $\chi^2$ test or *Fisher's exact* test

ETEC: Enterotoxigenic *Escherichia coli*; EIEC: Enteroinvasive *Escherichia coli*;

EPEC: Enteropathogenic *Escherichia coli*; EHEC: Enterohemorrhagic *Escherichia coli*;

STEC: Shiga-toxin producing *Escherichia coli*

Figure 4.1 shows the distribution of DEC in diarrhoeal and healthy groups. Mono-infection with EPEC or EAEC were high in both groups. EIEC mono-infection was more common in the diarrhoeal patient group than the controls. The distribution of DEC co-infection among both groups was complex and highly variable.

The most common co-infections in the diarrhoeal group were EAEC + EIEC (3.8%, 12/319) and EAEC + EIEC + ETEC (2.2%, 7/319); whereas EPEC + EAEC (3.4%, 17/498) was common in the healthy control group. Co-infection with DEC was more likely to be associated with diarrhoeal diseases (16.3%, 52/319 vs. 9.6%, 48/498, $p$=0.005, $\chi^2$ test). However, due to the predominant presence of EIEC in the diarrhoeal group, I suspected that EIEC infection is a confounder. Therefore, I conducted further analysis on these co-infection data to disaggregate the potential effect of EIEC.

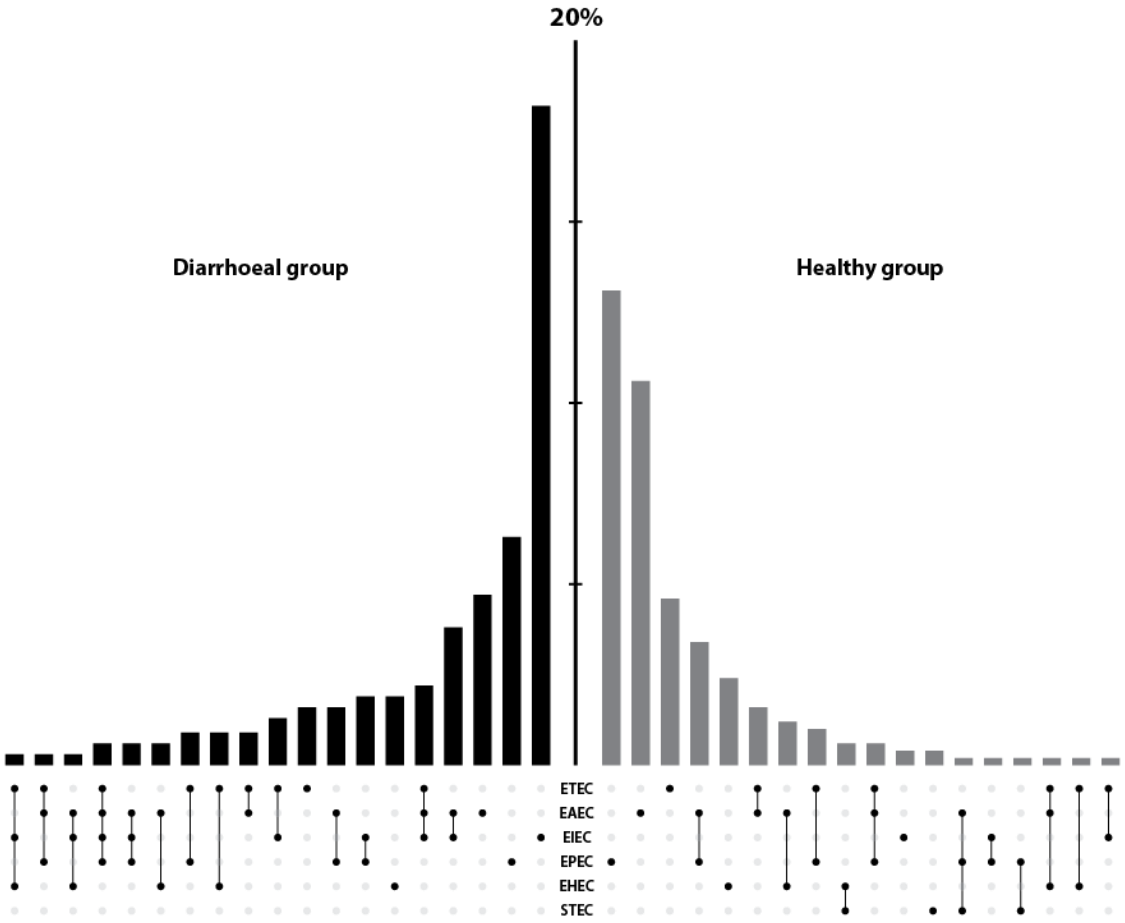



**Figure 4.1.** Mono- and co-infection with diarrhoeagenic *Escherichia coli* in the 24-60-month-old diarrhoeal and healthy groups

Diarrhoeagenic *Escherichia coli* mix-infection patterns among diarrhoea group (N=319) (in black) and healthy group (N=498) (in grey) are shown. The bar chart demonstrates the percentage of infectious cases in each group with the most frequency located near the middle. The dots and lines between dots show mixed infection status for six pathotypes of diarrhoeagenic *E. coli* ETEC: Enterotoxigenic *Escherichia coli*; EIEC: Enteroinvasive *Escherichia coli*; EPEC: Enteropathogenic *Escherichia coli*; EHEC: Enterohemorrhagic *Escherichia coli*; STEC: Shiga-toxin producing *Escherichia coli.*

Lastly, I performed both binary univariate and multivariate logistic regression to identify variables and DEC that were associated with diarrhoeal disease in children aged 24-60 months (Table 4.5).

In a univariate model, co-infection with ETEC, mono-infection with EIEC, co-infection with EIEC, and co-infection without EPEC, EHEC, and STEC were found to be significantly associated with diarrhoea. However, after controlling for confounders, I found that mono or co-infection with EIEC only and wasting were significantly associated with diarrhoea. Correspondingly, mono-infection with ETEC, EAEC, and being obese were significantly more common in the non-diarrhoeal children.

**Table 4.5.** Univariate and multivariate analysis of diarrhoeagenic *Escherichia coli* mono-infection and co-infection associated with diarrhoeal disease among children from 24-60 months of age using binary logistic regression model

| Variable | Univariate Model | | | Multivariate Model | | |
|---|---|---|---|---|---|---|
| | **Odds Ratio** | **95% Confidence Interval** | ***P*-value**[c] | **Odds Ratio** | **95% Confidence Interval** | ***P*-value**[d] |
| Types of infection [a] | | | | | | |
| Mono-infection with ETEC | 0.39 | 0.15 – 1.05 | 0.062 | 0.32 | 0.11 – 0.94 | **0.037** |
| Co-infection with ETEC | 2.27 | 1.21 – 4.27 | **0.011** | | | |
| Co-infection without ETEC | 1.74 | 1.00 – 3.02 | 0.051 | | | |
| Mono-infection with EAEC | 0.51 | 0.28 – 0.93 | 0.028 | 0.45 | 0.24 – 0.86 | **0.015** |
| Co-infection with EAEC | 1.75 | 1.06 – 2.89 | 0.030 | | | |
| Co-infection without EAEC | 2.55 | 1.19 – 5.46 | 0.016 | | | |
| Mono-infection with EIEC | 52.13 | 12.57 – 216.21 | **<0.001** | 49.66 | 11.90 – 207.24 | **<0.001** |
| Co-infection with EIEC | 31.46 | 7.47 – 132.47 | **<0.001** | 35.60 | 8.28 – 153.12 | **<0.001** |
| Co-infection without EIEC | 0.66 | 0.37 – 1.20 | 0.173 | 0.63 | 0.34 – 1.18 | 0.149 |
| Mono-infection with EPEC | 0.55 | 0.32 – 0.95 | 0.031 | 0.59 | 0.34 – 1.02 | 0.057 |
| Co-infection with EPEC | 1.22 | 0.66 – 2.25 | 0.525 | | | |
| Co-infection without EPEC | 2.97 | 1.65 – 5.34 | **<0.001** | | | |
| Mono-infection with EHEC | 0.90 | 0.33 – 2.44 | 0.834 | 0.96 | 0.35 – 2.64 | 0.937 |
| Co-infection with EHEC | 1.14 | 0.44 – 3.01 | 0.785 | | | |
| Co-infection without EHEC | 2.19 | 1.36 – 3.52 | **0.001** | | | |
| Mono-infection with STEC | - | | | | | |
| Co-infection with STEC | - | | | | | |
| Co-infection without STEC | 2.17 | 1.39 – 3.40 | **0.001** | | | |

| Variable | Univariate Model | | | Multivariate Model | | |
|---|---|---|---|---|---|---|
| | **Odds Ratio** | **95% Confidence Interval** | ***P*-value**[c] | **Odds Ratio** | **95% Confidence Interval** | ***P*-value**[d] |
| Gender | | | | | | |
| Female | 0.81 | 0.61 – 1.07 | 0.142 | 0.65 | 0.47 – 0.91 | **0.013** |
| Growth [b] | | | | | | |
| Obese | 0.57 | 0.35 – 0.92 | 0.022 | 0.51 | 0.29 – 0.89 | **0.019** |
| Overweight | 0.88 | 0.57 – 1.36 | 0.560 | 1.00 | 0.62 – 1.62 | 0.998 |
| Risk of overweight | 0.68 | 0.46 – 1.01 | 0.053 | 0.83 | 0.54 – 1.27 | 0.390 |
| Wasted | 19.13 | 2.48 – 147.54 | **0.005** | 19.58 | 2.43 – 157.92 | **0.005** |

[a] Odds Ratio with the reference was non-infection status

[b] Odds Ratio with the reference was normal growth status

[c] *P*-value considered significant when $p<0.01$

[d] *P*-value considered significant when $p<0.05$

## 4.4. Discussion

*E. coli*, a common bacterial species which colonises the human gastrointestinal has pathogenic variants that harbour virulence genes and cause diarrhoeal or extra-intestinal disease. Pathogenic *E. coli* are classified into different pathotypes based on infecting niche, specific virulence determinants, or adherence patterns[141]. Collectively, DEC are the most common bacterial pathogens that are identified in diarrhoeal diseases[262,264,265]. However, comparatively little is known about the prevalence and distribution of DEC in children in Vietnam due to a lack of routine diagnostic tests. Here, I developed an efficient real-time PCR assay that was standardised and applied on a large number of MC sweeps. This approach (real-time PCR on bacterial sweeps) save materials, labour and has a comparatively short turn-around time[266]. The primers and probes I used and developed were highly sensitive and specific. Ultimately, the four multiplex real-time PCR reactions detected nine target sequences in six pathotypes of DEC, which permitted detection of these pathogens with a high degree of accuracy and utility.

This combination of multiplex real-time PCR assays provides a reliable and convenient method to detect samples containing the main DEC virulence genes that can aid the diagnosis of infection caused . However, there are some limitations of the assay. Firstly, the gene for the invasion plasmid antigen H (*ipaH*) and the *uidA* (the internal control gene for *E. coli*) are present in both *Shigella*[150] and EIEC. Therefore, this approach cannot differentiate between EIEC and *Shigella*. Secondly, both EPEC and EHEC carry the *eae* gene; using the classification of atypical EPEC (*eae*), EHEC (*eae* and *rfbO157*); therefore, mixed infections with EPEC and EHEC are likely to be missed. For the comparable reason, hybrid pathotypes such as ST-EAEC would likely be misclassified as co-infections. This combination of PCRs is unable to conclude which pathotypes are associated with the Shiga toxin if more than one pathotype in the sample produces an amplicon for the *stx* gene. Lastly, this protocol could not sub-classify non-O157 EHEC, as only *rfbE_O157* was used as a target. In addition to the methodological constraints of the study, as

control children did not include children under two years of age, this study could not evaluate associations with diarrhoea in those more vulnerable younger age groups.

ETEC is the most common DEC internationally. However, the prevalence of ETEC in this setting was found to be lower than other areas in Africa[146,266]. This result may be due to the study entrance criteria, as only children presenting with bloody and/or mucoid diarrhoeal illness were recruited; theoretically ETEC is more commonly associated with watery diarrhoea[141]. In a comparable manner of this study, previous research on ETEC infections in children identified a higher percentage of LT-ETEC than ST-ETEC and outlines only weak associations between LT-ETEC infection and diarrhoea[146,266]. In contrast, a recent study in Shenzhen, China found that the majority of ETEC infections occurred in adults and ST-ETEC were more common than LT-ETEC[144].

The EAEC pathotype is highly heterogeneous; with the ability to produce a stacked-brick appearance on epithelial surface[140]. Notwithstanding with discrepancy, EAEC was the most commonly detected pathotype in faecal samples from children with diarrhoea in this study, consistent with earlier studies that have reported high detection rates for EAEC compared to other DEC in Vietnam[263,267]. A range of reports in the literature suggest that some EAEC are pathogens, but variants may have different propensities to cause disease[229,268–272]. Several outbreaks and human volunteer studies have unequivocally shown that at least some EAEC can cause disease. Here, infection with EAEC was associated being wasted growth; alternatively, one third of EAEC mono-infection required antimicrobial IV treatment i.e. the third generation cephalosporins or imipenem (data not shown). Notably, three children in this study produced positive PCR amplicons for both EAEC and *stx*. These cases may represent mixed infections EAEC and STEC or even hybrid strains that were associated with an extensive outbreak in Europe several years ago[273]. Methodological limitations make it impossible to determine whether hybrids were found

in this case but I noted that no specific sequelae were observed in these cases. These samples would be available for further studying and characterisation.

EAEC was not associated with disease in children within the 24-60-month age group in this study, but I found that EAEC was the most commonly detected pathotype from children with wasting. The potential nutritional impact of EAEC in healthy children, as well as those with symptomatic diarrhoea is of interest and potential concern. The issue of EAEC has been highlighted recently in the MAL-ED study, which reported that irrespective of associations with disease, EAEC are associated with growth shortfalls[274].

Mixed or co-infection was found to be associated with diarrhoeal diseases and more severe symptoms[275,276]. Co-infection among DEC or with other enteric pathogens were found to be significantly associated with the diarrhoeal group[268,276–278]. This study demonstrated that mixed infection among DEC was not associated with diarrhoea and was also common in apparently healthy children. Notably, only co-infection with EIEC was significantly associated with diarrhoeal disease. The fact that EIEC infection was highly significantly associated with diarrhoeal illness and EIEC infection was high in diarrhoeal subset children, lead to question to impact of multiple aetiologies in combination with EIEC infection. A multivariate logistic regression model confirmed that DEC co-infection without EIEC was not associated with diarrhoea.

Typical EPEC is commonly identified in the African continent[257,266]. In other regions and as observed in this study, atypical EPEC tends to predominate[279]. DEC were found to colonise in the intestines within the first year of life and asymptomatic infection with ETEC, EAEC, and EPEC were associated with the introduction of weaning food and the termination of breastfeeding[280]. Here, EPEC was also the most common DEC isolated from faecal samples of diarrhoea and non-

diarrhoea children, partially due to the analysing age group (24-60 months of age). According to the recent published data originating from the GEMS showed that typical EPEC and ST-ETEC infections were significantly responsible for diarrhoea-related mortality[14].

EHEC_O157 is endemic serotype that causes severe diarrhoea and is associated with a high mortality rate in western countries[281]. EHEC_O157 in this setting had a low prevalence and more than half the positive samples were positive for the *rfbE_O157* gene only, which suggests these are likely to be of lower pathogenicity. Two samples that tested positive for EHEC_O157 also produced amplicons for the Shiga-toxin gene (*stx*2), denoting that this is a comparatively minor pathogen in this location. The fact that EHEC/STEC were also isolated from the healthy group is comparable to the data observed in Indonesia, where researchers investigated the distribution of DEC infection in children aged 1-10 years[282].

### 4.5. Conclusions

I found that the panel of multiplex real-time PCR applied on a collection of bacterial sweeps from MC media was an efficient method for detecting six major pathotypes of DEC. I suggested that this method should be further validated and compared to more conventional approaches. This methodology may prove to be more sensitive and cost effective than picking individual organisms from plates and then screening them independently. Using these methods, I found that the prevalence of DEC infection was high in both healthy children and children with diarrhoea. Most significantly, EAEC and atypical EPEC were commonly detected in both healthy and diseased children; EIEC was significantly associated with diarrhoeal diseases. This substantial study is the largest study of DEC in children with and without diarrhoea from Vietnam. My data shows that DEC are major pathogens in Vietnam and provides the methods and baseline data for further clinical, epidemiological, AMR, and genomic studies. The screening for DEC pathogens should become integrated into any future disease surveillance system in Vietnam.

# 5. An evaluation of the Luminex xTAG Gastrointestinal Pathogen Panel assay for the detection of multiple diarrhoeal pathogens in faecal samples

## 5.1. Abstract

Diarrhoeal disease is a complex syndrome that remains a leading cause of childhood morbidity and mortality globally. Diagnosis of enteric pathogens in a timely and precise manner is important for treatment decisions and informing public health policy, but accurate diagnosis is a major challenge in industrialising countries. Multiplex molecular diagnostic techniques may represent a significant improvement over classical approaches. Here, I evaluated the Luminex xTAG GPP assay for the detection of common enteric bacterial and viral pathogens in Vietnam. Microbiological culture and real-time PCR were used as gold standards. Tests were performed on 479 stool samples collected from people admitted to hospital for diarrhoeal disease throughout Vietnam. Sensitivity and specificity were calculated for the xTAG GPP for the seven principal diarrhoeal aetiologies. The sensitivity and specificity for the xTAG GPP were >88% for *Shigella* spp., *Campylobacter* spp., rotavirus, norovirus genotype 1/2 GI/GII and adenovirus compared to those of microbiological culture and/or real-time PCR. However, the specificity was low (~60%) for *Salmonella* spp. Additionally, a number of important pathogens that are not identified in routine hospital procedures in this setting such as *Cryptosporidium* spp. and *Clostridium difficile* were detected with the GPP. The use of the Luminex xTAG GPP for the detection of enteric pathogens in settings like Vietnam would dramatically improve the diagnostic accuracy and capacity of hospital laboratories, allowing for timely and appropriate therapy decisions as well as a wider understanding of the epidemiology of pathogens associated with severe diarrhoeal disease in low resource settings.

## 5.2. Introduction

Diarrhoeal disease remains a considerable public health challenge and is responsible for 0.8 million deaths and close to 90,000 DALYs annually[249,261], the majority of which occur in children <5 years of age in industrialising regions[1]. Infectious diarrhoea (caused by a pathogenic agent) can be caused by a number of different viruses, bacteria and parasites. Rotavirus remains the most common cause of diarrhoea in children <2 years of age[14,15], although the introduction of rotavirus vaccines in many countries has led to a fall in incidence in locations where uptake has been significant[11,12]. Bacterial pathogens such as *Shigella* spp., ETEC, *Campylobacter* spp., *Salmonella* spp., norovirus and the parasitic pathogens *Giardia* and *Cryptosporidium* spp. are also known to be present frequently in the stools of young children with diarrhoea in resource poor regions[14,15,213]. As the aetiology of diarrhoeal disease is diverse and complex, sensitive and specific diagnostics are required for prompt and accurate identification and treatment.

The detection of pathogens from stool specimens is challenging. In settings such as HCMC Vietnam, hospital laboratories rarely perform routine microbiological culture, and if they do they only aim to isolate a limited range of bacterial pathogens. Antimicrobial consumption in the community further reduces the sensitivity of microbiological culture for bacterial pathogens[28]. Microscopy is used to detect occult white blood cells, red blood cells and parasites such as *Entamoeba histolytica*, *Cryptosporidium* spp., *Giardia lamblia*; however microscopic detection of parasites has poor sensitivity[283], and presents difficulties due to the requirement of a fresh specimen, the requirement for an experienced microscopist and a lack of methodological standardisation. In addition, real-time PCR systems are not used routinely in HCMC (or indeed across Vietnam) due to the cost, lack of equipment and paucity of trained staff. As such, a number of important pathogens such as rotavirus, norovirus, pathovars of *E. coli* and *Clostridium difficile* are not routinely detected by procedures currently in place. The lack of appropriate diagnostics

hinders not only clinical treatment decisions but also a wider understanding of the epidemiology and the public health implications of severe paediatric diarrhoeal disease in locations like HCMC.

Given the limitations of traditional diagnostics, a multi-panel pathogen identification system that is sensitive and specific and easy to operate represents a significant improvement on classical techniques. The Luminex xTAG GPP assay (Luminex Molecular Diagnostics, Austin, TX, USA) – is a qualitative multiplex test cleared by the FDA that is able to identify 19 enteric pathogens in one reaction in 6h, however the assay has a prohibitive cost in Vietnam of approximately US$75 per sample (as of December 2015). The Luminex platform has been shown to be highly sensitive and specific for a variety of pathogens in several clinical setting globally[51–53] . In this chapter I aimed to evaluate the sensitivity and specificity of the xTAG GPP platform against those of conventional diagnostic techniques on clinical samples isolated from patients hospitalised with diarrhoeal disease in Vietnam.

## 5.3. Results

### 5.3.1. Baseline results

Overall, 479 stool samples were collected from 92 adults (>15 years) and 387 children (≤15 years old) admitted to hospital with diarrhoeal disease in Vietnam between 2009 and 2014. The median age of adult patients was 50 years (interquartile range [IQR]: 33-64 years) and 39% (36/92) were male. In children, the median age was 16.5 months (interquartile range [IQR]: 6.7-20 months) with 57% (221/387) being male. A total of 105 samples (22%) were positive for a bacterial pathogen by microbiological culture, 268 (56%) were positive for a pathogen by real-time PCR and 404 samples (84%) were positive for a pathogen by the xTAG GPP.

From the 479 tested samples, the most commonly identified pathogens through the xTAG GPP platform were *Salmonella* (n=210, 44%), rotavirus (121, 25%), norovirus GII (89, 19%),

*Shigella* (91, 19%) and *Cryptosporidium* (83, 17%). Though less common, the xTAG GPP also detected *C. difficile* (45, 9%), ETEC (40, 8%), *Giardia* (15, 3%), norovirus GI (15, 3%), *E. coli* O157 (11, 2%), STEC (8, 2%) and *Entamoeba histolytica* (4, 1%).

### 5.3.2. Validation

To initially validate the xTAG GPP, 40 stool samples positive for *Shigella* spp., 40 samples positive for *Salmonella* spp. and 30 samples positive for *Campylobacter* spp. by microbiological culture were retested using the xTAG GPP as shown in Table 5.1. The proportion of positive agreement of xTAG GPP and microbiological culture was high, ranging from 90 to 100%. Furthermore, using stool samples a previously detected viral pathogen by real-time PCR, the xTAG GPP had similarly high agreement (90.5 to 93.0%) in 68 samples positive for rotavirus, 58 samples positive norovirus GI/GII and 17 samples positive for adenovirus 40/41 infections.

**Table 5.1.** Number of positive Luminex xTAG GPP assay results on samples known to be positive by microbiological culture and real-time PCR

| Pathogen detected by method | No. detected by culture/ real-time PCR | No. detected by xTAG GPP | % positive agreement |
|---|---|---|---|
| **Culture** | | | |
| *Shigella* | 40 | 40 | 100 |
| *Salmonella* | 40 | 38 | 95.0 |
| *Campylobacter* | 30 | 27 | 90.0 |
| | | | |
| **Real-time PCR** | | | |
| Rotavirus | 68 | 63 | 92.6 |
| Norovirus GI/GII | 58 | 54 | 93.1 |
| Adenovirus | 20 | 17 | 85.0 |
| *Shigella* | 44 | 44 | 100 |
| *Salmonella* | 48 | 45 | 93.8 |
| *Campylobacter* | 40 | 36 | 90.0 |

### 5.3.3. Negative specimen evaluation

A total of 307 faecal samples that were negative by microbiological culture and real-time PCR were tested by xTAG GPP. *Salmonella* were the most frequently pathogen, presented in 45% (138/307) of the initially negative specimens. Further, rotavirus and *Shigella* spp. were identified in 19% (57/307) and 16% (48/307) of the negative specimens, respectively. Additionally, several significant enteric pathogens that are not well studied (or even previously described in Vietnam) due to lack of diagnostic capacity were also identified, including *Cryptosporidium* (15%, 45/307), *C. difficile* (12%, 36/307) and pathogenic *E. coli* (14%, 42/307), as shown in Figure 5.1.

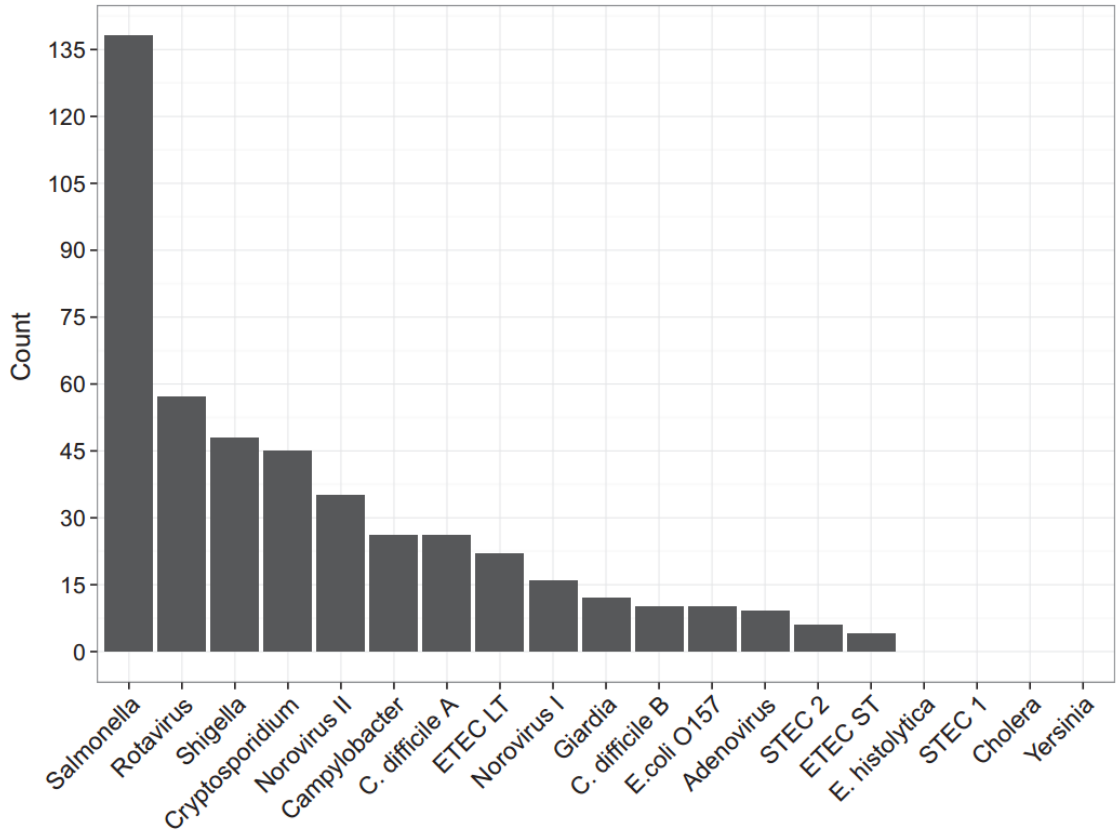



**Figure 5.1.** Count of pathogens detected by the Luminex xTAG GPP assay in 307 specimens that tested negative by microbiological culture and real-time PCR.

### 5.3.4. Performance evaluation on clinical specimens

Table 5.2 shows the performance of xTAG GPP in comparison to both a microbiological culture positive and a real-time PCR positive gold standard for seven enteric pathogens including *Shigella* spp., *Campylobacter* spp., and *Salmonella* spp. and also rotavirus, norovirus I/II and adenovirus. The xTAG GPP had high sensitivity for all pathogens, ranging from 88.2% to 100% using both culture and real-time PCR as gold standards. The specificity of xTAG GPP was also moderately high for most of the detected bacterial and viral pathogens, ranging from 88.4% to 99.3%, with the notable exception of *Salmonella* spp. A total of 128 and 172 stool specimens were negative for *Salmonella* spp. by real-time PCR and culture, respectively, but were positive by the xTAG GPP assay, resulting in the specificity of the xTAG GPP assay to be 60.8% and 66.8% for *Salmonella* in comparison to real-time PCR and culture, respectively. However, when I attempted to redefine the cut-off values to improve the specificity, I found no adjusted cut-off rules that could improve significantly the sum of the sensitivity and specificity for probe 1 and probe 2 combined as compared to that of the originally defined cut-offs by the manufacturer's instructions.

**Table 5.2.** Sensitivity and Specificity of Luminex xTAG GPP in comparison to real-time PCR and microbiological culture

| | xTAG GPP compared to real-time PCR | | | | | xTAG GPP compared to culture | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Real-time PCR | | | | | Microbial culture | | | |
| Organism detected | GPP | Pos | Neg | Sensitivity (% [95%CI]) | Specificity (% [95%CI]) | GPP | Pos | Neg | Sensitivity (% [95%CI]) | Specificity (% [95%CI]) |
| *Shigella* | Pos | 86 | 6 | 95.6 (89.0-98.8) | 98.5 (96.7-99.4) | Pos | 40 | 51 | 100 (91.2-100) | 88.4 (85.0-91.2) |
| | Neg | 4 | 383 | | | Neg | 0 | 388 | | |
| *Salmonella* | Pos | 84 | 128 | 90.3 (82.4-95.5) | 66.8 (61.9-71.5) | Pos | 38 | 172 | 95.0 (83.1-99.4) | 60.8 (56.1-65.4) |
| | Neg | 9 | 258 | | | Neg | 2 | 267 | | |
| *Campylobacter* | Pos | 59 | 3 | 90.8 (81.0-96.5) | 99.3 (97.9-99.8) | Pos | 27 | 35 | 90.0 (73.5-97.9) | 92.2 (89.3-94.5) |
| | Neg | 6 | 411 | | | Neg | 3 | 414 | | |
| Adenovirus | Pos | 23 | 5 | 92.0 (74.0-99.0) | 98.9 (97.4-99.6) | | | | | |
| | Neg | 2 | 449 | | | | | | | |
| Norovirus GI | Pos | 15 | 5 | 88.2 (63.6-98.5) | 98.9 (97.5-99.6) | | | | | |
| | Neg | 2 | 457 | | | | | | | |
| Norovirus GII | Pos | 85 | 4 | 96.6 (90.4-99.3) | 99.0 (97.4-99.7) | | | | | |
| | Neg | 3 | 387 | | | | | | | |
| Rotavirus | Pos | 117 | 4 | 92.9 (86.9-96.7) | 98.9 (97.1-99.7) | | | | | |
| | Neg | 9 | 349 | | | | | | | |

GPP, Gastrointestinal Pathogen Panel; Pos = positive; Neg = negative; 95%CI = Confidence Interval

### 5.3.5. Co-infection

Of all evaluated stool samples with a detected pathogen, 58% (233/404) had more than one enteric pathogen identified by the xTAG GPP assay. The majority of co-infections had two pathogens detected, although the number of detected pathogens was as high as seven in one sample. The value of correlation coefficient was shown in Table 5.3 and illustrated in Figure 5.2. According to the Table 5.3 and Figure 5.2, *Cryptosporidium* spp. and ETEC were the most common pathogens to be detected in stool samples with other pathogens including *E. histolytica*, *Salmonella* spp., rotavirus and norovirus with *Cryptosporidium* spp. and STEC, *C. difficile*, and adenovirus with ETEC. Additionally, norovirus and rotavirus were commonly often identified in the same sample. Notably, *Campylobacter* patients tended to have negative correlations with most other pathogens, suggesting a higher prevalence of mono infection.

**Table 5.3.** The value of correlation matrix of co-infections from 479 diarrhoeal stool samples

| | *Shigella* n=91 | *Campylobacter* n=62 | *Rotavirus* n=121 | *Norovirus* n=104 | *Cryptosporidium* n=83 | *Adenovirus* n=28 | *E. coli O157* n=11 | *ETEC* n=40 | *STEC* n=8 | *E. histolytica* n=3 | *Giardia* n=15 | *C. difficile* n=45 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Giardia* n=15 | | | | | | | | | | | | 1<br>-0.02 |
| *E. histolytica* n=3 | | | | | | | | | | | 0<br>-0.02 | 0<br>-0.03 |
| *STEC* n=8 | | | | | | | | | | 0<br>-0.01 | 0<br>-0.02 | 2<br>0.07 |
| *ETEC* n=40 | | | | | | | | | 6<br>0.31 | 0<br>-0.03 | 1<br>-0.01 | 12<br>0.21 |
| *E. coli O157* n=11 | | | | | | | | 2<br>0.05 | 1<br>0.09 | 0<br>-0.01 | 2<br>0.13 | 2<br>0.05 |
| *Adenovirus* n=28 | | | | | | | 1<br>0.02 | 8<br>0.18 | 2<br>0.11 | 0<br>-0.02 | 0<br>-0.04 | 3<br>0.01 |
| *Cryptosporidium* n=83 | | | | | | 6<br>0.03 | 1<br>-0.03 | 11<br>0.08 | 1<br>-0.02 | 3<br>0.14 | 1<br>-0.05 | 13<br>0.1 |
| *Norovirus* n=104 | | | | | 32<br>0.19 | 5<br>-0.02 | 3<br>0.02 | 11<br>0.04 | 2<br>0.01 | 1<br>0.01 | 2<br>-0.04 | 13<br>0.06 |
| *Rotavirus* n=121 | | | | 36<br>0.11 | 31<br>0.13 | 10<br>0.06 | 3<br>0.01 | 13<br>0.05 | 1<br>-0.04 | 1<br>0 | 4<br>0.01 | 11<br>-0.01 |
| *Campylobacter* n=62 | | | 16<br>0 | 8<br>-0.08 | 9<br>-0.03 | 3<br>-0.02 | 2<br>0.02 | 3<br>-0.05 | 0<br>-0.05 | 1<br>0.03 | 2<br>0 | 10<br>0.09 |
| *Shigella* n=91 | | 7<br>-0.08 | 24<br>0.01 | 14<br>-0.07 | 21<br>0.07 | 9<br>0.08 | 2<br>0 | 8<br>0.01 | 1<br>-0.02 | 3<br>0.13 | 4<br>0.04 | 5<br>-0.06 |
| *Salmonella* n=210 | 29<br>-0.12 | 11<br>-0.2 | 58<br>0.05 | 53<br>0.08 | 55<br>0.21 | 12<br>0 | 8<br>0.09 | 24<br>0.1 | 5<br>0.05 | 1<br>-0.03 | 8<br>0.03 | 29<br>0.13 |

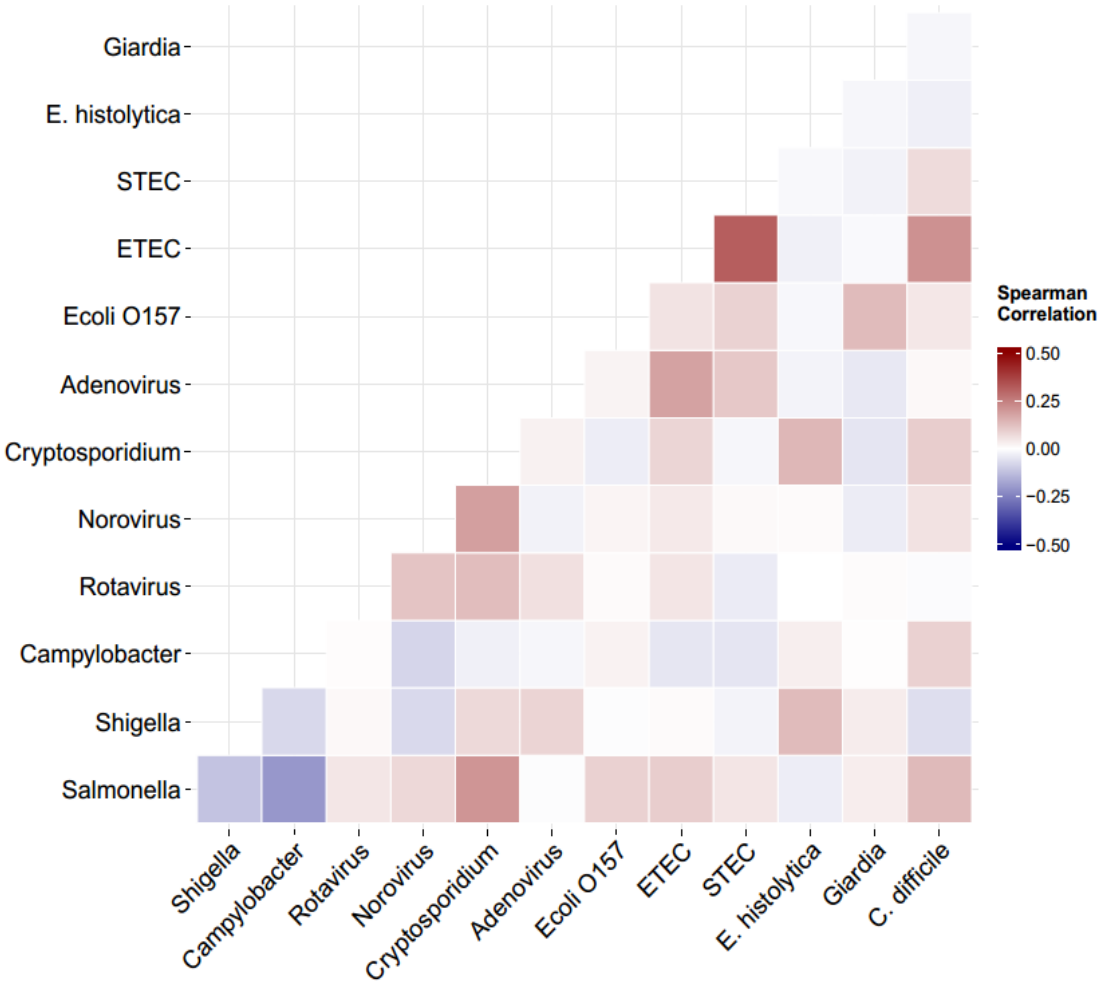



**Figure 5.2.** Correlation matrix of co-infections from 479 diarrhoeal stool samples.

Common pathogens detected by the Luminex xTAG Gastrointestinal Pathogen Panel are listed in a matrix format along the x and y-axes. The colour within each square represents the Spearman correlation coefficient for each pairwise co-infection. The darker the red, the more positive the correlation and the darker the blue, the more negative the correlation. Note that the range of the colour scale spans -0.50 to 0.50.

## 5.4. Discussion

Diarrhoeal disease is the second most common cause of death in children <5 years of age globally[1]. The need for rapid and reliable diagnostics is important for treatment decisions and is even more pressing given the alarming trends of AMR in many of the most common bacterial enteric pathogens[168], which may be in part due to the unregulated and excessive use of antimicrobials worldwide. In this study I demonstrated that the multiplex molecular Luminex xTAG GPP detection system is both highly sensitive and specific compared to both microbiological culture and real-time PCR for the majority of surveyed pathogens. Additionally, the xTAG GPP system allowed for identification of a number of important pathogens including ETEC and *Cryptosporidium* spp., which are not detected through routine procedures in hospitals in Vietnam, or across other parts of Southeast Asia.

The sensitivity and specificity of the xTAG GPP were >88% for all compared pathogens (three bacteria and four viruses) using both culture and real-time PCR; however, a notable exception was *Salmonella*. Our sensitivity and specificity findings are similar to, if somewhat lower than, those of other studies conducted in the North America, Europe and China[51–53,284]. The low specificity (~60%) of *Salmonella* detection in our study has not been commonly reported in other studies, though Wessel *et al.*[285] found a higher rate of false positive *Salmonella* detection in a small study from the Netherlands. This may be due to a high rate of asymptomatic carriage with *Salmonella* in Vietnamese children[213], or cross-reactivity with an alternative component of the gastrointestinal microbiota present in this population. The low specificity of the xTAG for detecting *Salmonella* likely led to an artificially inflated rate of overall co-infection in our samples (58% of positive samples) compared to work conducted elsewhere[49,53,284,286]. However, the rates of pairwise co-infections such as *C. difficile* and *Cryptosporidium* spp. are similar to those found in a multicentre Italian study[287]. An obvious issue raised by the high rate of co-infection and the use of the xTAG GPP in general is pathogenicity. Platts-Mills and colleagues[15]

recently found that *Cryptosporidium* spp., STEC and *Shigella* spp. were more commonly associated with severe diarrhoea than other pathogens through a birth cohort that sampled healthy and diarrhoeal stools. In order to investigate relative pathogenicity in the future, use of the xTAG GPP in combination with a quantitative technique[288], may provide more insight into the relationship between presence of a pathogen and clinical disease.

Through the use of the xTAG GPP platform I was able to identify a far wider range of pathogens than those normally screened in hospitals and in research studies in Vietnam. *Cryptosporidium* for example, was the fifth most common pathogen detected in our sample set (17%), although almost nothing is known about the epidemiology of this parasite in Vietnam. Globally, *Cryptosporidium* is known to be common in young children with moderate-to-severe diarrhoea and can be commonly identified in a very high rate (15-25%) of children with persistent diarrhoea[14,289]. Because the intestinal damage due to infection with *Cryptosporidium* can result severe long-term sequelae[290], specific studies on this parasite, and its epidemiology, are warranted. Additionally, 9% of patients in our cross-sectional sample had *C. difficile* detected in their stool. *C. difficile* is known to be much less common in children than adults, and asymptomatic infection with toxigenic strains is common[291–293], and may represent a reservoir for infection[294]. Additional investigation into the epidemiology and clinical significance of *C. difficile* infection in children in this setting is warranted. The xTAG GPP assay allows for detection of such important pathogens and may aid in not only treatment decisions but also in generation of hypotheses for further epidemiological and etiological studies.

While the benefits of the xTAG GPP system are numerous, the significant drawback for use in a country such as Vietnam is the cost. The price of a single xTAG GPP reaction is currently prohibitive for Vietnam, although recent work from the United Kingdom has demonstrated that routine use of the multiplex diagnostic led to massive savings at the hospital level due to rapid

identification of non-infectious diarrhoeal disease and subsequent reduction of the need for single-patient rooms that far exceeded the cost of implementing the xTAG GPP itself[295]. Whether or not such economic analyses are applicable to Vietnam is questionable, and future such analyses tailored to industrialising regions are recommended. An additional drawback is the lack of an organism for antimicrobial susceptibility testing. Given the rampant AMR found in pathogens such as *Shigella* spp. in Vietnam[213], this is a significant limitation.

This study has limitations. First, the microbiological culture was performed at different laboratories across Vietnam and may have introduced variability into our results. Secondly, I was not able to identify pathogens such as *Vibrio cholerae* or *Yersinia* so I was unable to evaluate the performance of the xTAG GPP against these pathogens, which may be more relevant in other setting across the region. Notwithstanding these limitations, our results shed valuable insight into the accuracy of the xTAG GPP system and its utility in an industrialising setting.

### 5.5. Conclusion

In conclusion, the Luminex xTAG GPP diagnostic platform is highly sensitive and specific for common bacterial and viral enteric pathogens in Vietnam, with the notable exception of *Salmonella* species. Furthermore, the xTAG GPP allowed for detection of a number of important pathogens that are not routinely identified through current hospital laboratory procedures in Vietnam. While there are a number of benefits to the xTAG GPP platform, the high cost and lack of an organism for antimicrobial susceptibility testing may represent barriers to widespread use in low-income regions. However, use of such a diagnostic in locations, like HCMC, Vietnam, would dramatically improve the ability of clinicians to appropriately treat gastrointestinal infections in a timely manner, provide evidence for a more prudent use of antimicrobials, and allow for an unprecedented level of epidemiological granularity to aid in prevention and control efforts for diarrhoeal disease. Furthermore, given that this approach is standardised it would be suitable for

any programs that aim to perform countrywide (and regional) surveillance. The development of newer, smaller and more cost effective Luminex machines means that this approach could be rolled out through a network of provincial hospitals. I propose that a multiplex system such as this to be used as a component of the national disease surveillance and reporting.

# 6. Genoserotyping and antimicrobial resistance determinant detection of non-typhoidal *Salmonella* isolated from children hospitalised with gastroenteritis

## 6.1. Abstract

Diarrhoeal disease associated with NTS infection has a global distribution, but the focus of infection is in young children resident in LMICs. *Salmonella enterica* subspecies *enterica* includes more than 2,500 serovars, of which the majority are associated with animals and can cause disease in humans. This diverse collection of serovars is potentially associated with differing pathologies, epidemiology, clinical presentations, and AMR profiles. In section 3.1 I isolated 478 NTS, as a component of my hospital observation study. I aimed to investigate this collection of pathogens in more detail. Therefore, I performed WGS using an Illumina HiSeq platform and the resulting sequence data was subject to molecular serotyping and the AMR profiles of various NTS isolates were determined. This is the first major study on the *Salmonella* serovars associated with diarrhoeal disease in children admitted to tertiary hospitals in HCMC. Firstly, *in silico* molecular serotyping for *Salmonella* was performed for individual genome assembly using the *Salmonella* in Silico Typing Resource (SISTR). The AMR gene content of each organism was determined by analysing the genome sequences using ARIBA via CARD read. Among the 450 NTS infections, the clinical manifestations were generally severe with 60% (274/450) experiencing acute bloody diarrhoea, and high diarrhoea output (10 episodes/24 hrs.). Antimicrobials were frequently prescribed, with 92.4% (423/450) of patients receiving empirical antimicrobial treatment. Genoserotyping identified 45 different serovars from 15 serogroups. *Salmonella* Typhimurium was the most common serovar among the confirmed cases of NTS in hospitalised children (bloody and/or mucoid diarrhoea), accounting for 41.8% (188/450). Other common serovars were *S.* Stanley (63/450, 14.0%), *S.* Weltevreden (34/450, 7.6%), *S.* Newport (29/450, 6.4%) and *S.* Enteritidis (17/450, 3.8%). Each NTS serovar demonstrated a different

antimicrobial susceptibility profile. A high MDR rate was observed in *S.* Typhimurium, *S.* Newport, *S.* Kentucky, *S.* Rissen and *S.* Saintpaul. In contrast, *S.* Stanley and *S.* Weltevreden were generally broadly susceptible. The prevalence of AMR genes for >10 classes of antimicrobial agents were determined, which exhibited good concordance with conventional phenotypic antimicrobial susceptibility testing. I found that WGS is optimal method for serotyping and studying the genetic basis of AMR in NTS. *S.* Typhimurium was predominant in this setting and carried a variety of AMR genes leading to a high rate of MDR in this serovar. For untested antimicrobial agents, the presence of AMR genes may predict the AMR phenotype i.e. lincomycin, rifampicin and colistin resistance in NTS. Phylogeny investigation of these NTS isolates vs. a collection of global isolates including local invasive NTS isolates is the next stage of analysis required to understand the circulation of NTS in Vietnam.

## 6.2. Introduction

NTS are amongst the most common aetiological agents causing diarrhoea diseases worldwide[91]. More than 90 million cases of gastroenteritis due to *Salmonella* species are estimated to occur globally per annum, of which 155,000 cases lead to death[91]. The main focus of this burden of NTS is in young children in Asia[6,91].

The *Salmonellae* are a collection of Gram-negative bacteria belonging to the *Enterobacteriaceae* family. The *Salmonella* genus is composed of two species: *Salmonella enterica* and *Salmonella bongori*, with *S. enterica* being divided into an additional six subspecies[296]. *Salmonella enterica* subspecies *enterica* contains >2,500 serovars, which cause a variety of diseases in humans and animals. The diverse collection of serovars represented potentially differing pathology, epidemiology, clinical presentations, and AMR profiles. Clinically, NTS infections are usually associated with acute gastroenteritis with the onset of fever, vomiting, abdominal cramp, and diarrhoea[297]. The symptoms are usually self-limiting and resolve within 5-7 days. Therefore, antimicrobial treatment is generally considered unnecessary and is not recommended[108,298]. However, salmonellosis can also result in invasive diseases (i.e. bloodstream infection), which has a high mortality rate, which often occurs in immunocompromised patients[99,299]. Therefore, antimicrobials may be critical for treatment of NTS gastroenteritis in high-risk patients[108,109].

In Vietnam, NTS is now the predominant enteric bacteria detected among children hospitalised with diarrhoea[94,300]. However, due to the difficulty in serovar determination, the clinical manifestations, antimicrobial susceptibility profiles and management for each specific serovar are unknown. Recent advances in WGS and analysis offer an opportunity for the molecular serotyping of *Salmonella* (genoserotyping) with high degree of accuracy and specificity[245]. Here, by utilising genome sequence data, I aimed to characterise the common NTS serovars isolated

from diarrhoeal children in three tertiary hospitals in HCMC, Vietnam. I additionally investigated the AMR mechanisms of each specific serovar by analysing their components of AMR genes.

## 6.3. Results

### 6.3.1. Study baseline information

Between May 2014 and April 2016, 478 paediatric patients were identified to be infected with NTS from the collaborating hospitals[300]. However, due to the loss during storage and sample transfer, only 450 organisms were available for analysis. More than half of the children with NTS infections were male (271/450; 60.2%), with the age ranging from 1 month to 135 months (median age 9 months, IQR 6.4 – 14.9 months). More than 20% (101/450) of children were under 6 month of age. These children were ill for a median of 2 days (IQR: 2-4 days) before hospitalisation.

One third of this population had abnormal growth status, including 22.2% (100/450) being overweighed/obese/risk of overweight and 12.5% (56/450) being wasted/severe wasted (Table 6.1). The measurement of haemoglobin concentration, according to WHO cut-off, showed that 32.9% the children in this study additionally suffered from anaemia (148/450).

### 6.3.2. Clinical manifestations and empirical treatment of NTS infections

Sixty per cent (274/450) of children diagnosed with an NTS infection were hospitalised with acute bloody diarrhoea. The remainder (176) were hospitalised with mucoid diarrhoea (Table 6.1). Profuse diarrhoea was commonly recorded with an average of 10 episodes in the primary 24-hour period (IQR: 6-10 episodes). Other symptoms, including fever (294/450, 65.3%) and vomiting (190/450, 42.2%), were also frequently reported. Most children were found not to be dehydrated (with 25 cases assessed as some dehydration); and no specific severe sequelae were recorded.

I recorded the treatment regimens of the enrolled patients, which included oral rehydration therapy (ORS), intravenous rehydration, zinc, probiotics, and antimicrobials (Table 6.1). More than 90% of patients were administered ORS and zinc supplementation. Antimicrobials were frequently prescribed, with 92.4% (423/450) of patients receiving empirical antimicrobial treatment following admission to hospital and prior to obtaining a laboratory susceptibility test result.

Fluoroquinolones were the most commonly used antimicrobials (310/423; 73.3%). Third generation cephalosporins were the first prescribed antimicrobial in 85 cases and were the second most common choice after fluoroquinolones; used in 21 cases. Imipenem was given to two patients as a last resort antimicrobial. The two young infants (3 month of age and 7 months of age) receiving imipenem presented with bloody/mucoid diarrhoea and high fever. Both were eventually found to be infected with *Salmonella* Newport ST46. These *S*. Newport isolates were ESBL positive and resistant to all tested antimicrobial agents (only susceptible to imipenem and amikacin and reduced susceptible to ciprofloxacin).

**Table 6.1.** Demographic and clinical manifestations of diarrhoeal paediatric patients infected with non-typhoidal *Salmonella* (N=450)

| **Characteristics** | **n** | **%** |
|---|---|---|
| Socio-demographic | | |
| Male | 271 | 60.2 |
| Age in months, *median* [IQR] | 9 | 6.4-14.9 |
| Growth [a] | | |
| Obese / Overweight / Risk of overweight | 100 | 22.2 |
| Normal | 271 | 63.5 |
| Wasted / Severely wasted | 56 | 12.5 |
| Clinical symptoms | | |
| Bloody diarrhoea | 274 | 60.9 |
| Mucoid diarrhoea | 176 | 39.1 |
| Number of episodes per day, *median* [IQR] | 10 | 6-10 |
| Dehydration, [b] | 25 | 5.6 |
| Abdominal pain | 115 | 25.6 |
| Fever (≥37.5°C) at enrolment | 294 | 65.3 |
| Vomit | 190 | 42.2 |
| Haematology | | |
| Anaemia (Haemoglobin level=70-109g/L) [c] | 148 | 32.9 |
| Neutrophil count ($10^3$/μL), *median* [IQR] | 4.9 | 3.2-7.2 |
| C-reactive protein (mg/L), *median* [IQR] | 29 | 11.0-50.0 |
| Treatment | | |
| Low-osmolarity oral rehydration solution | 418 | 92.9 |
| IV Rehydration | 31 | 6.9 |
| Antimicrobials | 423 | 94.0 |
| Fluoroquinolones, *n* (% of all prescribed antimicrobials) | 310 | 73.3 |
| Zinc | 412 | 91.6 |
| Probiotics | 300 | 66.7 |
| Outcome | | |
| Hospital stay in days, *median* [IQR] | 5 | 3-7 |
| Recovered / Improved after 3 days [d] | 393 | 87.3 |
| Not improved / worse after 3 days | 57 | 12.7 |

[a] Obese: weight for length z score >3SD in children age < 24months; BMI for age z score >3SD in children age ≥24months, Overweight: weight for length z score >2SD in children age < 24months; BMI for age z score >2SD in children age ≥24months, Wasted: weight for length z score <-2SD in children age < 24months; BMI for age z score <-2SD in children age≥24months, Severely wasted: weight for length z score <-3SD in children age < 24months); BMI for age z score <-3SD in children age ≥24months [251]

[b] All of the dehydrated cases classified as some dehydration according to Integrated Management of Childhood Illness [60]

[c] Haemoglobin level cut-off according to World Health Organization guidelines [247]

[d] Defined as "recovered" if patient had <3 passages of loose stool in the 24 hours or "improved" if patient had less episodes of diarrhoea and less mucus and/or bloody in comparison to the condition of the patient at enrolment.

### 6.3.3. *Salmonella* genoserotyping

The distribution of the infecting NTS serovars as determined via WGS data on the SISTR platform are presented in Table 6.2. *S.* Typhimurium was the most common serovar among the confirmed cases of NTS in hospitalised children (bloody or mucoid diarrhoea), accounting for 41.8% (188/450) of all NTS cases. Two thirds (112/188) of the *S.* Typhimurium infection was *S.* Typhimurium ST34 (monophasic variant). Other common serovars were *Salmonella* Stanley (63/450, 14.0%), *S.* Weltevreden (34/450, 7.6%), *S.* Newport (29/450, 6.4%) and *S.* Enteritidis (17/450, 3.8%). Other serovars such as Kentucky, Bovismorbificans, Rissen, Saintpaul, Virchow, etc. were less commonly reported.

No significant difference was found in the clinical manifestation between NTS serovars. In general, NTS infections were more commonly associated with young infants; the median age of infection was 9 months. Notably, *S.* Weltevreden infection was associated with disease in very young infants (median age 4.5 months, IQR 3.4-9.6 months); whereas *S.* Enteritidis infections were more common in older age groups (median age 14.2 months, IQR 12.3-33.8), $P$=0.0001, $\chi^2$ test.

**Table 6.2.** Non-typhoidal *Salmonella* serovars isolated from children with diarrhoeal diseases in three tertiary hospitals in Ho Chi Minh City

| Serogroup | Serovar | ST | Count | Serogroup | Serovar | ST | Count |
|---|---|---|---|---|---|---|---|
| **B** | Typhimurium (monophasic) | 34 | 112 | **E1** | Weltevreden | 365 | 34 |
| | Typhimurium (biphasic) | 19; 34; 36 | 76 | | London | 155 | 5 |
| | Stanley | 29; 2615 | 63 | | Give | 516 | 5 |
| | Saintpaul | 27; 50 | 8 | | Anatum | 64 | 2 |
| | Paratyphi B | 135; 423 | 8 | | Lexington | 1542 | 1 |
| | Agona | 13 | 4 | | | | |
| | Indiana | 17 | 2 | **E4** | Meleagridis | 3248 | 1 |
| | Chester | 343; 2063 | 2 | | | | |
| | Schleissheim | 1578; 2397 | 2 | **D1** | Enteritidis | 11; 74 | 17 |
| | Sandiego | 20 | 1 | | Javiana | 1547 | 4 |
| | | | | | Panama | 48; 2494 | 4 |
| **C1** | Rissen | 469 | 9 | | Dublin | 74 | 1 |
| | Virchow | 197; 359 | 6 | | | | |
| | Bareilly | 203; 909 | 5 | **G** | Kedougou | 1543 | 3 |
| | Thompson | 26; 2417 | 2 | | Agbeni | 2606 | 1 |
| | Ohio | 329 | 1 | | | | |
| | Mbandaka | 1602 | 1 | **I** | Hvittingfoss | 446 | 2 |
| | | | | | Orientalis | 558 | 1 |
| **C2-C3** | Newport | 31; 46; 2366; 2855 | 29 | | | | |
| | Kentucky | 198 | 10 | **K** | Cerro | 367 | 1 |
| | Bovismorbificans | 1499 | 9 | **N** | Johannesburg | 512 | 1 |
| | Corvallis | 1541 | 4 | **O** | Alachua | 1298 | 1 |
| | Albany | 292 | 2 | **Q** | Wandsworth | 1498 | 3 |
| | Hadar | 33 | 1 | | | | |
| | Emek | 76 | 1 | **U** | Houtenae | 958 | 1 |
| | Litchfield | 214 | 1 | **Y** | Salamae | 3200 | 1 |
| | Muenchen/Virginia | 2424 | 1 | | | | |
| | Brunei | 2809 | 1 | | | | |

### 6.3.4. Antimicrobial resistance in non-typhoidal *Salmonella*

Laboratory antimicrobial susceptibility testing was performed for each NTS isolate and is summarised in this section. Generally, the NTS isolates exhibited a high rate of AMR. NTS remained exhibiting susceptibling to imipenem and aminoglycoside with the susceptible rates as 98.9% (445/450) and 99.3% (447/450), respectively; whereas, over half of the isolates exhibited resistance against ampicillin and chloramphenicol. Approximately 38% (171/450) of the NTS additionally exhibited resistance to trimethoprim-sulfamethoxazole. The prevalence of resistance against ciprofloxacin was comparatively low at 8.7% (39/450). However, the proportion of NTS exhibiting reduced susceptibility to ciprofloxacin was substantially higher (221/450, 49.1%). This percentage contributed to a total non-susceptible proportion to ciprofloxacin of 57.8% (260/450). The prevalence of resistance against nalidixic acid was 21.4% (96/450); intermediate resistance was 20% (90/450), making the proportion nalidixic acid non-susceptible organisms 41.4% (186/450). Furthermore, approximately 13% of NTS exhibited resistance against third generation cephalosporins (ceftriaxone and ceftazidime). Notably, 18% (81/450) of the NTS isolates were resistant to azithromycin. Concurrent with this high rate of resistance, more than half of NTS isolates were determined to be MDR (non-susceptible to any antimicrobial agent in ≥ 3 classes of tested antimicrobials) (53.8%, 242/450).

Next, I investigated the AMR profiles of the ten most common NTS serovars (Figure 6.1). Each NTS serovar demonstrated different antimicrobial susceptibility profiles. *S.* Typhimurium displayed a high rate of MDR (149/188, 79.3%). Specifically, 68.4% (52/76) of the biphasic *S.* Typhimurium variants were resistant to 4 antimicrobial classes (ampicillin/amoxicillin-clavulanate, ciprofloxacin, trimethoprim-sulfamethoxazole and chloramphenicol) due to acquisition of *blaTEM-95*, *qnrS1*, *dfrA12 + sul2 + sul3* and *floR* (Figure 6.2). Moreover, ST34 of biphasic *S.* Typhimurium variant had acquired more AMR genes in comparison to ST19 and ST36 including *aac(3)-IIa*, *mphA + mrx*, *tetA + tetR*. Although the monophasic variant had a

similar rate of MDR (75%, 84/112), these profiles were variable with 35.7% (40/112) resistance to 3 classes, 25.9% (29/112) to 4 classes and 13.4% (15/112) to 5 classes. Other serovars which displayed a high MDR rate were *S.* Newport (51.7%, 15/29), *S.* Kentucky (70%, 7 /10), *S.* Rissen (66.7%, 6/9) and *S.* Saintpaul (100%, 8/8). Of note, the majority of *S.* Newport isolates were ST46 (22/29), and half of these (n=11) were non-susceptible to 10 of the tested antimicrobial agents (nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole, ceftriaxone, ceftazidime, ampicillin, amoxicillin-clavulanate, azithromycin, chloramphenicol and gentamicin). Alternatively, the remaining 10 isolates of this serovar (ST46 (n=6), ST31 (n=3) and ST2366 (n=1)) exhibited susceptibility to all tested antimicrobials (Figure 6.1). Further analysis for AMR genes in the *S.* Newport isolates is presented in Figure 6.3. The *S.* Newport ST46 isolates that were non-susceptible had acquired several AMR genes including *blaTEM-95*, *blaCTX-M-55*, *aac(3)-IIa*, *aac(6)-Iy*, *aph(6)-Id*, *aph(3')-Ia*, *aadA22/24*, *mphA + mrx*, *qnrS1*, *dfrA14 + sul3*, *floR*, *linG*, and *arr2/arr3* (Figure 6.3). *S.* Stanley and *S.* Weltevreden generally remained susceptible to all classes of tested antimicrobials with the susceptibility proportions at 50.8% (32/63) and 73.5% (25/34), respectively. While all *S.* Weltevreden isolates acquired only one AMR gene (*aac(6)-Iy*); 2 isolates acquired additionally *qnrS1*.

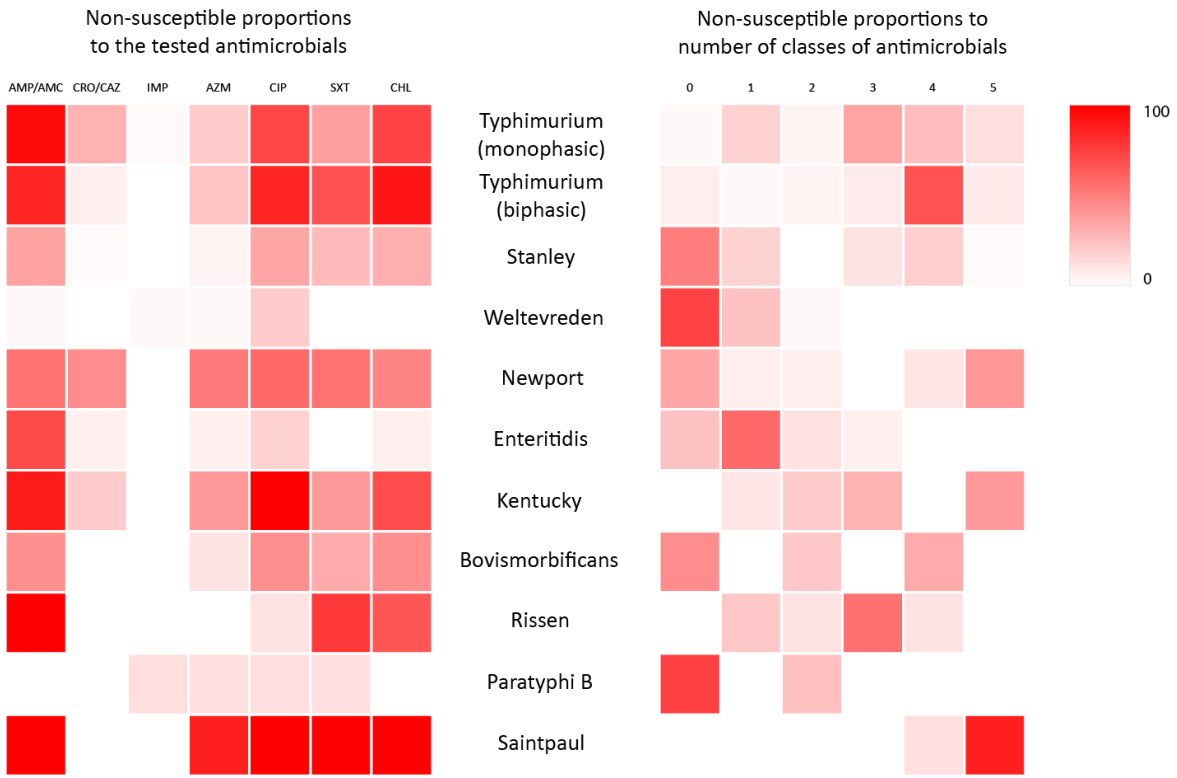



**Figure 6.1.** Heat map of antimicrobial resistance profiles of ten most prevalent non-typhoidal *Salmonella* serovars

The heat map consists of two parts: **(1)** The left panel displays the susceptibility of NTS isolates to 7 classes of tested antimicrobial agents including ampicillin/amoxicillin-clavulanate (AMP/ AMC); ceftriaxone/ ceftazidime (CRO/ CAZ); imipenem (IMP); azithromycin (AZM); ciprofloxacin (CIP); trimethoprim-sulfamethoxazole (SXT) and chloramphenicol (CHL). **(2)** The right panel displays the susceptibility of NTS isolates to the number of tested antimicrobial classes including β-lactam (AMP/AMC/CRO/CAZ); IMP; AZM; CIP; SXT and CHL. Isolates were classified as non-susceptible to an antimicrobial class if they were non-susceptible to any agent of that class. The column of number "0" shows the proportion of NTS isolates which were susceptible to six antimicrobial classes. The colour intensity in a cell is proportional to the percentage of non-susceptible NTS isolates to the tested antimicrobial class. The NTS serovars are arranged in decreasing order of prevalence (from top to bottom).

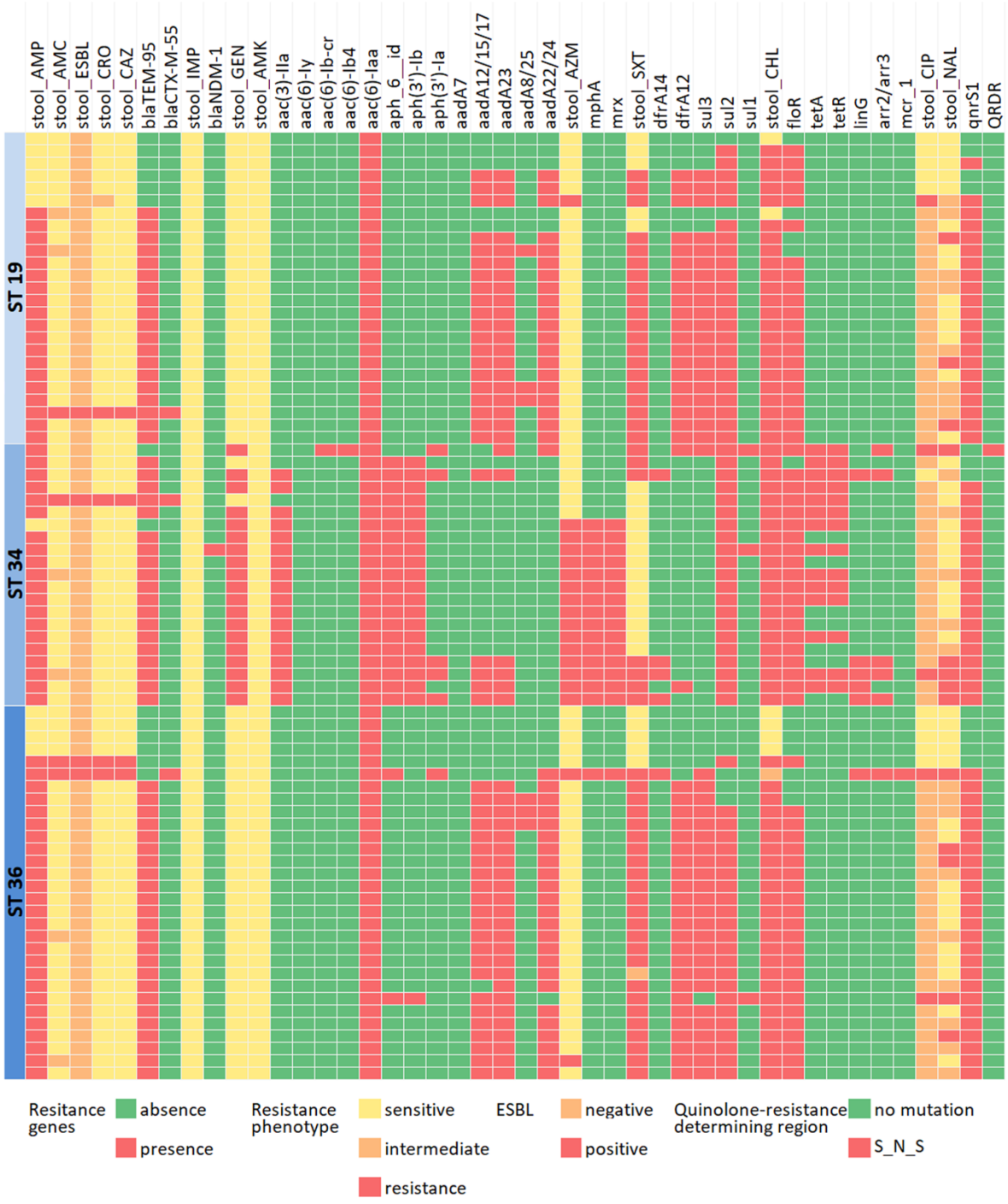



**Figure 6.2.** Antimicrobial resistance phenotype and genotype of biphasic variant of *Salmonella* Typhimurium (n=76)

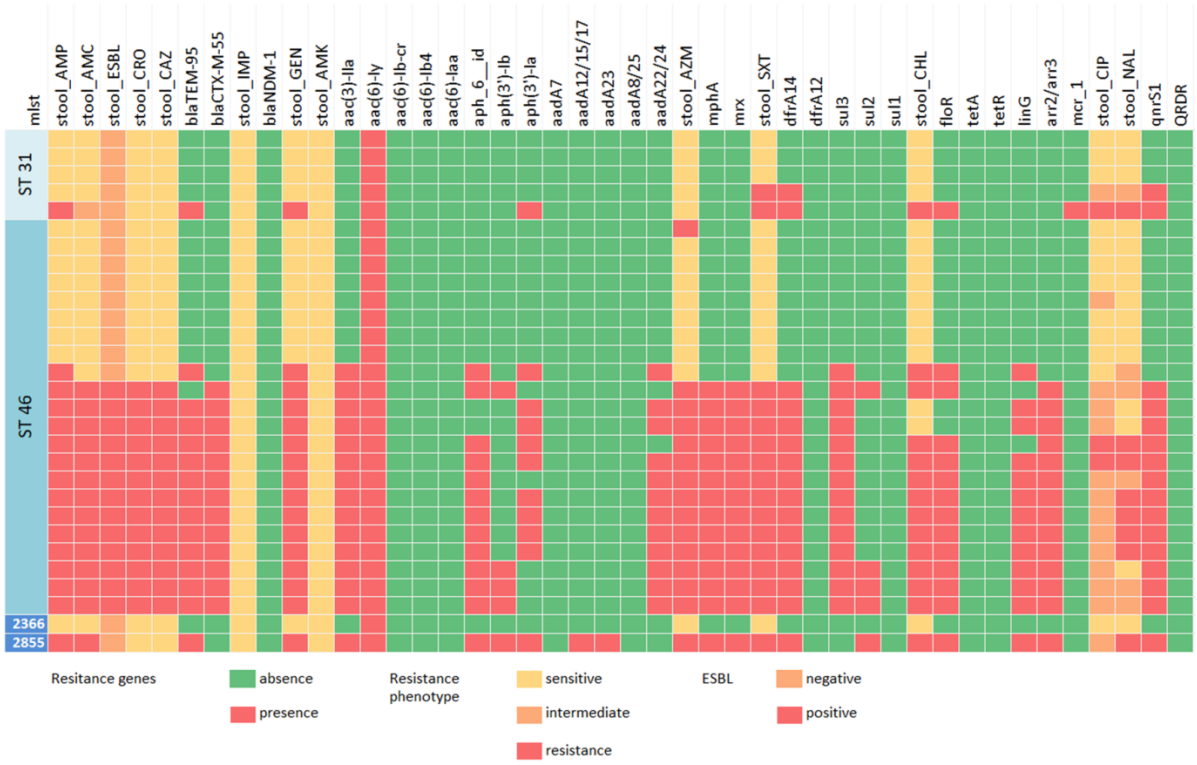



**Figure 6.3.** Antimicrobial resistance phenotype and genotype of *Salmonella* Newport (n=29)

### 6.3.5. Antimicrobial resistance gene content and genotype-phenotype concordance

The acquisition of multiple AMR genes were common characteristics of the accessory genomes of the NTS isolates. Firstly, I analysed prevalence of individual AMR genes from all NTS isolates (N=450) (Table 6.3). This approach permitted me to document the prevalence of all AMR genes as well as the corresponding resistance mechanisms to various antimicrobial classes. Subsequently, I proceeded to compare the concordance between genotype and phenotype for each tested antimicrobial class by calculating the sensitivity and specificity of determining AMR genes with WGS using zone diameter disk diffusion tests as a gold standard (Table 6.4).

#### 6.3.5.1. Quinolone resistance

Quinolone resistance is mediated by quinolone resistance determining region (QRDR) mutations and/or the acquisition of plasmid-mediated quinolone resistance genes (PMQR)[301]. Among the 450 NTS isolates, 38 isolates (8.4%) had at least one mutation in QRDR (*gyrA*-83, *gyrA*-87 and *parC*-80). Any one of these *gyrA* mutations resulted in full resistance to nalidixic acid (Table 6.4); whereas PMQR carriage predicted a sensitivity of 93.5% and the specificity of 73.4% in nalidixic acid resistance. Seven variants of QRDR mutations were identified, with 11 isolates carrying triple mutations and exhibiting full resistance to ciprofloxacin (Table 6.5). Specifically, all ten *S*. Kentucky isolates harboured triple QRDR mutations (*gyrA*S83F, *gyrA*D87N, and *parC*S80I) and exhibited full resistance to ciprofloxacin. Among the PMQR genes, *qnrS1* was the most prevalent, present in 47.8% of the isolates in this collection (215/450). For isolates without the aforementioned QRDR mutations (n=412), the presence of PMQR explained non-susceptibility to ciprofloxacin with 89.5% sensitivity and 96.7% specificity. A combination of a single QRDR mutation and PMQR gene(s) always resulted in full resistance to fluoroquinolones (Table 6.5).

#### 6.3.5.2. β-lactam resistance

The most common beta-lactamase encoded genes identified were $bla_{\text{TEM-95}}$ (58.7%, 264/450) and $bla_{\text{CTX-M-55}}$ (11.6%, 52/450). These were followed by other less common genes (less than 2% of total isolates). For β-lactam resistance, genotypes predicted phenotypes with 58.2-94.4% sensitivity (different sensitivity by groups of β-lactam drug) and >99.4% specificity. One isolate of biphasic *S.* Typhimurium carried $bla_{\text{NDM-1}}$; however, the phenotype was determined to be susceptible to imipenem.

#### 6.3.5.3. Sulphonamide and trimethoprim resistance

Sulphonamide resistance was predominantly conferred by *sul2* or *sul3* (present in 52.7%, 237/450 and 25.8%, 116/450 of NTS isolates, respectively). In addition, *sul1* was found in 33/450 isolates (7.3%). Resistance to trimethoprim was explicable via the acquisitions of dihydrofolate reductase variants (*dfrA*), with *dfrA12* (21.6%, 97/450) and *dfrA14* (15.3%, 69/450) being the most common. Genotypic prediction for trimethoprim-sulfamethoxazole resistance was calculated by combining the presence of both *sul* and *dfr* genes, resulted in a sensitivity of 99.4% and with the specificity of 57.4%. The high sensitivity denotes that resistance is almost exclusively explained by the presence of *sul-dfr* combination. However, their presence does not always translate to phenotypic resistance, probably due to the genes' disruptive mutations or silent transcription, which results in the observed low specificity.

#### 6.3.5.4. Macrolide resistance

Azithromycin- resistant *Salmonella* isolates was determined by a macrolide inactivation gene cluster, *mphA-mrx-mphr(A)* within a novel *Salmonella* azithromycin resistance genomic island (SARGI)[184]. Here I found 58 NTS isolates carried *mphA-mrx*, which were the most prevalent

macrolide resistance genes. Other, less common macrolide resistance genes included *ermF*, *T*, *B*; and *mefB*.

#### 6.3.5.5. Aminoglycoside resistance

A high number of NTS carried aminoglycosides resistance genes i.e. *aac*, *aph*, *aad*, and each of these are responsible to resistance to different classes of aminoglycosides. In some instances, the NTS genotype does not always fit with their phenotypes. *aac(6')-Iy* is chromosomally encoded and specific to *Salmonella*, but normally it is not transcribed due to the absence of adjacent upstream promoter[302]. This results in the observed genotype-phenotype mismatch in resistance against amikacin.

**Table 6.3.** Prevalence of antimicrobial genes (AMR genes) among non-typhoidal *Salmonella* isolates from children hospitalised with diarrhoeal diseases in Ho Chi Minh City (N=450)

| Classes | Antimicrobial agents | AMR genes | Count |
|---|---|---|---|
| **β-lactams** | **Ampicillin** | *blaTEM-95* | 264 |
| | | *blaOXA-1* | 6 |
| | | *blaCARB-3* | 2 |
| | | *blaCTX-M-15 + blaSHV-66* | 1 |
| | **Cephalosporins III** | *blaCTX-M-55* | 52 |
| | | *blaCMY-2/ blaCMY-42* | 5 |
| | | *blaCTX-M-14* | 2 |
| | **Imipenem** | *blaNDM-1* | 1 |
| **Aminoglycosides** | **Gentamicin** | *aac(3)-IIa* | 86 |
| | | *aac(3)-IV* | 5 |
| | | *aac(6)-IIa* | 1 |
| | **Amikacin** | *aac(6)-Iy* | 237 |
| | | *aac(6)-Iaa* | 213 |
| | | *aac(6)-Ib4* | 10 |
| | | *aac(6)-Ib-cr* | 10 |
| | **Streptomycin** | *aph(6)-Id* (*strB*) | 186 |
| | | *aadA2/ aadA3* | 132 |
| | | *aadA12/ aadA15 / aadA17* | 130 |
| | | *aadA22-24* | 115 |
| | | *aadA8/ aadA25* | 13 |
| | | *aadA7* | 10 |
| | | *aadA16* | 4 |
| | | *aadA5* | 2 |
| | **Kanamycin** | *aph(3')-Ib* (*strA*) | 168 |
| | | *aph(3')-Ia* | 56 |
| | **Hygromycin B** | *aph(4)-Ia* | 5 |
| **Macrolides** | **Azithromycin** | *mphA + mrx* | 58 |
| | | *ermF* | 7 |
| | | *mphA* | 2 |
| | | *ermT* | 2 |
| | | *ermB* | 1 |
| | | *mefB* | 1 |

| Classes | Antimicrobial agents | AMR genes | Count |
|---|---|---|---|
| **Quinolones** | **Ciprofloxacin/ Nalidixic acid** | *qnrS1* | 215 |
| | | *qnrS2* | 4 |
| | | *qnrB6* | 3 |
| | | *qnrD1* | 1 |
| | | *oqx_AB* | 1 |
| | | *patA* | 1 |
| **Folate Pathway Inhibitors** | **Trimethoprim** | *dfrA12* | 97 |
| | | *dfrA14* | 69 |
| | | *dfrA1* | 3 |
| | | *dfrA17* | 2 |
| | | *dfrA5* | 1 |
| | **Sulfonamide** | *sul2* | 237 |
| | | *sul3* | 116 |
| | | *sul1* | 33 |
| **Phenicols** | **Chloramphenicol** | *floR* | 225 |
| | | *catB3* | 6 |
| | | *catA1* | 1 |
| **Tetracyclines** | **Tetracycline** | *tetA + tetR* | 87 |
| | | *tetA + tetR + tetD* | 1 |
| **Lincosamides** | **Lincomycin** | *linG* | 48 |
| **Rifamycin** | **Rifampicin** | *arr2/ arr3* | 50 |
| **Cyclic peptides** | **Colistin** | *mcr_1* | 6 |
| **Antineoplastic** | **Bleomycin** | *ble* | 1 |

**Table 6.4.** The performance of whole genome sequencing in determine the antimicrobial susceptibility of non-typhoidal *Salmonella*

| Antimicrobials | Antimicrobial resistance genes | | Phenotype | | Sensitivity (%[95%CI]) | | Specificity (%[95%CI]) | | PPV | NPV |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Non-Susceptible | Susceptible | | | | | | |
| ampicillin | *blaTEM-95/ blaOXA_1/ blaCARB-3/ blaCTX-M15 + blaSHV-66* | yes | 271 | 1 | 94.4 | (91.1-96.8) | 99.4 | (96.6-100) | 99.6 | 91.0 |
| | | no | 16 | 162 | | | | | | |
| amoxicillin-clavulanate | *blaCTX-M-55/ blaCTX-M-14/ blaCMY-2/ blaCMY-42* | yes | 53 | 2 | 58.2 | (47.4-68.5) | 99.4 | (98.0-99.9) | 96.4 | 90.4 |
| | | no | 38 | 357 | | | | | | |
| ceftriaxone/ ceftazidime | *blaCTX-M-55/ blaCTX-M-14/ blaCMY-2/ blaCMY-42* | yes | 53 | 2 | 88.3 | (77.4-95.2) | 99.5 | (98.2-99.9) | 96.4 | 98.2 |
| | | no | 7 | 388 | | | | | | |
| imipenem | *blaNDM_1* | yes | 0 | 1 | 0.0 | (0.0-52.2) | 99.8 | (98.8-100) | 0.0 | 98.9 |
| | | no | 5 | 444 | | | | | | |
| gentamicin | *aa(3)-IIa / aac(3)-IV / aac(6)-IIa* | yes | 89 | 2 | 100 | (95.9-100) | 99.4 | (98.0-99.9) | 97.8 | 100 |
| | | no | 0 | 358 | | | | | | |
| azithromycin | *mphA + mrx / mphA mefB / ermT / ermF / ermB* | yes | 59 | 7 | 72.8 | (61.8-82.1) | 98.1 | (96.1-99.2) | 89.4 | 94.3 |
| | | no | 22 | 362 | | | | | | |
| ciprofloxacin | Wild type QRDR PMQR genes | yes | 205 | 6 | 89.5 | (84.8-93.2) | 96.7 | (93.0-98.8) | 97.2 | 88.1 |
| | | no | 24 | 177 | | | | | | |
| nalidixic acid | Wild type QRDR PMQR genes | yes | 141 | 70 | 95.3 | (90.5-98.1) | 73.4 | (67.6-78.6) | 66.8 | 96.5 |
| | | no | 7 | 193 | | | | | | |
| ciprofloxacin | Mutation on QRDR | yes | 31 | 7 | 100 | (88.8-100) | 0.0 | (0.0-41.0) | 81.6 | . |
| | | no | 0 | 0 | | | | | | |
| nalidixic acid | Mutation on QRDR | yes | 38 | 0 | - | | | | | |
| | | no | 0 | 0 | | | | | | |
| trimethoprim-sulfamethoxazole | *dfr + sul* | yes | 172 | 118 | 99.4 | (96.8-100) | 57.4 | (51.3-63.3) | 59.3 | 99.4 |
| | | no | 1 | 159 | | | | | | |
| chloramphenicol | *catA1 / catB3 / floR* | yes | 224 | 2 | 93.3 | (89.4-96.1) | 99.0 | (96.6-99.9) | 99.1 | 92.9 |
| | | no | 16 | 208 | | | | | | |

Quinolone resistance determining region (QRDR): *gyrA*_83(S) + *gyrA*_87(D) + *parC*_80(S); Plasmid-mediated Quinolone Resistance genes (PMQR): *qnrB6/ qnrD1/ qnrS1/ qnrS2/ oqx_AB/ patA/ aac(6)-Ib-cr*

**Table 6.5.** The prevalence of mutations in the quinolone resistance determining region (QRDR) in non-typhoidal *Salmonella* isolated from children hospitalised with diarrhoeal diseases and the association with fluoroquinolone resistance

| ***gyrA*_83** | ***gyrA*_87** | ***parC*_80** | **CIP**[a] | **Serovar** | **ST**[b] | **Count** | **Note** |
|---|---|---|---|---|---|---|---|
| **Y** | D | S | R | Saintpaul | 50 | 6 | all isolates carried *qnrS1* |
| | | | I | Give | 516 | 5 | 4/6 carried *qnrS1* |
| | | | I | Emek | 76 | 1 | |
| **F** | **N** | **I** | R | Kentucky | 198 | 10 | 4/10 isolates carried *qnrS1* |
| S | **N** | S | S | Enteritidis | 11 | 7 | |
| | | | R | Typhimurium (biphasic) | 34 | 1 | carried *oqxAB* and *aac(6)-Ib-cr* |
| **F** | D | S | I | Albany | 292 | 2 | |
| | | | I | Hadar | 33 | 1 | |
| S | **G** | S | I | Typhimurium (monophasic) | 34 | 1 | |
| | | | I | Indiana | 17 | 1 | |
| S | **Y** | S | I | Enteritidis | 11 | 1 | |
| **F** | **G** | **R** | R | Indiana | 17 | 1 | carried *aac(6)-Ib-cr* |

[a] Inhibition zone diameter interpretive criteria for ciprofloxacin susceptibility according to CLSI standard[224] as followed ≤20mm means resistance (R), 21-30mm means intermediate (I) and ≥31mm means sensitive (S)

[b] Multilocus sequence typing

Amino acids letter code: tyrosine (Y), aspartic acid (D), serine (S), phenylalanine (F), asparagine (N), isoleucine (I), glycine (G), arginine (R)

## 6.4. Discussion

NTS has become the most common cause of bacterial diarrhoeal diseases globally[91]. The clinical presentation of NTS infection ranges from mild diarrhoea to life threatening invasive diseases, depending on the interplay of the pathogen's biology and the host susceptibility[298]. Serotyping for *Salmonella* is currently not routinely performed due to the shortage of trained personnel and dedicated resources. Genoserotyping (i.e. *in silico* serotyping using genomic data), has become a desirable alternative with high throughput and accurate performance. Specifically, the burden of NTS diseases and its serotype prevalence have not been previously well characterised in Vietnam. Therefore, my study has provided the first opportunity not only to document the prevalence of NTS infection in diarrhoeal paediatric population but also to identify the most common *Salmonella* serovars in this setting.

Vietnam has undergone (is undergoing) an economic transition to become a LMIC. This has been followed by an epidemiology shift in bacterial enteric infections, in which NTS has become the most common bacterial aetiology for diarrhoeal diseases. This now resembles diarrhoeal epidemiology reported in high income countries[97,256]. While *S.* Enteritidis was found to be the most widespread in Greece[303], China[103] or Tunisa[304], *S.* Typhimurium was the most common serovar in this study. This serovar is more commonly associated with MDR[297,303] and invasive disease[99,299]. This analysis on AMR profile of *S.* Typhimurium showed substantial agreement with previous findings from other regions. The rates of resistance were substantially higher in antimicrobial classes of first line treatments, such as amoxicillin, trimethoprim-sulfamethoxazole or azithromycin compared to other areas[103,303,304].

I observed a high concurrence between the presence of AMR genes and antimicrobial resistance phenotypes with the tested antimicrobial agents. In NTS isolates, any mutation on QRDR leaded to fully resistance to nalidixic acid and reduced susceptibility to ciprofloxacin. More than one

mutation on QRDR increased the resistance to ciprofloxacin, which has also been observed in *E. coli*[301]. The acquisition of PMQR genes was associated with high sensitivity and specificity with non-susceptibility to ciprofloxacin and nalidixic acid; although the specificity for nalidixic acid was low and associated with a high false positive rate. This trend was also observed with trimethoprim-sulfamethoxazole susceptibility and in 22 NTS isolates that did not harbour azithromycin resistance genes but exhibited resistance. The presence of genes but a lack of resistance is likely associated with either a lack of gene expression or expression of the gene is not sufficient to stimulate resistance i.e. the gene stimulates an increase in MIC, which is not sufficient to induce resistance. Clearly this discrepancy between genotype and phenotype is worthy of further investigation and suggests that classical microbiology is still important for antimicrobial susceptibility testing.

The Vietnamese healthcare system currently follows the WHO guidelines for treatment of paediatric diarrhoea. These guidelines recommend the use of low osmolarity ORS, zinc, and antimicrobials for all patients with bloody diarrhoea, irrespective of their age[16,19]. The drug of choice is ciprofloxacin or one of the three second-line alternatives: pivmecillinam, azithromycin, or ceftriaxone. The results presented in this chapter outline a situation where there is a high prevalence of resistance in NTS to the most commonly used and recommended antimicrobials. These data suggest that resistance in these organisms can be attributed to the sustained use of these antimicrobials. One such example is the fluoroquinolones, which are the most frequently prescribed antimicrobials; the majority of organisms here exhibited reduced susceptibility to ciprofloxacin. The most mechanism of ciprofloxacin resistance among these NTS isolates were DNA gyrase mutations in combination with the wide spread distribution of *qnrS1*.

In this investigation, I found that NTS infections most commonly occurred in very young age group, particularly children under 6 months of age. Since NTS are widely distributed in human

and animal reservoirs, the source of NTS infections in these infections is uncertain. These cases could be associated with zoonotic transmissions through the food chain or via sustained human-to-human transmissions distinct from the NTS reservoirs in animals[305,306]. This observational study was limited to the description of hospitalised diarrhoea, so it was not possible to identify the possible source of infection without more detailed epidemiological data (i.e. dietary habit, household living conditions, animal contact, and household cases). Previous studies have shown that NTS transmissions may occur through human-to-human contact in the home[94]. Besides the young age, the NTS infected children presented with many severe clinical symptoms, including high faecal bloody output, fever, and vomiting. These observations suggest the highly infectious nature of this disease. However, updated guidelines advise that antimicrobials should not be used routinely in NTS infections, except for the immunocompromised, the neonates and the elderly[108,109,298]. The treatment of these patients requires careful attention.

### 6.5. Conclusions

In the setting where NTS has become the most common enteric bacteria detected in the stools of children hospitalised with bloody or mucoid diarrhoeal diseases in HCMC[300]. There is an increasing trend of AMR, especially against the first line antimicrobial classes recommended by the WHO guidelines, i.e. ciprofloxacin, ceftriaxone. Therefore, the first choice of empirical antimicrobials for infectious diarrhoeal diseases should be critically re-evaluated. WGS is an alternative method for serotyping and AMR genetic investigations in NTS. *S.* Typhimurium was the predominant serovar in this setting and associated with a variety of AMR genes leading to a high rate of MDR in this organism. For untested antimicrobial agents, the presence of AMR genes may predict the AMR trend i.e. lincomycin, rifampicin and colistin resistance in NTS. A phylogeny investigation of these NTS isolates vs. a collection of global isolates and/or iNTS isolates is the next stage of this investigation and required to better understand transmission dynamics and the evolutionary processes underpinning the circulation of these organisms.

# 7. General discussion

My work represents very personal insights into diarrhoeal disease in Vietnam. Prior to this work, the bacterial aetiology of this disease was poorly described; as shown here there is a significant burden of diarrhoea associated with AMR bacteria in Vietnam. Additionally, my work investigated the treatment and outcome in these infections, again for which there are little data. My research was conducted over two years using standard microbiological methods and newer experimental techniques, these approaches produced new clinical and epidemiological insights into the most common bacteria causing diarrhoea in paediatric patients attending three tertiary hospitals in HCMC. The first aim of my thesis was to describe the prevalence of common enteric bacteria. In chapter 3 and chapter 4 I measured the prevalence of NTS, *Campylobacter*, *Shigella* and DEC using microbiological culture and by optimising a new collection of multiplex real-time PCR assays. This provided the epidemiology of diarrhoeal diseases in 2014-2016 period including children with diarrhoea in the hospitals and healthy children from a cohort study. The clinical manifestations of mono-infection and co-infection with these bacteria were also investigated. However, most importantly, and for the first time in Vietnam, I measured the usage and benefit of antimicrobial therapy for diarrhoeal diseases.

For the prevalence of enteric bacteria I found that NTS has become the most common in diarrhoea in children. NTS was followed by *Campylobacter* and *Shigella*; this observation likely reflects economic development. Emerging foodborne pathogens are not well described in this setting and my result highlights a significant burden of NTS infection. Additionally, my data also found a substantial rate of AMR and MDR among these three major enteric pathogens. These resistance rates were high in comparison with Europe and North America and analogous to other LMICs in Asia.

The most significant finding from my thesis was in studying antimicrobial usage in paediatric hospitals in HCMC. Hospitals in this location follow the standard of care as recommend by WHO guidelines for the management of dysentery. Here, antimicrobial treatment for children with diarrhoea not only contradicted to the antimicrobial susceptibility profiles of the bacterial isolates but also prolonged the duration of hospitalisation. Additionally, and regardless of the antimicrobial use if the child had an AMR or MDR infection, the majority of patients recovered or improved after three days follow-up. Despite using a proxy outcome, which limited my ability to measure the direct effect of antimicrobial use, the magnitude of the study and by controlling for confounding I was able to conclude that antimicrobial treatment was not associated with a clinical benefit among children hospitalised with diarrhoeal diseases. In an editorial commentary, Timothy Jones highlighted that AMR and MDR enteric bacteria are a problem worldwide and noted high antimicrobial usage in US[307]. The author concluded that the "first do no harm" antimicrobial treatment approach for treating diarrhoea must be seriously considered. Therefore, improving diagnostics for diarrhoea should be an effective approach for supporting clinical treatment decisions[307].

DEC are a common group of bacterial pathogens associated with diarrhoea in children in LMICs. Due to the morphological similarities with commensal *E. coli*, the burden of DEC infection has been greatly underestimated in Vietnam. Hence, I developed and optimised a set of multiplex real-time PCR assays to detect the six pathotypes of DEC in faecal samples. These assays were specific and a suitably high throughput method, permitting me to generate data for DEC on >3,000 clinical samples in a few days. The utility of these real-time PCR assays permitted a breakdown of the distribution of the various DEC pathotypes in this setting. Principally, I found that the role of DEC in paediatric diarrhoea was controversial as most pathotypes were found in stool samples from healthy children and children with diarrhoea. Atypical EPEC and EAEC were the most common DEC variants in both diarrhoea and non-diarrhoea groups, demonstrating that

these pathotypes were not correlated with diarrhoea in this population. This observation was similar with findings from the GEMS study and other studies in LMICs from Asia and Africa. Some studies have questioned whether mixed DEC infections may be associated with diarrhoea in children. My analysis found that infection with EIEC/*Shigella* was significantly associated with diarrhoea. Although the real-time PCR assays had some limitations (i.e. unable to differentiate EIEC from *Shigella* and difficult to interpret when several virulence genes where detected in a single sample), this approach was still useful for investigating DEC in a large population.

The next aim of my thesis was to apply variety of molecular diagnostic methods to diagnose and further analyse some enteric pathogens. In chapter 5, I applied multiplex panel assays (xTAG GPP) to detect multiple enteric pathogens in one reaction. Most of the tested samples were from the main project (12EN). In order to have a variety range of samples, I collected faecal samples from paediatric acute diarrhea study (2009-2010) and aldult diarrhoeal episodes (2007-2014). In 2015, although Luminex xTAG GPP has been used in industrial areas[48,49,51,52,284], the evaluation of this assay in LMICs was limited. My research regarding the utility of Luminex xTAG GPP on clinical faecal samples was the first such publication from Vietnam and Southeast Asia. This assay was convenient, had a short turnaround time, high output and the ability to detect several enteric pathogens. However, one obvious limitation of this assay was the cost. Furthermore, I found that xTAG GPP generated a high false positive result for *Salmonella* detection. Lastly, with a high proportion of people carriers enteric-pathogen[15], mixed infections, as observed by xTAG GPP, were difficult to interpret.

As NTS became were the most frequent enteric bacteria associated with diarrhoea in children, I further investigated this group of pathogens. Several studies have applied WGS for studying NTS [308–312]. Here, I had an exclusive opportunity to describe the complexity of NTS serovars currently

circulating in Vietnam. *S.* Typhimurium was predominant and associated with a complex array of AMR mechanisms. Additionally, WGS provided a large amount of supporting epidemiological data. I focused on describing the AMR (genotype and phenotype) of the common NTS serovars. I additionally analysed the clinical manifestations of each NTS serovar; however, there was significant difference among clinical symptoms and locations between NTS serovars. An exception was *S.* Weltevreden, which was significantly associated with very young infants. Further work on the phylogeny of these organisms would expand our knowledge of each NTS serovar.

In conclusion, my work added various new insights into diarrhoeal disease in Vietnam. In an era where we are exhausting our antimicrobial resources, we need new approaches for reducing antimicrobial usage. My approach of using cutting edge diagnostics and WGS demonstrated some utility in studying AMR pathogens. AMR bacterial pathogens are rampant in this location and it appears that antimicrobial usage has no clinical benefit. Future studies in Vietnam should focus on trying to introduce a more streamlined approach to diagnostics, aiming to provide enough clinical evidence to reduce or stop empirical antimicrobial usage for paediatric diarrhoea. I aim to continue my research in this setting and provide the data to support better clinical decision making and to educate clinicians to use antimicrobials in a more concise manner.

## 8. References


1 Walker CLF, Rudan I, Liu L, *et al.* Global burden of childhood pneumonia and diarrhoea. *Lancet* 2013; **381**: 1405–16.

2 Fischer Walker CL, Perin J, Aryee MJ, Boschi-Pinto C, Black RE. Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review. *BMC Public Health* 2012; **12**: 220.

3 Walker CLF, Aryee MJ, Boschi-Pinto C, Black RE. Estimating diarrhea mortality among young children in low and middle income countries. *PLoS One* 2012; **7**: e29151.

4 GBD Diarrhoeal Diseases Collaborators. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect Dis* 2017; **17**: 909–48.

5 Machado C. Netter's Pediatrics. Philadelphia: Elsevier, 2011.

6 WHO. WHO estimates of the global burden of foodborne diseases. *Who* 2015; : 1–255.

7 Guandalini S, Dhawan A, Branski D, editors. Textbook of Pediatric Gastroenterology, Hepatology and Nutrition. Springer International Publishing, 2016 DOI:10.1007/978-3-319-17169-2.

8 Kirk MD, Pires SM, Black RE, *et al.* World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. *PLoS Med* 2015; **12**: 1–21.

9 Tate JE, Burton AH, Boschi-Pinto C, *et al.* Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013. *Clin Infect Dis* 2016; **62**: S96–105.

10 Nguyen VM, Nguyen VT, Huynh PL, *et al.* The epidemiology and disease burden of rotavirus in Vietnam: sentinel surveillance at 6 hospitals. *J Infect Dis* 2001; **183**: 1707–12.

11 Tate JE, Patel MM, Cortese MM, *et al.* Remaining issues and challenges for rotavirus vaccine in preventing global childhood diarrheal morbidity and mortality. *Expert Rev*

*Vaccines* 2012; **11**: 211–20.

12 Patel MM, Steele D, Gentsch JR, Wecker J, Glass RI, Parashar UD. Real-world impact of rotavirus vaccination. *Pediatr Infect Dis J* 2011; **30**: S1-5.

13 Desselberger U, Goodfellow I. Noroviruses: A global cause of acute gastroenteritis. *Lancet Infect Dis* 2014; **14**: 664–5.

14 Kotloff KL, Nataro JP, Blackwelder WC, *et al.* Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): A prospective, case-control study. *Lancet* 2013; **382**: 209–22.

15 Platts-Mills J a, Babji S, Bodhidatta L, *et al.* Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *Lancet Glob Heal* 2015; **3**: e564-75.

16 World Health Organization. The Treatment of Diarrhoea. 2005 http://www.who.int/maternal_child_adolescent/documents/9241593180/en/.

17 Guarino A, Dupont C, Gorelov A V, *et al.* The management of acute diarrhea in children in developed and developing areas: from evidence base to clinical practice. *Expert Opin Pharmacother* 2012; **13**: 17–26.

18 Guandalini S, Vaziri H, editors. Diarrhea. Totowa, NJ: Humana Press, 2011 DOI:10.1007/978-1-60761-183-7.

19 D. Legros. Guidelines for the control of shigellosis, including epidemics due to Shigella dysenteriae type 1. *World Heal Organ* 2005; : 1–70.

20 Pfeiffer ML, DuPont HL, Ochoa TJ. The patient presenting with acute dysentery - A systematic review. *J Infect* 2012; **64**: 374–86.

21 Pawlowski SW, Warren CA, Guerrant R. Diagnosis and Treatment of Acute or Persistent Diarrhoea. *Gastroenterology* 2010; **136**: 1874–86.

22 Guerrant RL, Van Gilder T, Steiner TS, *et al.* Practice guidelines for the management of infectious diarrhea. *Clin Infect Dis* 2001; **32**: 331–51.

23 Dupont HL. Systematic review: The epidemiology and clinical features of travellers' diarrhoea. *Aliment Pharmacol Ther* 2009; **30**: 187–96.

24 Schiller LR. Secretory diarrhea. *Curr Gastroenterol Rep* 1999; **1**: 389–97.

25 Hammer HF, Fine KD, Santa Ana CA, Porter JL, Schiller LR, Fordtran JS. Carbohydrate malabsorption. Its measurement and its contribution to diarrhea. *J Clin Invest* 1990; **86**: 1936–44.

26 V.Sigh O. Foodborne Pathogens and Antibiotic Resistance. Hoboken, NJ, USA: John Wiley & Sons, Inc., 2016 DOI:10.1002/9781119139188.

27 Platts-Mills JA, Liu J, Houpt ER. New concepts in diagnostics for infectious diarrhea. *Mucosal Immunol* 2013; **6**: 876–85.

28 Nga DTT, Chuc NTK, Hoa NPQPQ, *et al.* Antibiotic sales in rural and urban pharmacies in northern Vietnam: an observational study. *BMC Pharmacol Toxicol* 2014; **15**: 6.

29 BioMérieux Inc. API 20 E Instruction. 2002; : 1–5.

30 John P. Anhalt and Catherine. Fenselau. Identification of bacteria using mass spectrometry. *Anal Chem* 1975; **47**: 219–25.

31 Vestling MM. Using Mass Spectrometry for Proteins. *J Chem Educ* 2003; **80**: 122.

32 Holland RD, Wilkes JG, Rafii F, *et al.* Rapid Identification of Intact Whole Bacteria Based on Spectral Patterns using Matrix-assisted Laser Desorption/Ionization with Time-of-flight Mass Spectrometry. *Rapid Commun Mass Spectrom* 1996; **10**: 1227–32.

33 Claydon MA, Davey SN, Edwards-Jones V, Gordon DB. The rapid identification of intact microorganisms using mass spectrometry. *Nat Biotechnol* 1996; **14**: 1584–6.

34 Krishnamurthy T, Ross PL. Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Commun Mass Spectrom* 1996; **10**: 1992–6.

35 Biswas S, Rolain JM. Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. *J Microbiol Methods* 2013; **92**: 14–24.

36 Croxatto A, Prod'hom G, Greub G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol Rev* 2012; **36**: 380–407.

37 Vlek ALM, Bonten MJM, Boel CHE. Direct Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Improves Appropriateness of Antibiotic Treatment of Bacteremia. *PLoS One* 2012; **7**: e32589.

38 He Y, Li H, Lu X, Stratton CW, Tang YW. Mass spectrometry biotyper system identifies enteric bacterial pathogens directly from colonies grown on selective stool culture media. *J Clin Microbiol* 2010; **48**: 3888–92.

39 Bessède E, Solecki O, Sifré E, Labadi L, Mégraud F. Identification of Campylobacter species and related organisms by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. *Clin Microbiol Infect* 2011; **17**: 1735–9.

40 Martiny D, Visscher A, Catry B, Chatellier S, Vandenberg O. Optimization of Campylobacter growth conditions for further identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). *J Microbiol Methods* 2013; **94**: 221–3.

41 Dieckmann R, Malorny B. Rapid screening of epidemiologically important Salmonella enterica subsp. enterica serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl Environ Microbiol* 2011; **77**: 4136–46.

42 Brooks, George F., Ernest Jawetz, Joseph L Melnick and EAA. Jawetz, Melnick & Adelberg's Medical Microbiology. 26th edition, 26th editi. New York : London: McGraw-Hill Medical, 2013.

43 Nwabor OF, Dickson ID, Ajibo QC. Epidemiology of *Salmonella* and *Salmonellosis*. *Int Lett Nat Sci* 2015; **47**: 54–73.

44 Jones TF, Gerner-Smidt P. Nonculture diagnostic tests for enteric diseases. *Emerg Infect Dis* 2012; **18**: 513–4.

45 Platts-Mills JA, Operario DJ, Houpt ER. Molecular diagnosis of diarrhea: Current status

and future potential. *Curr Infect Dis Rep* 2012; **14**: 41–6.

46 Loftis AD, Reeves WK, Health P, Service I. Veterinary PCR Diagnostics. BENTHAM SCIENCE PUBLISHERS, 2012 DOI:10.2174/97816080534831120101.

47 Liu J, Platts-Mills J a, Juma J, *et al.* Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. *Lancet* 2016; **388**: 1291–301.

48 Halligan E, Edgeworth J, Bisnauthsing K, *et al.* Multiplex molecular testing for management of infectious gastroenteritis in a hospital setting : a comparative diagnostic and clinical utility study. 2013.

49 Mengelle C, Mansuy JM, Prere MF, *et al.* Simultaneous detection of gastrointestinal pathogens with a multiplex Luminex-based molecular assay in stool samples from diarrhoeic patients. *Clin Microbiol Infect* 2013; **19**: E458–65.

50 Patel a., Navidad J, Bhattacharyya S. Site-Specific Clinical Evaluation of the Luminex xTAG Gastrointestinal Pathogen Panel for Detection of Infectious Gastroenteritis in Fecal Specimens. *J Clin Microbiol* 2014; **52**: 3068–71.

51 Claas EC, Burnham CAD, Mazzulli T, Templeton K, Topin F. Performance of the xTAG gastrointestinal pathogen panel, a multiplex molecular assay for simultaneous detection of bacterial, viral, and parasitic causes of infectious gastroenteritis. *J Microbiol Biotechnol* 2013; **23**: 1041–5.

52 Navidad JF, Griswold DJ, Gradus MS, Bhattacharyya S. Evaluation of Luminex xTAG gastrointestinal pathogen analyte-specific reagents for high-throughput, simultaneous detection of bacteria, viruses, and parasites of clinical and public health importance. *J Clin Microbiol* 2013; **51**: 3018–24.

53 Deng J, Luo X, Wang R, *et al.* A comparison of Luminex xTAG® Gastrointestinal Pathogen Panel (xTAG GPP) and routine tests for the detection of enteropathogens circulating in Southern China. *Diagn Microbiol Infect Dis* 2015.

DOI:10.1016/j.diagmicrobio.2015.07.024.

54 Corporation L. (Gastrointestinal Pathogen Panel) For use with Luminex® 100/200$^{TM}$ and MAGPIX® Systems. Luminex Molecular Diagnostics, Inc., 2013.

55 Lefterova MI, Suarez CJ, Banaei N, Pinsky BA. Next-Generation Sequencing for Infectious Disease Diagnosis and Management: A Report of the Association for Molecular Pathology. *J Mol Diagn* 2015; **17**: 623–34.

56 Zankari E, Hasman H, Cosentino S, *et al*. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 2012; **67**: 2640–4.

57 McArthur AG, Waglechner N, Nizam F, *et al*. The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother* 2013; **57**: 3348–57.

58 Baker S, Hanage WP, Holt KE. Navigating the future of bacterial molecular epidemiology. *Curr Opin Microbiol* 2010; **13**: 640–5.

59 WHO. Preventing diarrhoea through better water, sanitation and hygiene. *WHO Libr Cat Data* 2014; : 1–48.

60 World Health Organization. Handbook IMCI: Integrated management of childhood illness. Geneva, 2005 DOI:10.1093/heapol/czh001.

61 World Health Organization. Pocket Book of Hospital Care for Children: Guidelines for the Management of Common Childhood Illnesses. 2nd edition. Geneva: Available from: https://www.ncbi.nlm.nih.gov/books/NBK154447/, 2013.

62 Guarino A, Ashkenazi S, Gendrel D, *et al*. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition/European Society for Pediatric Infectious Diseases Evidence-Based Guidelines for the Management of Acute Gastroenteritis in Children in Europe: Update 2014. *Jpgn* 2014; **59**: 132–52.

63 Lewis K. VESIKARI CLINICAL SEVERITY SCORING SYSTEM MANUAL. PATH. 2011. http://www.path.org/publications/files/VAD_vesikari_scoring_manual.pdf.

64 UNICEF. Water, Sanitation and Hygiene Annual Report 2013. 2014; : 1–52.

65 De Vos B, Han HH, Bouckenooghe A, *et al.* Live attenuated human rotavirus vaccine, RIX4414, provides clinical protection in infants against rotavirus strains with and without shared G and P genotypes: integrated analysis of randomized controlled trials. *Pediatr Infect Dis J* 2009; **28**: 261–6.

66 World Health Organization. Rotavirus Vaccination. Meeting of the immunization Strategic Advisory Group of Experts, April 2009. *Wkly Epidemiol Rec* 2009; **84**: 220–36.

67 Loharikar A, Dumolard L, Chu S, Hyde T, Goodman T MC. Status of New Vaccine Introduction — Worldwide. *MMWR Morb Mortal Wkly Rep*; **65**: 1136–1140.

68 Administrative T, Wong SCK, Lee N, *et al.* Effectiveness of Rotavirus Vaccination: A Systematic Review of the First Decade of Global Postlicensure Data, 2006-2016. *Clin Infect Dis* 2017; **65**: 840–50.

69 Tu HAT, Rozenbaum MH, Coyte PC, Li SC, Woerdenbag HJ, Postma MJ. Health economics of rotavirus immunization in Vietnam: Potentials for favorable cost-effectiveness in developing countries. *Vaccine* 2012; **30**: 1521–8.

70 Griffiths PL, Park RWA. Campylobacters associated with human diarrhoeal disease. *J Appl Bacteriol* 1990; **69**: 281–301.

71 Bessède E, Delcamp A, Sifré E, Buissonnière A, Mégraud F. New methods for detection of campylobacters in stool samples in comparison to culture. *J Clin Microbiol* 2011; **49**: 941–4.

72 Sarah S. Long CGP and MF. Principles and practice of pediatric infectious diseases, fourth edi. Elsevier, 2012.

73 Ban Mishu Allos, M.D., Martin J. Blaser, M.D., James A. Platts-Mills, M.D., Margaret N. Kosek M. Campylobacter species. 2013; : 1–11.

74 Fernández-Cruz A, Muñoz P, Mohedano R, *et al.* Campylobacter bacteremia: clinical characteristics, incidence, and outcome over 23 years. *Medicine (Baltimore)* 2010; **89**: 319–30.

75 Facciolà A, Riso R, Avventuroso E, Visalli G, Delia SA, Laganà P. Campylobacter: From microbiology to prevention. *J Prev Med Hyg* 2017; **58**: E79–92.

76 World Health Organization. The global view of Campylobacteriosis. 2012 www.who.int/iris/bitstream/10665/80751/1/9789241564601_eng.pdf.

77 Geissler AL, Bustos Carrillo F, Swanson K, *et al.* Increasing Campylobacter Infections, Outbreaks, and Antimicrobial Resistance in the United States, 2004-2012. *Clin Infect Dis* 2017; **65**: 1624–31.

78 Clark CG, Price L, Ahmed R, *et al.* Characterization of Waterborne Outbreak – associated Walkerton , Ontario. *Emerg Infect Dis* 2003; **9**: 1232–41.

79 Jay-Russell MT, Mandrell RE, Yuan J, *et al.* Using major outer membrane protein typing as an epidemiological tool to investigate outbreaks caused by milk-borne Campylobacter jejuni isolates in California. *J Clin Microbiol* 2013; **51**: 195–201.

80 Humphries RM, Linscott AJ. Laboratory diagnosis of bacterial gastroenteritis. *Clin Microbiol Rev* 2015; **28**: 3–31.

81 Li Y, Zhang S, He M, *et al.* Prevalence and molecular characterization of Campylobacter spp. Isolated from patients with Diarrhea in Shunyi, Beijing. *Front Microbiol* 2018; **9**: 1–8.

82 Lluque A, Riveros M, Prada A, Ochoa TJ, Ruiz J. Virulence and antimicrobial resistance in campylobacter spp. From a peruvian pediatric cohort. *Scientifica (Cairo)* 2017; **2017**. DOI:10.1155/2017/7848926.

83 Mukherjee P, Ramamurthy T, Mitra U, Mukhopadhyay AK. Emergence of high-level azithromycin resistance in Campylobacter jejuni isolates from pediatric diarrhea patients in Kolkata, India. *Antimicrob Agents Chemother* 2014; **58**: 4248.

84 Ghosh R. Increasing Antimicrobial Resistance of Campylobacter Jejuni Isolated from Paediatric Diarrhea Cases in A Tertiary Care Hospital of New Delhi, India. *J Clin Diagnostic Res* 2013; **7**: 247–9.

85 Guyard-Nicodème M, Rivoal K, Houard E, *et al.* Prevalence and characterization of Campylobacter jejuni from chicken meat sold in French retail outlets. *Int J Food Microbiol* 2015; **203**: 8–14.

86 Ternhag A, Asikainen T, Giesecke J, Ekdahl K. A meta-analysis on the effects of antibiotic treatment on duration of symptoms caused by infection with Campylobacter species. *Clin Infect Dis* 2007; **44**: 696–700.

87 Skerman V, McGowan V, Sneath P. Approved Lists of Bacterial Names. *Int J Syst Evol Microbiol* 1980; **30**: 225–420.

88 Eng SK, Pusparajah P, Ab Mutalib NS, Ser HL, Chan KG, Lee LH. Salmonella: A review on pathogenesis, epidemiology and antibiotic resistance. *Front Life Sci* 2015; **8**: 284–93.

89 Popoff MY, Bockemühl J, Gheesling LL. Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Res Microbiol* 2003; **154**: 173–4.

90 Reeves MW, Evins GM, Heiba AA, Plikaytis BD, Farmer JJ. Clonal nature of Salmonella typhi and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis and proposal of Salmonella bongori comb. nov. *J Clin Microbiol* 1989; **27**: 313–20.

91 Majowicz SE, Musto J, Scallan E, *et al.* The Global Burden of Nontyphoidal Gastroenteritis. *Clin Infect Dis* 2010; **50**: 882–9.

92 Shimoni Z, Pitlik S, Leibovici L, *et al.* Nontyphoid Salmonella bacteremia: age-related differences in clinical presentation, bacteriology, and outcome. *Clin Infect Dis* 1999; **28**: 822–7.

93 Collard JM, Bertrand S, Dierick K, *et al.* Drastic decrease of Salmonella Enteritidis isolated from humans in Belgium in 2005, shift in phage types and influence on foodborne outbreaks. *Epidemiol Infect* 2008; **136**: 771–81.

94 Thompson CN, Phan VTM, Le TPT, *et al.* Epidemiological features and risk factors of Salmonella gastroenteritis in children resident in Ho Chi Minh City, Vietnam. *Epidemiol*

*Infect* 2013; **141**: 1604–13.

95 Leung DT, Das SK, Malek MA, *et al.* Non-typhoidal Salmonella gastroenteritis at a diarrheal hospital in Dhaka, Bangladesh, 1996-2011. *Am J Trop Med Hyg* 2013; **88**: 661–9.

96 Jones TF, Ingram LA, Fullerton KE, *et al.* A case-control study of the epidemiology of sporadic Salmonella infection in infants. *Pediatrics* 2006; **118**: 2380–7.

97 Scallan E, Mahon BE, Hoekstra RM, Griffin PM. Estimates of Illnesses, Hospitalizations, and Deaths Caused By Major Bacterial Enteric Pathogens in Young Children in the United States. *Pediatr Infect Dis J* 2012; **32**: 1.

98 Gordon M a, Graham SM, Walsh AL, *et al.* Epidemics of invasive Salmonella enterica serovar enteritidis and S. enterica Serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clin Infect Dis* 2008; **46**: 963–9.

99 Phu Huong Lan N, Le Thi Phuong T, Nguyen Huu H, *et al.* Invasive Non-typhoidal Salmonella Infections in Asia: Clinical Observations, Disease Outcome and Dominant Serovars from an Infectious Disease Hospital in Vietnam. *PLoS Negl Trop Dis* 2016; **10**: 1–13.

100 Kariuki S, Revathi G, Kariuki N, Kiiru J, Mwituria J, Hart CA. Characterisation of community acquired non-typhoidal Salmonella from bacteraemia and diarrhoeal infections in children admitted to hospital in Nairobi, Kenya. *BMC Microbiol* 2006; **6**: 1–10.

101 Sjölund-Karlsson M, Joyce K, Blickenstaff K, *et al.* Antimicrobial susceptibility to azithromycin among Salmonella enterica isolates from the United States. *Antimicrob Agents Chemother* 2011; **55**: 3985–9.

102 Huang IF, Kao CH, Lee WY, *et al.* Clinical manifestations of nontyphoid salmonellosis in children younger than 2 years old - Experiences of a tertiary hospital in Southern Taiwan. *Pediatr Neonatol* 2012; **53**: 193–8.

103 Li Y, Xie X, Xu X, *et al.* Nontyphoidal salmonella infection in children with acute gastroenteritis: prevalence, serotypes, and antimicrobial resistance in Shanghai, China. *Foodborne Pathog Dis* 2014; **11**: 200–6.

104 Wei ZQ, Chang HL, Li YF, Xu XB, Zeng M. [Clinical epidemiology and antimicrobial resistance of nontyphoidal Salmonella enteric infections in children: 2012-2014]. *Zhonghua er ke za zhi = Chinese J Pediatr* 2016; **54**: 489–95.

105 Acheson D, Hohmann EL. Nontyphoidal Salmonellosis. *Clin Infect Dis* 2001; **32**: 263–9.

106 Chiappini E, Galli L, Pecile P, Vierucci A, de Martino M. Results of a 5-year prospective surveillance study of antibiotic resistance among Salmonella enterica isolates and ceftriaxone therapy among children hospitalized for acute diarrhea. *Clin Ther* 2002; **24**: 1585–94.

107 Parry CM, Threlfall EJ. Antimicrobial resistance in typhoidal and nontyphoidal salmonellae. *Curr Opin Infect Dis* 2008; **21**: 531–8.

108 Onwuezobe I a, Oshun PO, Odigwe CC. Antimicrobials for treating symptomatic non-typhoidal Salmonella infection. *Cochrane Libr issue* 2012; **11**: 1–50.

109 Wen SCH, Best E, Nourse C. Non-typhoidal Salmonella infections in children: Review of literature and recommendations for management. *J Paediatr Child Health* 2017; **53**: 936–41.

110 James Cherry & Gail J. Demmler-Harrison & Sheldon L. Kaplan & William J. Steinbach & Peter Hotez. Feigin and Cherry's Textbook of Pediatric Infectious Diseases. Philadelphia: Elsevier, 2014.

111 Jakaitis BM, Denning PW. Human breast milk and the gastrointestinal innate immune system. *Clin Perinatol* 2014; **41**: 423–35.

112 Sánchez-Vargas FM, Abu-El-Haija MA, Gómez-Duarte OG. Salmonella infections: An update on epidemiology, management, and prevention. *Travel Med Infect Dis* 2011; **9**: 263–77.

113 World Health Organization. Information Sheet observed rate of Vaccine reactions - Typhoid vaccine. 2014 http://www.who.int/vaccine_safety/initiative/tools/Typhoid_vaccine_rates_information_sheet.pdf.

114 Trofa AF, Ueno-Olsen H, Oiwa R, Yoshikawa M. Dr. Kiyoshi Shiga: Discoverer of the Dysentery Bacillus. *Clin Infect Dis* 1999; **29**: 1303–6.

115 Kotloff KL, Riddle MS, Platts-Mills JA, Pavlinac P, Zaidi AKM. Shigellosis. *Lancet* 2017. DOI:10.1016/S0140-6736(17)33296-8.

116 Levine MM, DuPont HL, Formal SB, *et al.* Pathogenesis of Shigella dysenteriae 1 (Shiga) dysentery. *J Infect Dis* 1973; **127**: 261–70.

117 Sack D a, Sack D a, Lyke C, *et al.* Antimicrobial resistance in shigellosis, cholera and campylobacteriosis. *World Heal Organ* 2001; : 1–56.

118 Khan WA, Griffiths JK, Bennish ML. Gastrointestinal and Extra-Intestinal Manifestations of Childhood Shigellosis in a Region Where All Four Species of Shigella Are Endemic. *PLoS One* 2013; **8**. DOI:10.1371/journal.pone.0064097.

119 Avital A, Maayan C, Goitein KJ. Incidence of convulsions and encephalopathy in childhood Shigella infections. Survey of 117 hospitalized patients. *Clin Pediatr (Phila)* 1982; **21**: 645–8.

120 Khan WA, Dhar U, Salam MA, Griffiths JK, Rand W, Bennish ML. Central nervous system manifestations of childhood shigellosis: prevalence, risk factors, and outcome. *Pediatrics* 1999; **103**: E18.

121 Struelens MJ, Patte D, Kabir I, Salam A, Nath SK, Butler T. Shigella septicemia: prevalence, presentation, risk factors, and outcome. *J Infect Dis* 1985; **152**: 784–90.

122 Keddy KH, Sooka A, Crowther-Gibson P, *et al.* Systemic shigellosis in South Africa. *Clin Infect Dis* 2012; **54**: 1448–54.

123 Jung IS, Kim HS, Park H, Lee SI. The clinical course of postinfectious irritable bowel

syndrome: a five-year follow-up study. *J Clin Gastroenterol* 2009; **43**: 534–40.

124 Collaborators GB of DS 2013. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015; **386**: 743–800.

125 Gupta A, Polyak CS, Bishop RD, Sobel J, Mintz ED. Laboratory-confirmed shigellosis in the United States, 1989-2002: epidemiologic trends and patterns. *Clin Infect Dis* 2004; **38**: 1372–7.

126 von Seidlein L, Kim DR, Ali M, *et al.* A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. *PLoS Med* 2006; **3**: e353.

127 Khatun F, Faruque ASG, Koeck JL, *et al.* Changing species distribution and antimicrobial susceptibility pattern of Shigella over a 29-year period (1980-2008). *Epidemiol Infect* 2011; **139**: 446–52.

128 Vinh H, Nhu NTK, Nga TVTT, *et al.* A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation. *BMC Infect Dis* 2009; **9**: 204.

129 Thompson CN, Duy PT, Baker S. The Rising Dominance of Shigella sonnei: An Intercontinental Shift in the Etiology of Bacillary Dysentery. *PLoS Negl Trop Dis* 2015; **9**: e0003708.

130 Qiu S, Xu X, Yang C, *et al.* Shift in serotype distribution of Shigella species in China, 2003-2013. *Clin Microbiol Infect* 2015; **21**: 252.e5-252.e8.

131 Sack RB, Rahman M, Yunus M, Khan EH. Antimicrobial resistance in organisms causing diarrheal disease. *Clin Infect Dis* 1997; **24 Suppl 1**: S102–5.

132 Kahsay AG, Muthupandian S. A review on Sero diversity and antimicrobial resistance patterns of Shigella species in Africa, Asia and South America, 2001-2014. *BMC Res*

*Notes* 2016; **9**: 1–6.

133 Niyogi SK. Shigellosis. *J Microbiol* 2005; **43**: 133–43.

134 Nüesch-Inderbinen M, Heini N, Zurfluh K, Althaus D, Hächler H, Stephan R. Shigella antimicrobial drug resistance mechanisms, 2004-2014. *Emerg Infect Dis* 2016; **22**: 1083–5.

135 Puzari M, Sharma M, Chetia P. Emergence of antibiotic resistant Shigella species: A matter of concern. *J Infect Public Health* 2017. DOI:10.1016/j.jiph.2017.09.025.

136 Darton TC, Thanh Tuyen H, Chung The H, *et al.* Azithromycin resistance in *Shigella* spp. in Southeast Asia. *Antimicrob Agents Chemother* 2018; : AAC.01748-17.

137 Hosangadi D, Smith PG, Kaslow DC, Giersing BK, WHO ETEC & Shigella Vaccine Consultation Expert Group. WHO consultation on ETEC and Shigella burden of disease, Geneva, 6-7th April 2017: Meeting report. *Vaccine* 2018; : 6–7.

138 Pitisuttithum P, Islam D, Chamnanchanunt S, *et al.* Clinical trial of an oral live shigella sonnei vaccine candidate, WRSS1, in Thai adults. *Clin Vaccine Immunol* 2016; **23**: 564–75.

139 Croxen M a., Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. Recent advances in understanding enteric pathogenic Escherichia coli. *Clin Microbiol Rev* 2013; **26**: 822–80.

140 Gomes TAT, Elias WP, Scaletsky ICA, *et al.* Diarrheagenic Escherichia coli. *Brazilian J Microbiol* 2016; **47**: 3–30.

141 Nataro JP, Kaper JB. Diarrheagenic Escherichia coli Strains. *Clin Microbiol Rev* 1998; **11**: 142–201.

142 Qadri F, Svennerholm A-M, Faruque ASG, Sack RB. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev* 2005; **18**: 465–83.

143 Wennerås C, Erling V. Prevalence of enterotoxigenic Escherichia coli-associated

diarrhoea and carrier state in the developing world. *J Health Popul Nutr* 2004; **22**: 370–82.

144 Li Y, Luo Q, Shi X, *et al.* Phenotypic and Genotypic Characterization of Clinical Enterotoxigenic Escherichia coli Isolates from Shenzhen, China. *Foodborne Pathog Dis* 2017; **14**: 333–40.

145 Begum Y a., Talukder K a., Azmi IJ, *et al.* Resistance pattern and molecular characterization of enterotoxigenic Escherichia coli (ETEC) strains isolated in Bangladesh. *PLoS One* 2016; **11**: 1–11.

146 Trainor E, Iturriza-Gómara M, Ngwira B, Cunliffe N. Detection of enterotoxigenic *E. coli* in hospitalised children with and without diarrhoea in Blantyre, Malawi. *Paediatr Int Child Health* 2016; **36**: 102–5.

147 Sack RB. Human diarrheal disease caused by enterotoxigenic Escherichia coli. *Annu Rev Microbiol* 1975; **29**: 333–53.

148 Nataro JP, Yikang D, Yingkang D, Walker K. AggR, a transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative Escherichia coli. *J Bacteriol* 1994; **176**: 4691–9.

149 Hicks S, Candy DC, Phillips AD. Adhesion of enteroaggregative Escherichia coli to pediatric intestinal mucosa in vitro. *Infect Immun* 1996; **64**: 4751–60.

150 Ud-Din A, Wahid S. Relationship among Shigella spp. And enteroinvasive Escherichia coli (EIEC) and their differentiation. *Brazilian J Microbiol* 2014; **45**: 1131–8.

151 Harris JR, Mariano J, Wells JG, Payne BJ, Donnell HD, Cohen ML. Person-to-person transmission in an outbreak of enteroinvasive Escherichia coli. *Am J Epidemiol* 1985; **122**: 245–52.

152 Hyland RM, Sun J, Griener TP, *et al.* The bundlin pilin protein of enteropathogenic Escherichia coli is an N-acetyllactosamine-specific lectin. *Cell Microbiol* 2008; **10**: 177–87.

153 Trabulsi L, Keller R, Tardelli Gomes T. Typical and atypical enteropathogenic Escherichia coli. *Emerg Infect Dis* 2002; **8**: 508–13.

154 Bielaszewska M, Friedrich AW, Aldick T, Schürk-Bulgrin R, Karch H. Shiga toxin activatable by intestinal mucus in Escherichia coli isolated from humans: predictor for a severe clinical outcome. *Clin Infect Dis* 2006; **43**: 1160–7.

155 CDC. Foodborne Diseases Active Surveillance Network ( FoodNet ): FoodNet Surveillance Report for 2014 (Final Report). 2014; : 1–25.

156 Rovid Spickler A. Enterohemorrhagic Escherichia coli Infections. *Cent Secur Public Heal* 2016; : 1–15.

157 Orth D, Würzner R. What makes an enterohemorrhagic Escherichia coli? *Clin Infect Dis* 2006; **43**: 1168–9.

158 Clements A, Young JC, Constantinou N, Frankel G. Infection strategies of enteric pathogenic Escherichia coli. *Gut Microbes* 2012; **3**: 71–87.

159 Tarr PI, Gordon C a, Chandler WL. Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome. *Lancet (London, England)* 2005; **365**: 1073–86.

160 Rivas L, Mellor GE, Gobius K, Fegan N. Detection and Typing Strategies for Pathogenic Escherichia coli. New York, NY: Springer New York, 2015 DOI:10.1007/978-1-4939-2346-5.

161 Eigner U, Hiergeist a., Veldenzer a., Rohlfs M, Schwarz R, Holfelder M. Evaluation of a new real-time PCR assay for the direct detection of diarrheagenic Escherichia coli in stool specimens. *Diagn Microbiol Infect Dis* 2017; : 1–5.

162 Fleming A. Nobel Lecture: Penicillin. *Nobel Lect Physiol or Med 1942-1962* 1945; : 83–93.

163 Powers JH. Antimicrobial drug development – the past, the present, and the future. *Clin Microbiol Infect* 2004; **10**: 23–31.

164 Coates A, Hu Y, Bax R, Page C. The future challenges facing the development of new

antimicrobial drugs. *Nat Rev Drug Discov* 2002; **1**: 895–910.

165 Coates AR, Halls G, Hu Y. Novel classes of antibiotics or more of the same? *Br J Pharmacol* 2011; **163**: 184–94.

166 World Health Organization. ANTIBACTERIAL AGENTS IN CLINICAL DEVELOPMENT. Geneva, 2017 http://www.who.int/medicines/news/2017/IAU_AntibacterialAgentsClinicalDevelopment_webfinal_2017_09_19.pdf.

167 O'Neill J. Tackling drug-resistant infections globally: final report and recommendations. *Rev Antimicrob Resist* 2016; : 84.

168 World Health Organization, The World Health Organization. Antimicrobial resistance: global report on surveillance 2014. *Bull World Health Organ* 2014; **61**: 383–94.

169 Zellweger RM, Carrique-Mas J, Limmathurotsakul D, *et al.* A current perspective on antimicrobial resistance in Southeast Asia. *J Antimicrob Chemother* 2017; **72**: 2963–72.

170 Kapoor G, Saigal S, Elongavan A. Action and resistance mechanisms of antibiotics: A guide for clinicians. *J Anaesthesiol Clin Pharmacol* 2017; **33**: 300–5.

171 Jessica M. A. Blair, Mark A. Webber, Alison J. Baylay DOO& LJVP. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015; **13**: 42–51.

172 Jacoby GA, Munoz-Price LS. The new beta-lactamases. *N Engl J Med* 2005; **352**: 380–91.

173 Roberts MC, Schwarz S, Aarts HJM. Erratum: Acquired antibiotic resistance genes: An overview. *Front Microbiol* 2012; **3**: 1–17.

174 Drawz SM, Bonomo RA. Three decades of beta-lactamase inhibitors. *Clin Microbiol Rev* 2010; **23**: 160–201.

175 James H Jorgensen; Michael A Pfaller; Karen C Carroll. Manual of Clinical Microbiology, 11th Edition. American Society of Microbiology, 2015 DOI:10.1128/9781555817381.

176 Nordmann P, Naas T, Poirel L. Global spread of carbapenemase producing

Enterobacteriaceae. *Emerg Infect Dis* 2011; **17**: 1791–8.

177 Glupczynski Y. MINIREVIEW Aminoglycosides : Activity and Resistance. 1999; **43**: 727–37.

178 Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* 1993; **57**: 138–63.

179 Magnet S, Blanchard JS. Molecular Insights into Aminoglycoside Action and Resistance. *Chem Rev* 2005; **105**: 477–98.

180 Vannuffel P, Cocito C. Mechanism of action of streptogramins and macrolides. *Drugs* 1996; **51 Suppl 1**: 20–30.

181 Roberts MC. Resistance to tetracycline, macrolide-lincosamide-streptogramin, trimethoprim, and sulfonamide drug classes. *Appl Biochem Biotechnol - Part B Mol Biotechnol* 2002; **20**: 261–83.

182 Maravić G. Macrolide resistance based on the Erm-mediated rRNA methylation. *Curr Drug Targets Infect Disord* 2004; **4**: 193–202.

183 Van Hoek AH a M, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJM. Acquired antibiotic resistance genes: An overview. *Front Microbiol* 2011; **2**: 1–27.

184 Nair S, Ashton P, Doumith M, *et al.* WGS for surveillance of antimicrobial resistance: a pilot study to detect the prevalence and mechanism of resistance to azithromycin in a UK population of non-typhoidal *Salmonella*. *J Antimicrob Chemother* 2016; **15**: dkw318.

185 Fàbrega A, Madurga S, Giralt E, Vila J. Mechanism of action of and resistance to quinolones. *Microb Biotechnol* 2009; **2**: 40–61.

186 Hawkey PM. Mechanisms of quinolone action and microbial response. *J Antimicrob Chemother* 2003; **51**: 29–35.

187 Kato J ichi, Nishimura Y, Imamura R, Niki H, Hiraga S, Suzuki H. New topoisomerase essential for chromosome segregation in E. coli. *Cell* 1990; **63**: 393–404.

188 Li XZ. Quinolone resistance in bacteria: Emphasis on plasmid-mediated mechanisms. *Int J Antimicrob Agents* 2005; **25**: 453–63.

189 Vila J, Ruiz J, Goñi P, De Anta MT. Detection of mutations in parC in quinolone-resistant clinical isolates of Escherichia coli. *Antimicrob Agents Chemother* 1996; **40**: 491–3.

190 Jacoby GA. Mechanisms of resistance to quinolones. *Clin Infect Dis* 2005; **41 Suppl 2**: S120-6.

191 Jacoby G, Strahilevitz J, Hooper D. Plasmid-mediated quinolone resistance. *Microbiol Spectr* 2014; **2**: 997–1003.

192 Domínguez-Herrera J, Velasco C, Docobo-Pérez F, *et al.* Impact of qnrA1, qnrB1 and qnrS1 on the efficacy of ciprofloxacin and levofloxacin in an experimental pneumonia model caused by Escherichia coli with or without the GyrA mutation Ser83Leu. *J Antimicrob Chemother* 2013; **68**: 1609–15.

193 Robicsek A, Strahilevitz J, Jacoby GA, *et al.* Fluoroquinolone-modifying enzyme: A new adaptation of a common aminoglycoside acetyltransferase. *Nat Med* 2006; **12**: 83–8.

194 Huovinen P, Sundstrom L, Swedberg G, Skold O. Trimethoprim and sulfonamide resistance. *Antimicrob Agents Chemother* 1995; **39**: 279–89.

195 Byarugaba DK. Antimicrobial Resistance in Developing Countries. 2009 DOI:10.1007/978-0-387-89370-9.

196 Enne VI, Livermore DM, Stephens P, Hall LMC. Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction. *Lancet* 2001; **357**: 1325–8.

197 Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev* 2004; **28**: 519–42.

198 Leclercq R. Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. *Clin Infect Dis* 2002; **34**: 482–92.

199 Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular

biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001; **65**: 232–60.

200 Wehrli W. Rifampin: mechanisms of action and resistance. *Rev Infect Dis* 1983; **5 Suppl 3**: S407-11.

201 Arlet G, Nadjar D, Herrmann J, *et al.* Plasmid-Mediated Rifampin Resistance Encoded by an arr-2 -Like Gene Cassette in Klebsiella pneumoniae Producing an ACC-1 Class C β -Lactamase Plasmid-Mediated Rifampin Resistance Encoded by an arr-2 -Like Gene Cassette in Klebsiella pneumoniae Producing a. 2001; **45**: 2971–3.

202 Andrews JM, Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001; **48 Suppl 1**: 5–16.

203 Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 2008; **3**: 163–75.

204 American Society for Microbiology. Manual of antimicrobial susceptibility testing. 2005 DOI:10.1007/s13398-014-0173-7.2.

205 Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. CLSI supplement M100 (ISBN 1-56238-839-8). Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2018.

206 EUCAST. European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters. *Eucast* 2013; **7.1**: 3–7.

207 Revez J, Espinosa L, Albiger B, Leitmeyer KC, Struelens MJ. Survey on the Use of Whole-Genome Sequencing for Infectious Diseases Surveillance: Rapid Expansion of European National Capacities, 2015–2016. *Front Public Heal* 2017; **5**. DOI:10.3389/fpubh.2017.00347.

208 Schürch AC, van Schaik W. Challenges and opportunities for whole-genome sequencing–based surveillance of antibiotic resistance. *Ann N Y Acad Sci* 2017; **1388**: 108–20.

209 The World Factbook. Washington DC. Central Intelligence Agency. 2017. https://www.cia.gov/library/publications/the-world-factbook/geos/vm.html.

210 Isenbarger DW, Hien BT, Ha HT, *et al.* Prospective study of the incidence of diarrhoea and prevalence of bacterial pathogens in a cohort of Vietnamese children along the Red River. *Epidemiol Infect* 2001; **127**: 229–36.

211 Lee HY, Van Huy N, Choi S. Determinants of early childhood morbidity and proper treatment responses in Vietnam: Results from the Multiple Indicator Cluster Surveys, 2000-2011. *Glob Health Action* 2016; **9**: 1–8.

212 WHO and UN partners. Vietnam: WHO Statistical profile 2015. 2015. http://www.who.int/countries/vnm/en/.

213 Thompson CN, Phan MVTMVT, Hoang NVM, *et al.* A Prospective Multi-Center Observational Study of Children Hospitalized with Diarrhea in Ho Chi Minh City, Vietnam. *Am J Trop Med Hyg* 2015; **92**: 1045–52.

214 Vu Nguyen T, Le Van P, Le Huy C, Nguyen Gia K, Weintraub A. Etiology and epidemiology of diarrhea in children in Hanoi, Vietnam. *Int J Infect Dis* 2006; **10**: 298–308.

215 Forman DW, Tong MJ, Murrell KD, Cross JH. Etiologic study of diarrheal disease in Vietnam. *Am J Trop Med Hyg* 1971; **20**: 598–601.

216 Nishio O, Matsui K, Lan DTP, Ushijima H, Isom S. Rotavirus infection among infants with diarrhea in Vietnam. *Pediatr Int* 2000; **42**: 422–4.

217 Holt KE, Thieu Nga TV, Thanh DP, *et al.* Tracking the establishment of local endemic populations of an emergent enteric pathogen. *Proc Natl Acad Sci U S A* 2013; **110**: 17522–7.

218 Kelly-hope L a, Alonso WJ, Thiem VUD, *et al.* Geographical Distribution and Risk

Factors Associated With Enteric Diseases in Vietnam. 2007; **76**: 706–12.

219 Kozyreva VK, Jospin G, Greninger AL, Watt JP, Eisen J a., Chaturvedi V. Recent Outbreaks of Shigellosis in California Caused by Two Distinct Populations of Shigella sonnei with either Increased Virulence or Fluoroquinolone Resistance. *mSphere* 2016; **1**: 1–18.

220 Kim JS, Kim JJ, Kim SJ, *et a*l. Outbreak of Ciprofloxacin-Resistant Shigella sonnei Associated with Travel to Vietnam, Republic of Korea. Emerg Infect Dis 2015; 21: 1247–50.

221 The HC, Thanh DP, Holt KE, Thomson NR, Baker S. The genomic signatures of Shigella evolution, adaptation and geographical spread. *Nat Rev Microbiol* 2016; **14**: 235–50.

222 Hong Chau TT, Minh Chau NN, Hoang Le NT, *et al.* A Double-blind, Randomized, Placebo-controlled Trial of Lactobacillus acidophilus for the Treatment of Acute Watery Diarrhea in Vietnamese Children. *Pediatr Infect Dis J* 2018; **37**: 35–42.

223 Daltonics B. MALDI Biotyper 3.1 User Manual. 2012; **1**: 1–212.

224 The Clinical and Laboratory Standards Institute. M100S Performance Standards for Antimicrobial Susceptibility Testing. 2016.

225 Wickham H. Hadley Wickham. *Media* 2009; **35**: 211.

226 de Onis M, Onyango AW, Borghi E, Garza C, Yang H. Comparison of the World Health Organization (WHO) Child Growth Standards and the National Center for Health Statistics/WHO international growth reference: implications for child health programmes. *Public Health Nutr* 2007; **9**: 942–7.

227 Fitch A, Fox C, Bauerly K, Gross A, Heim C, Judge-Dietz J, Kaufman T, Krych E, Kumar S, Landin D, Larson J, Leslie D, Martens N, Monaghan-Beery N, Newell T, O'Connor P, Spaniol A, Thomas A WB. Prevention and Management of Obesity for Children and Adolescents. *Inst Clin Syst Improv* 2013; : 10.

228 Thompson CN, Anders KL, Nhi LTQ, *et al.* A cohort study to define the age-specific

incidence and risk factors of Shigella diarrhoeal infections in Vietnamese children: a study protocol. *BMC Public Health* 2014; **14**: 1289.

229 Bashir A, Ph D, Boisen N, *et al.* Origins of the E.coli Strain Causing an Outbreak of Hemolytic Uremic Syndrome in Germany. *N Engl J Med* 2011; **365**: 1–9.

230 Chattaway MA, Jenkins C, Ciesielczuk H, *et al.* Evidence of evolving extraintestinal enteroaggregative escherichia coli ST38 clone. *Emerg Infect Dis* 2014; **20**: 1935–7.

231 Chattaway M a, Schaefer U, Tewolde R, Dallman TJ, Jenkins C. Identification of Escherichia coli and Sequences. *J Clin Microbiol* 2017; **55**: 616–23.

232 Jenkins C, Lawson AJ, Cheasty T, Willshaw G a. Assessment of a real-time PCR for the detection and characterization of verocytotoxigenic Escherichia coli. *J Med Microbiol* 2012; **61**: 1082–5.

233 Frahm E, Obst U. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of Enterococcus spp. and Escherichia coli in water samples. *J Microbiol Methods* 2003; **52**: 123–31.

234 Conway JR, Lex A, Gehlenborg N. UpSetR: An R package for the visualization of intersecting sets and their properties. *Bioinformatics* 2017; **33**: 2938–40.

235 Rabaa M a et al., Tue NT, Phuc TM, *et al.* The Vietnam Initiative on Zoonotic Infections ( VIZIONS ): A Strategic Approach to Studying Emerging Zoonotic Infectious Diseases. *Ecohealth* 2015; **in press**. DOI:10.1007/s10393-015-1061-0.

236 Vu DT, Sethabutr O, Von Seidlein L, *et al.* Detection of Shigella by a PCR assay targeting the ipaH gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. *J Clin Microbiol* 2004; **42**: 2031–5.

237 LaGier MJ, Joseph L a., Passaretti T V., Musser K a., Cirino NM. A real-time multiplexed PCR assay for rapid detection and differentiation of Campylobacter jejuni and Campylobacter coli. *Mol Cell Probes* 2004; **18**: 275–82.

238 Anders KL, Thompson CN, Thuy NT Van, *et al.* The epidemiology and aetiology of

diarrhoeal disease in infancy in southern Vietnam: a birth cohort study. *Int J Infect Dis* 2015; **35**: 3–10.

239 Dung TTN, Phat VV, Nga TVT, *et al.* The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting rotavirus A and norovirus. *J Virol Methods* 2013; **187**: 138–43.

240 Trujillo a A, Mccaustland K a, Zheng D, *et al.* Use of TaqMan Real-Time Reverse Transcription-PCR for Rapid Detection , Quantification , and Typing of Norovirus Use of TaqMan Real-Time Reverse Transcription-PCR for Rapid Detection , Quantification , and Typing of Norovirus. *J Clin Microbiol* 2006; **44**: 1405–12.

241 Youden WJ. Index for rating diagnostic tests. *Cancer* 1950; **3**: 32–5.

242 Corporation P. Wizard ® Genomic DNA Purification Kit Wizard ® Genomic DNA. *Solutions* 2009; : 1123–6.

243 Wood DE, Salzberg SLS, Venter C, *et al.* Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol* 2014; **15**: R46.

244 Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 2008; **18**: 821–9.

245 Yoshida CE, Kruczkiewicz P, Laing CR, *et al.* The salmonella in silico typing resource (SISTR): An open web-accessible tool for rapidly typing and subtyping draft salmonella genome assemblies. *PLoS One* 2016; **11**: 1–17.

246 Hunt M, Mather AE, Sánchez-Busó L, *et al.* ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. *Microb Genomics* 2017. DOI:10.1099/mgen.0.000131.

247 World Health Organization. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. *Geneva, Switz World Heal Organ* 2011; : 1–6.

248 Fletcher SM, McLaws M-L, Ellis JT. Prevalence of gastrointestinal pathogens in developed and developing countries: systematic review and meta-analysis. *J Public health*

*Res* 2013; **2**: 9.

249 Murray CJL, Vos T, Lozano R, *et al.* Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2197–223.

250 Harrison JW, Dung TTN, Siddiqui F, *et al.* Identification of possible virulence marker from campylobacter jejuni isolates. *Emerg Infect Dis* 2014; **20**: 1026–9.

251 Onis Bm de, Blössner M. WHO Global Database on Child Growth and Malnutrition. *World Heal Organ* 2016; **25**: 228–34.

252 Collignon PC, Conly JM, Andremont A, McEwen SA, Aidara-Kane A, members of the World Health Organization Advisory Group B meeting on IS of AR (WHO-A. World Health Organization Ranking of Antimicrobials According to their Importance in Human Medicine: A Critical Step for Developing Risk Management Strategies to Control Antimicrobial Resistance from Food Animal Production. *Clin Infect Dis* 2016; : 1–22.

253 Jean SS, Hsueh PR. High burden of antimicrobial resistance in Asia. *Int J Antimicrob Agents* 2011; **37**: 291–5.

254 Meng CY, Smith BL, Bodhidatta L, *et al.* Etiology of diarrhea in young children and patterns of antibiotic resistance in Cambodia. *Pediatr Infect Dis J* 2011; **30**: 331–5.

255 Pathania M, Rathaur VK, Pathania M, Jayara A, Yadav N. Clinical study of acute childhood diarrhoea caused by bacterial enteropathogens. *J Clin Diagnostic Res* 2014; **8**: 1–5.

256 Zhang H, Pan F, Zhao X, *et al.* Distribution and antimicrobial resistance of enteric pathogens in Chinese paediatric diarrhoea: a multicentre retrospective study, 2008-2013. *Epidemiol Infect* 2015; : 1–8.

257 Langendorf C, Le Hello S, Moumouni A, *et al.* Enteric Bacterial Pathogens in Children with Diarrhea in Niger: Diversity and Antimicrobial Resistance. *PLoS One* 2015; **10**: e0120275.

258 Pollett S, Rocha C, Zerpa R, *et al.* Campylobacter antimicrobial resistance in Peru: a ten-year observational study. *BMC Infect Dis* 2012; **12**: 1.

259 Chung The H, Rabaa M a, Pham Thanh D, *et al.* South Asia as a Reservoir for the Global Spread of Ciprofloxacin-Resistant Shigella sonnei: A Cross-Sectional Study. *PLoS Med* 2016; **13**: e1002055.

260 Traa BS, Fischer Walker CL, Munos M, *et al.* Antibiotics for the treatment of dysentery in children. *Int J Epidemiol* 2010; **39**: 70–4.

261 Liu L, Johnson HL, Cousens S, *et al.* Global, regional, and national causes of child mortality: An updated systematic analysis for 2010 with time trends since 2000. *Lancet* 2012; **379**: 2151–61.

262 Yu J, Jing H, Lai S, *et al.* Etiology of diarrhea among children under the age five in China: results from a five-year surveillance. *J Infect* 2015; published online March. DOI:10.1016/j.jinf.2015.03.001.

263 Nguyen T, Van P Le, Huy C Le. Detection and characterization of diarrheagenic Escherichia coli from young children in Hanoi, Vietnam. *J Clin …* 2005; **43**: 755–60.

264 Uppal B, Perween N, Aggarwal P, Kumar SK. A comparative study of bacterial and parasitic intestinal infections in India. *J Clin Diagn Res* 2015; **9**: DC01-4.

265 Shah M, Kathiiko C, Wada A, *et al.* Prevalence, seasonal variation, and antibiotic resistance pattern of enteric bacterial pathogens among hospitalized diarrheic children in suburban regions of central Kenya. *Trop Med Health* 2016; **44**: 39.

266 Iijima Y, Oundo JO, Hibino T, *et al.* High prevalence of diarrheagenic *Escherichia coli* among children with diarrhea in Kenya. *Jpn J Infect Dis* 2016. DOI:10.7883/yoken.JJID.2016.064.

267 Hien BTT, Scheutz F, Cam PD, *et al.* Diarrheagenic Escherichia coli and Shigella strains isolated from children in a hospital case-control study in Hanoi, Vietnam. *J Clin Microbiol* 2008; **46**: 996–1004.

268 Tobias J, Kassem E, Rubinstein U, *et al.* Involvement of main diarrheagenic Escherichia coli, with emphasis on enteroaggregative E. coli, in severe non-epidemic pediatric diarrhea in a high-income country. *BMC Infect Dis* 2015; **15**: 1–7.

269 Čobeljić M, Miljković-Selimović B, Paunović-Todosijević D, *et al.* Enteroaggregative Escherichia coli associated with an outbreak of diarrhoea in a neonatal nursery ward. *Epidemiol Infect* 1996; **117**: 11–6.

270 Itoh Y, Nagano I, Kunishima M, Ezaki T. Laboratory Investigation of Enteroaggregative Escherichia coli O Untypeable : H10 Associated with a Massive Outbreak of Gastrointestinal Illness. *J Clin Microbiol* 1997; **35**: 2546–50.

271 Smith H, Cheasty T, Rowe B. Enteroaggregative Escherichia coli and outbreaks of gastroenteritis in UK. *Lancet* 1997; **350**: 814–5.

272 Pai M, Kang G, Ramakrishna BS, Venkataraman A, Muliyil J. An epidemic of diarrhoea in south India caused by enteroaggregative Escherichia coli. *Indian J Med Res* 1997; **106**: 7–12.

273 Rohde H, Qin J, Cui Y, *et al.* Open-source genomic analysis of Shiga-toxin-producing E. coli O104:H4. *N Engl J Med* 2011; **365**: 718–24.

274 Lima AAM, Soares AM, Filho JQS, *et al.* Enteroaggregative Escherichia coli Subclinical Infection and Coinfections and Impaired Child Growth in the MAL-ED Cohort Study. *J Pediatr Gastroenterol Nutr* 2018; **66**: 325–33.

275 Valentini D, Vittucci a C, Grandin a, *et al.* Coinfection in acute gastroenteritis predicts a more severe clinical course in children. *Eur J Clin Microbiol Infect Dis* 2013; **32**: 909–15.

276 Zhang S-X, Zhou Y-M, Xu W, *et al.* Impact of co-infections with enteric pathogens on children suffering from acute diarrhea in southwest China. *Infect Dis poverty* 2016; **5**: 64.

277 Ochoa TJ, Ecker L, Barletta F, *et al.* Age-Related Susceptibility to Infection with Diarrheagenic Escherichia coli among Infants from Periurban Areas in Lima, Peru. *Clin Infect Dis* 2009; **49**: 1694–702.

278 Shrivastava AK, Kumar S, Mohakud NK, Suar M, Sahu PS. Multiple etiologies of infectious diarrhea and concurrent infections in a pediatric outpatient-based screening study in Odisha, India. *Gut Pathog* 2017; **9**: 16.

279 Araujo JM, Tabarelli GF, Aranda KRS, *et al.* Typical enteroaggregative and atypical enteropathogenic types of Escherichia coli are the most prevalent diarrhea-associated pathotypes among Brazilian children. *J Clin Microbiol* 2007; **45**: 3396–9.

280 Quiroga M, Oviedo P, Chinen I, *et al.* Asymptomatic infections by diarrheagenic Escherichia coli in children from misiones, Argentina, during the first twenty months of their lives. *Rev Inst Med Trop Sao Paulo* 2000; **42**: 9–15.

281 Byrne L, Jenkins C, Launders N, Elson R, Adak GK. The epidemiology, microbiology and clinical impact of Shiga toxin-producing Escherichia coli in England, 2009–2012. *Epidemiol Infect* 2015; : 1–13.

282 Osawa K, Raharjo D, Wasito EB, *et al.* Frequency of diarrheagenic Escherichia coli among children in Surabaya, Indonesia. *Jpn J Infect Dis* 2013; **66**: 446–8.

283 Elsafi SH, Al-Maqati TN, Hussein MI, Adam A a., Hassan MMA, Al Zahrani EM. Comparison of microscopy, rapid immunoassay, and molecular techniques for the detection of Giardia lamblia and Cryptosporidium parvum. *Parasitol Res* 2013; **112**: 1641–6.

284 Khare R, Espy MJ, Cebelinski E, *et al.* Comparative evaluation of two commercial multiplex panels for detection of gastrointestinal pathogens by use of clinical stool specimens. *J Clin Microbiol* 2014; **52**: 3667–73.

285 Wessels E, Rusman LG, van Bussel MJ a. WM, Claas ECJ. Added value of multiplex Luminex Gastrointestinal Pathogen Panel (xTAG® GPP) testing in the diagnosis of infectious gastroenteritis. *Clin Microbiol Infect* 2014; **20**: O182–7.

286 Perry MD, Corden SASA, Howe RARA. Evaluation of the Luminex xTAG Gastrointestinal Pathogen Panel and the Savyon Diagnostics Gastrointestinal Infection

Panel for the detection of enteric pathogens in clinical samples. *J Med Microbiol* 2014; **63**: 1419–26.

287 Vocale C, Rimoldi SG, Pagani C, *et al.* Comparative evaluation of the new xTAG® GPP multiplex assay in the laboratory diagnosis of acute gastroenteritis. Clinical assessment and potential application from a multicentric Italian study. *Int J Infect Dis* 2015; **34**: 33–7.

288 Liu J, Kabir F, Manneh J, *et al.* Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study. *Lancet Infect Dis* 2014; **14**: 716–24.

289 Checkley W, White a C, Jaganath D, *et al.* A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium. *Lancet Infect Dis* 2015; **15**: 85–94.

290 Guerrant DI, Moore SR, Lima A a, Patrick PD, Schorling JB, Guerrant RL. Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four-seven years later in a poor urban community in northeast Brazil. *Am J Trop Med Hyg* 1999; **61**: 707–13.

291 Bryant K, McDonald LC. Clostridium difficile infections in children. *Pediatr Infect Dis J* 2009; **28**: 145–6.

292 Larson HE, Barclay FE, Honour P, Hill ID. Epidemiology of Clostridium difficile in infants. *J Infect Dis* 1982; **146**: 727–33.

293 Al Jumaili IJ, Shibley M, Lishman a. H, Record CO. Incidence and origin of Clostridium difficile in neonates. *J Clin Microbiol* 1984; **19**: 77–8.

294 Hecker MT, Riggs MM, Hoyen CK, Lancioni C, Donskey CJ. Recurrent infection with epidemic Clostridium difficile in a peripartum woman whose infant was asymptomatically colonized with the same strain. *Clin Infect Dis* 2008; **46**: 956–7.

295 Goldenberg SD, Bacelar M, Brazier P, Bisnauthsing K, Edgeworth JD. A cost benefit analysis of the Luminex xTAG Gastrointestinal Pathogen Panel for detection of infectious

gastroenteritis in hospitalised patients. *J Infect* 2014; **44**: 504–11.

296 Ryan MP, O'Dwyer J, Adley CC. Evaluation of the Complex Nomenclature of the Clinically and Veterinary Significant Pathogen Salmonella. *Biomed Res Int* 2017; **2017**. DOI:10.1155/2017/3782182.

297 Chen HM, Wang Y, Su LH, Chiu CH. Nontyphoid Salmonella infection: Microbiology, clinical features, and antimicrobial therapy. *Pediatr Neonatol* 2013; **54**: 147–52.

298 World Health Organization. Salmonella ( non-typhoidal ). 2017; : 1–4.

299 Feasey N a., Dougan G, Kingsley R a., Heyderman RS, Gordon M a. Invasive non-typhoidal salmonella disease: An emerging and neglected tropical disease in Africa. *Lancet* 2012; **379**: 2489–99.

300 Duong VT, Tuyen HT, Minh P Van, *et al*. No clinical benefit of empirical antimicrobial therapy for pediatric diarrhea in a high usage, high resistance setting. *Clin Infect Dis* 2017; : 1–28.

301 Redgrave LS, Sutton SB, Webber MA, Piddock LJ V. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary succes. *Trends Microbiol* 2014; **22**.

302 Magnet S, Courvalin P. Activation of the Cryptic aac ( 6 ′ ) -Iy Aminoglycoside Resistance Gene of Salmonella by a Chromosomal Deletion Generating a Transcriptional Fusion Activation of the Cryptic aac ( 6 J ) -Iy Aminoglycoside Resistance Gene of Salmonella by a Chromosomal De. 1999; **181**: 6650–5.

303 Maraki S, Papadakis IS. Serotypes and antimicrobial resistance of human nontyphoidal isolates of salmonella enterica from crete, Greece. *Interdiscip Perspect Infect Dis* 2014; **2014**. DOI:10.1155/2014/256181.

304 Abbassi-Ghozzi I, Jaouani a., Aissa RB, Martinez-Urtaza J, Boudabous a., Gtari M. Antimicrobial resistance and molecular analysis of non-typhoidal Salmonella isolates from human in Tunisia. *Pathol Biol* 2011; **59**: 207–12.

305 Gharieb RM, Tartor YH, Khedr MHE. Non-Typhoidal Salmonella in poultry meat and diarrhoeic patients: prevalence, antibiogram, virulotyping, molecular detection and sequencing of class I integrons in multidrug resistant strains. *Gut Pathog* 2015; **7**: 34.

306 Mather AE, Reid SWJ, Maskell DJ, Parkhill J, Fookes MC, Harris SR. Europe PMC Funders Group Distinguishable Epidemics Within Different Hosts of the Multidrug Resistant Zoonotic Pathogen Salmonella Typhimurium. 2014; **341**: 1514–7.

307 Jones T. Overutilization of Antibiotics in Children With Diarrhea: First Do No Harm. *Clin Infect Dis* 2018; **66**: 512–3.

308 Ido N, Lee KI, Iwabuchi K, *et al.* Characteristics of Salmonella enterica serovar 4,[5],12:i:- As a monophasic variant of serovar Typhimurium. *PLoS One* 2014; **9**. DOI:10.1371/journal.pone.0104380.

309 Folster JP, Campbell D, Grass J, *et al.* Identification and characterization of multidrug-resistant Salmonella enterica serotype Albert isolates in the United States. *Antimicrob Agents Chemother* 2015; **59**: 2774–9.

310 Mather AE, Vaughan TG, French NP. Molecular approaches to understanding transmission and source attribution in nontyphoidal salmonella and their application in Africa. *Clin Infect Dis* 2015; **61**: S259–65.

311 McDermott PF, Tyson GH, Kabera C, *et al.* Whole-Genome Sequencing for Detecting Antimicrobial Resistance in Nontyphoidal Salmonella. *Antimicrob Agents Chemother* 2016; **60**: 5515–20.

312 Mather AE, Phuong TLT, Gao Y, *et al.* New Variant of Multidrug-Resistant Salmonella enterica Serovar Typhimurium Associated with Invasive Disease in Immunocompromised Patients in Vietnam. *MBio* 2018; **9**. DOI:10.1128/mBio.01056-18.

# 9. Appendices

## 9.1. Protocol of 12EN study

**PROTOCOL**

OXTREC No: 1045-13

Protocol No: 12EN

Version: Protocol V2.3 18AUG15

**Project**

The phylogeography and humoral immune responses of *Shigella* in children attending three hospitals in Ho Chi Minh City, Vietnam

**Principal Investigators:**

Dr Stephen Baker (OUCRU) sbaker@oucru.org

Dr Lu Lan Vi (Lead Investigator HTD)

**Other Investigators**:

Dr Hoang Le Phuc (Lead Investigator CH1), Dr Nguyen Minh Ngoc (Lead Investigator CH2), Ms Corinne Thompson (OUCRU), Dr Ha Vinh (HTD), Dr Nguyen Phu Huong Lan (HTD), Dr Nguyen Van Vinh Chau (HTD), Dr Vu Thuy Duong (CH1), Dr Ton Thi Thanh Ha (CH1), Dr Tang Le Chau Ngoc (CH2), Dr Nguyen Thi Ngoc Anh (CH2), Dr Nguyen Thanh Huong (CH2).

**Collaborating Institutions and Departments:**

Oxford University Clinical Research Unit, HCMC Vietnam

Children's Hospital No. 1, HCMC, Vietnam

Children's Hospital No. 2 HCMC, Vietnam

The Hospital for Tropical Diseases, HCMC, Vietnam

**Proposed Start Date**

May 2014

## 1. Introduction

This prospective investigation is designed to study the epidemiology, microbial population structure and host immune response of *Shigella* infections in children in Ho Chi Minh City (HCMC), Vietnam and the surrounding provinces. The overall aim of this study is understand the distribution and evolution of *Shigella* in Vietnam and to study the immunological responses and genetic susceptibilities to this infection. We know that *Shigella* has undergone a dramatic shift in Vietnam over the last fifteen years; *Shigella sonnei* (*S. sonnei*) has replaced *Shigella flexneri* (*S. flexneri*) as the leading cause of bloody and/or mucoid diarrhoea. We hypothesise that this shift is on-going and being driven by the emergence of organisms that exhibit resistance to antimicrobials. This species shift and disease burden highlights the potential need for specific *S. sonnei* vaccines. We additionally hypothesise that by studying the acute antibody response to novel *Shigella* antigens, we can identify potential vaccine candidates. This study will provide evidence for the changing epidemiology and bacteriology of *Shigella* in HCMC and the surrounding provinces, provide plasma and host DNA to allow us to understand the nature of the acute immune response to *Shigella* infections and investigate human genetic associations with disease and immunological responses.

## 2. Background

Diarrhoea remains a major cause of childhood morbidity and mortality globally[1,2], with the vast majority of the 2 billion annual infections and 2.5 million resultant deaths occurring in low and middle-income countries[3,4]. 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*)[5], are amongst the most common causes of dysenteric diarrhoea, with >164 million infections annually[6]. However, the burden of *Shigella* is disproportionately distributed amongst the species. *S. flexneri* has historically been responsible for the majority of disease in developing countries[7,8] , whilst *S. sonnei* has dominated in developed countries[9–11]. This pattern is changing, however, as *S. sonnei* is replacing *S. flexneri* as the primary agent of dysentery in many countries[8,12,13].

As diarrhoeal aetiology is not routinely evaluated in Vietnam, we performed a yearlong cross-sectional study of 1,500 cases of hospitalised diarrhoea in 2009-2010 (09AV study - A Descriptive and Molecular Epidemiological Study of Children with Diarrhoeal Disease in three Hospitals in HCMC, Vietnam - Protocol No: CTU09AVDEC08). Data originating from the 09AV study demonstrated that *S. sonnei* is undergoing a local clonal expansion across Vietnam

and we hypothesise the majority of this evolution is occurring in HCMC, driven predominantly by antimicrobial resistance. These data also suggest that this clonal expansion is occurring in regular time intervals and may be restricted to specific areas of HCMC. These data are published and have been combined with a global *S. sonnei* phylogeny, providing a key framework on which this current project is built[14].

Factors determining the distribution of the *Shigella* species are not understood but the *S. flexneri* / *S. sonnei* ratio is related to the economic status of the population[5]. The on-going collaborative work between OUCRU, CH1, CH2 and HTD in HCMC, Vietnam, has reaffirmed this association, demonstrating a transition from *S. flexneri* to *S. sonnei* over the past 15 years[13]. 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[15,16]. Yet, the increasing global dominance of *S. sonnei* is associated with resistance to last-resort antimicrobials such as fluoroquinolones and 3rd generation cephalosporins[17,18].

The *Shigellae* are members of the large bacterial family *Enterobacteriaceae*, they are Gram-negative, non-motile (aflagellated) rods and are closely related to *Escherichia coli*[19]. Once ingested, the organism invades the gut epithelial layer from the intestinal lumen through M-cells and is able to multiply intracellularly to then invade neighbouring enterocytes by polymerising actin and spreading cell-to-cell[20–22]. This ability to damage the gut mucosal surface results in host cell death and the characteristic mucosal ulcerations of dysentery[19,23]. After an incubation period of 12 hours to approximately 2 days, symptoms of shigellosis appear abruptly and include a short period of watery diarrhoea with intestinal cramps and general malaise, followed by fever, tenesmus and eventual emission of bloody, often mucopurulent stools[24,25]. While most healthy patients recover, *Shigella* infection can lead to complications. As antimicrobial resistance of *Shigella* to ampicillin, co-tromixazole and nalidixic acid is now widespread, the World Health Organisation (WHO) currently recommends ciprofloxacin for the treatment of shigellosis as first-line therapy[26]. Pivmecillinam, ceftriaxone and azithromycin are recommended as second-line therapies if resistance to ciprofloxacin is identified[26].

More than 100 years after the discovery of the agent of shigellosis, there is still neither a licensed vaccine nor agreement on the mechanisms of host immunity to *Shigella*[25]. Effective prototype vaccines have been prepared for oral use in humans but none to date have been both sufficiently

effective and safe[27]. There is agreement that vaccination still offers the greatest hope of an effective and sustainable strategy to control shigellosis[25]. Since type-specific immunity has been demonstrated in humans, vaccine protection to *Shigella* must also be serotype specific. However, serotype prevalence varies considerably in different regions of the world and is unknown in many areas[28,29]. This necessitates either tailor-made serotype combinations to suit each location specifically[29–31], or a global vaccine that protects against particularly common serotypes[27,32]. An alternate approach is to search for an antigen common to all pathogenic *Shigella* serotypes[8], which has shown promise in recent studies[33,34]. Vaccine development against *Shigella* has followed two strategies: the development of a conjugate vaccine in which *Shigella* polysaccharides are covalently linked to carrier proteins[35,36], and the development of live oral vaccines based on attenuated derivatives of wildtype *Shigella* spp.[37–39]. One of the most recently advanced vaccines relies on conjugation and has been shown to be safe in adults and young children, although efficacy levels are largely insufficient[40,41]. An ideal *Shigella* vaccine would be administered as a single dose infant vaccine, integrated into the Expanded Programme for Immunisation (EPI)[25,42]. However, the development of enteric vaccines that are able to induce a sufficient mucosal immune response in children in developing countries is a challenging exercise[43].

Advances in genotyping and sequencing technologies, and the availability of human genome sequence data, makes it feasible and valuable to investigate genetic variation in large numbers of individuals. Host genetics studies of *Shigella* are important to gain a more complete understanding of the disease process. Identification of susceptibility or resistance genes to dysentery within human populations provides insight into the interaction between the human host and bacterial pathogens. Currently, there are no data on how host genetics may play a role in susceptibility to enteric bacteria such as *Shigella*. OUCRU has a strong background in performing host genetics analyses to understand how specific mutations in host genes may correspond with an increased susceptibility with infection. Therefore, we hypothesise that patients with bloody and/or mucoid diarrhoea may have a genetic susceptibility for severe diarrhoea. This study will collect DNA from all subjects for future genetic screening. Control human DNA for these analyses will be provided from other OUCRU studies that have recruited members of the population into genetic association studies i.e. Control human DNA samples (and data) for genetic association studies are already available and have IRB approval.

In summary, there is a growing necessity to control *S. sonnei* and investigate the dramatic emergence of this pathogen in industrialising countries. Yet, there are extensive knowledge gaps regarding disease distribution, incidence and exposure. Furthermore, there is a limited understanding of the effect of uncontrolled antimicrobial usage on the international spread and dynamics of pathogens like *Shigella*. Vaccines and diagnostics are in development[35,37,38,44], but are compromised by a lack of understanding of the mechanisms behind the emergence of the immune response specifically against *Shigella* in children[27]. We aim to study the local evolution of *Shigella* in HCMC and surrounding provinces, study immunological responses to inform intelligent vaccine design and perform future host genetics studies to investigate genetic associations for diarrhoea with mucus and/or blood in stool.

**3. Study aims and outcomes**

This is an epidemiological study, designed to investigate the distribution, epidemiological characteristics, genetics (human and bacterial) and humoral immune response of *Shigella* and other agents of bloody and/or mucoid diarrhoea in HCMC and in the surrounding provinces. Our main interest lies in studying *Shigella* and other causative agents of diarrhoea with mucus and/or blood in stool among children attending hospital under the age of 16 years in this setting. This study will be performed over a three-year period starting in May 2014. We aim to investigate the spatial distribution of pathogens and disease, determine spatiotemporal hotspots of infection and identify regions were the organism is undergoing adaptive evolution in the city. Spatially stratified data will be used to the highlight relationships between infection and socio-economic status of affected households and communities. Additionally, blood will be taken from all enrolees to investigate the acute humoral immune response to *Shigella* O-antigen and a range of other *Shigella* antigens to evaluate immunogenicity. Patients with dysenteric aetiologies other than *Shigella* will be used as comparators. DNA will be collected and stored from all enrolled subjects to perform future genetic association studies for *Shigella* infections and dysenteric disease.

One of the aims of this project is to measure the acute serological responses induced during a *Shigella* infection in children. The hope is that these data will identify novel immunogenic proteins that may become candidate vaccines for *Shigella*. Whilst this study is a rational and vital step for identifying potential *Shigella* vaccine candidates it is unlikely that it will lead to a new *Shigella* vaccine directly; blood from these patients will be used to identify serological responses, but any potential vaccine (or *Shigella* intervention) will not be derived directly from these

samples. As there is currently no way of knowing what proteins may be immunogenic, this study will allow us to test the hypothesis that potential vaccine candidates can be identified in this manner. If we are able to identify immunogenic proteins during this project that may have potential as vaccine targets, then these will be patented by the principal investigator with all named contributing sites named on the patent application.

**3.1. Primary outcomes**

1. All *Shigella* isolates will be genome sequenced. These genetic data will be used to study the phylogeny of *Shigella* in HCMC and in the surrounding provinces and to investigate geographical disease associations, infer potential transmission networks, and identify potential pockets of microevolution.

**3.2. Secondary outcomes**

1. Using location data we will identify high-risk areas for *S. sonnei* and other agents of bloody and/or mucoid diarrhoea in HCMC and in the surrounding provinces.
2. Acute and follow up plasma samples with corresponding metadata that will be screened by serology to investigate the acute immune response and identify potential *Shigella* vaccine candidates.
3. DNA from patients with bloody and/or mucoid diarrhoea that will be used to investigate host genetic susceptibilities for dysenteric disease. These data will be compared to data from cross-sectional human genetics data that is already available for the Vietnamese population.

**3.3. Future outcomes after analysis**

1. A comprehensive understanding of the microbiological and genetic makeup and the temporal and phylogenetic relationships of *S. sonnei* in HCMC and in the surrounding provinces
2. Epidemiological data regarding the cause and seasonality of the various agents of bloody and/or mucoid diarrhoea
3. A data resource that will be made available to CH1, CH2, HTD, public health authorities in HCMC and in the surrounding provinces, NIHE, and the Vietnamese MoH
4. A measure of evolutionary rate, antimicrobial resistance development and the role of the accessory genome in the local evolution of *S. sonnei*
5. Data on the stability of potential vaccine candidates and loci that may be under selective pressure in *S. sonnei* in this population

6. ICD-10 data from all hospital admissions and out-patients within the same age group that can be denominator for temporal and spatial analyses of bloody and/or mucoid diarrhoea.

## 4. Research plan

### 4.1. Study design

A prospective, descriptive study over a period of three years

### 4.2. Study sites

1. Oxford University Clinical Research Unit
2. The Gastrointestinal Department and Out-Patient Department of Children's Hospital No. 1
3. The Gastrointestinal Department, Service Department 2, and Out-Patient Department of Children's Hospital No. 2
4. The Paediatric Ward B of The Hospital for Tropical Diseases

### 4.3. Hospital recruitment

To study the distribution and the evolution of *S. sonnei* across HCMC and in the surrounding provinces, with the assistance of clinical staff on the gastrointestinal wards, we are to enrol as many children (aged <16 years) with bloody and/or mucoid diarrhoea attending CH1, CH2, and HTD over a three-year period as possible. Retrospective data suggests that there are approximately 2,000 admissions for bloody diarrhoea in these three hospitals per year (from routine ICD-10 data from CH1, CH2 and HTD). This will result in approximately 6,000 bloody diarrhoeal episodes over the study period. This is a conservatively estimated sample size as it does not include estimated counts of admissions due to mucoid diarrhoea (which are not as common). Therefore, we aim to enrol all children from the participating centres, regardless of their province or town/city of origin. This study will also provide a diagnostic component to studying the cause or these infections.

### 4.4. Methods

#### 4.4.1. Inclusion criteria

**All children** from birth to 16 years of age with acute diarrhoea with visible blood and/or mucus in stool attending Children's Hospital No.1 (CH1), No.2 (CH2) and the Hospital for Tropical Diseases (HTD) will be eligible to participate in this study. Diarrhoeal disease is defined as a history of 1 or more loose stools (a loose stool being one that would take the shape of a stool container) (according to the definition of the WHO). Bloody and/or mucoid diarrhoea is diarrhoea

containing visible blood and/or mucus [45]. Children need to have bloody and/or mucoid diarrhoea within 72 hours of admission to hospital or whilst in hospital to be enrolled. Only those children with prior consent from a parent or guardian will be entered in the study.

#### 4.4.2. Exclusion criteria

Patients will not be recruited if they have suspected or confirmed intussusception at the time of enrolment.

### 4.5. Study size

As this is descriptive study the sample size is projected to be as many patients as is possible to recruit. From previous work, of the >2,000 annual cases of bloody diarrhoea reported to the three study sites annually, 20% of these are estimated to be due to *S. sonnei* [46]. Assuming a conservatively high non-consent rate of 10%, approximately 1,000 *S. sonnei* will be isolated over the 3 years from a total of 6,000 bloody and/or mucoid diarrhoea patients. From our previous genomics work, we estimate that 300 strains per year should provide sufficient statistical power to study the evolutionary rate throughout the districts of HCMC and provide adequate spatial resolution to identify areas with increased spatial risk of *Shigella* infections. Owing to biases induced by hospital referral patterns there may be insufficient power to study the distribution of dysenteric disease throughout the provinces surrounding HCMC. This study will provide pilot data on dysenteric disease in the provinces around HCMC.

### 4.6. Sampling

Due to feasibility, samples will be collected from the collaborating sites from Monday to Saturday, but patients will be recruited on all seven days of the week. Samples collected over the weekend will be transported to OUCRU laboratory on the following Monday.

### 4.7. Data entry and storage

Metadata, in the form of an electronic CRF (eCRF)/questionnaire or paper CRF/questionnaire (depending on site), will be recorded on a tablet on the day of recruitment with data being sent immediately to the server at OUCRU through a 3G internet connection. No information will be retained on the tablet once it is sent to the server. Only the named investigators or their designee(s) will have access to this information. Other investigators from CH1, CH2 and HTD will be updated regularly and will be granted access to data when requested. Patients will not be identified by their names.

### 4.8. Observations, investigations and data collection

#### 4.8.1. Flow of study procedures

1. Enrolment (at sites)
    a. Screening
    b. Informed consent
    c. Enrolment
2. Sample collection – Enrolment Day (at sites)
    a. Stool sample for culture and PCR (essential for all enrolees)
    b. Blood sample for optional CBC (if clinically indicated), serology and host genetics (essential for all enrolees). For children <6 months of age, 1 ml will be collected. For children ≥6 months, 2ml will be collected.
3. Data collection – Enrolment Day
    a. Completion of CRF/questionnaire (at sites)
        i. ICD-10 diagnostic code at admission or out-patient clinic
        ii. Phone number
        iii. Address (Town/City, District, ward)
        iv. Clinical information
        v. Epidemiological information
4. Microscopy, stool culturing and bacterial identification
    a. Microscopy
        i. Parasites
        ii. Red blood cells
        iii. White blood cells
    b. Stool culture for
        *i. Shigella*
        *ii. Salmonella*
        *iii. Campylobacter*
        iv. Pathogenic forms of *E. coli*
        v. Others
5. Antimicrobial susceptibility patterns
    a. Susceptibility testing to 11 antimicrobials by disc diffusion and/or MIC
6. DNA and RNA extraction
    a. Nucleic acid extraction from stool samples

i. PCR for Shigella and Salmonella
ii. Rotavirus and norovirus if requested
iii. 16s (Future work)

7. Blood separation
    a. Cells for future host genetics studies
    b. Plasma for serology
8. Sample storage
    a. Stool sample (-80$^{o}$C)
    b. Bacterial isolates (-80$^{o}$C and freeze dried)
    c. Nucleic acid from stool samples (-20$^{o}$C)
    d. Human blood cells for nucleic acid extraction (-20$^{o}$C)
    e. Plasma samples for serology (-20$^{o}$C)
9. Follow up
    a. After 28 days for all patients enrolled in this study (only if patient agrees to return)
        i. Blood for serology/host genetics in EDTA tube (1ml)
        ii. Rectal swab for *Shigella* PCR
10. Outcome
    a. Outcome will be recorded for all patients at 72hrs after presentation
        i. A study nurse will assess outcome (in patients) or a study nurse will call the parent/guardian of the child (out-patients). Outcome will fall under the following criteria.
            1. Recovered
            2. Diarrhoea improved (less episodes/no blood or mucus)
            3. Diarrhoea not-improved (same episodes/severity)
            4. Infection more severe (more episodes/still blood or mucus)
            5. Other
            6. Not Known
11. ICD-10 diagnostic data for all hospital admissions and out-patients
    a. Over the same period as a denominator for bloody diarrhoea as well as historic retrospective data from previous years as available

**4.8.2. Screening, informed consent and enrolment**

**4.8.2.1. Screening**

1. Potential patients will be identified in the outpatient clinic or on the gastrointestinal ward of each participating hospital according to standard operating procedures at each site.
2. Patients will be screened for eligibility according to the inclusion/exclusion criteria based on available clinical information only.

#### 4.8.2.2. Informed consent

**1.** No research procedures will be undertaken before the patient agrees to enrol in the study and signs an informed consent form. Consent is required from a parent, guardian or family member. If patients meet the criteria for enrolment, the parent, guardian or family member will be invited to discuss the details of the study. The study staff will give the patient/representative a copy of the informed consent form and explain the details of the study including the study procedures, risks and benefits, financial and confidentiality considerations, alternatives to participating and how to obtain more information.

**2.** The study staff will invite the parent, guardian or family member to ask questions and will endeavour to ensure that they understand the information given. The study staff will then ask the parent, guardian or family member to consider study participation. Participants will have time to consider participation after hospital admission. Participants enrolled at the out-patient clinic will be eligible for the duration of the visit.

**3.** Those who consent to the study will sign and date two copies of the informed consent form. The study staff will sign and date the two copies.

**4.** If the parent, guardian or family member is illiterate, a witness who is not a member of the study staff will be present during the informed consent discussion. The informed consent form will be read to the patient/representative in the presence of the witness. If the patient/representative agrees to participate, the form will be signed and dated by the witness. In this case, the participant or their representative will still be invited to make a mark on the signature line of the form.

#### 4.8.2.3. Enrolment

All patients will receive the best available standard of care, regardless of enrolment. Those who refuse consent will not have any study related procedures performed. Only consented patients will be enrolled.

### 4.8.3. Sample collection

#### 4.8.3.1. Stool samples

Stool specimens will be collected in sterile containers as soon as possible on the day of hospital admission or attendance at the Out Patient Department. If possible, samples should be collected before hospital prescribed antimicrobial treatment is started. However, a stool sample may also be collected after treatment is started. A total of 2-5 ml of stool must be collected by the study nurse/clinician in a sterile container (a rectal catheter may be used if recommended by study site). A collection form must be completed for each sample, and the patient will be given a unique personal identification number, which will be written on the sample collection container, the collection form, and the CRF/questionnaire. All participants will be anonymous and identified only by the ID number in the database. All collected samples from each hospital should be stored at room temperature (or 4 degrees in the ward refrigerator) and will be transported on the same day (apart from Sundays, whose samples will be transported on the following Monday) to OUCRU Microbiology laboratory by a member of OUCRU staff for analysis. The stool samples will be subjected to classical microbiological bacterial identification and parasite microscopy. Two aliquots of each stool sample will be stored at -80$^{o}$C as 10% suspensions in distilled PBS for batched viral identification and secondary analysis.

#### 4.8.3.2. Blood samples

The blood sample will be taken with a single needle puncture. A total of 2ml will be collected from all children > six months of age and 1ml for children < six months of age. Blood will be used for CBC at the hospital site if requested by the treating clinician. Any remaining blood sample will be used for research purposes.

### 4.9. Data collection

#### 4.9.1. Questionnaire

At enrolment a study nurse will ask questions of the child's parent or guardian and complete a simple questionnaire in electronic tablet or paper format (depending on site preferences).

#### 4.9.2. Case report form

To understand the symptoms and duration of disease, the treating clinician or study nurse will complete a basic standard Case Report Form (CRF).

### 4.10. Microscopy, stool culturing and bacterial identification

#### 4.10.1. Parasite screening

For parasitic examination, a fresh smear of faecal specimens will be prepared in phosphate buffered saline; 10µl of this solution will be examined at 400 times magnification to identify *Giardia lamblia, Entamoeba histolytica* at the OUCRU microbiology laboratory. *Cryptosporidium* cysts will be detected by ZN stain.

**4.10.2. Blood cells screening**

A fresh faecal smear of each sample will be screened under microscope at the OUCRU microbiology laboratory (HPF (× 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 cells/HPF, scale + = 1 to 10 cells/HPF, scale ++ = 11 to 20 cells/HPF, scale +++ = 21 to 30 cells/HPF and scale ++++ = >40 cells/HPF.

**4.10.3. Bacterial culture**

Culturing and Bacterial Identification will be performed at OUCRU Microbiology department. Stool specimens 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*, and *Escherichia coli*. The MC, XLD, and Selenite broth will be incubated at 37$^{o}$C overnight and the Campylobacter plates will be incubated micro-aerophically at 42$^{o}$C. Identification of potential pathogens will be performed using conventional biochemical (API E20 tests) or by VITEK automated machine 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 data.

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$^{o}$C at OUCRU. Samples will be subjected to PCR to identify pathogenic *E. coli* variants after DNA extraction.

**4.11. Antimicrobial susceptibility testing**

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 or by VITEK automated machine. Antimicrobial susceptibilities tests will be performed against nalidixic acid,

ciprofloxacin, ceftriaxone, ceftazidime, augmentin, ampicillin, trimethoprim-sulfamethoxazole, azithromycin, chloramphenicol, imipenem 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.

### 4.12. Isolation of nucleic acids and PCR

#### 4.12.1. 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.

#### 4.12.2. Bacterial PCR

To potentially increase the diagnosis rate of *Shigella* and *Salmonella* a multiplex real-time PCR will be performed on extracted nucleic acid to detect these pathogens, these methods will be performed following an OUCRU SOP.

#### 4.12.3. Viral PCR

Extracted RNA (cDNA) will be used as template in a multiplex real-time PCR to detect rotavirus and norovirus, these methods will be performed following an OUCRU SOP[47]. This step will not be necessarily carried out immediately after isolation of nucleic acids, but will be performed at a later date, or if requested by the site enrolling the patient.

### 4.13. Blood separation and sample storage

#### 4.13.1. Blood for full blood count/procalcitonin

A part of the blood sample will be subjected to a full blood count if this is standard of care at the collaborating sites. Additional a subset of samples may be used to assess procalcitonin levels (a marker of inflammation induce by a bacterial infection (>10 mg/L).

**4.13.2. Plasma for serology**

A part of the blood 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 cyrovial tubes for storage at -80$^{o}$C or -20$^{o}$C until required for serology.

**4.13.3. Cells for host genetics studies**

The cells (red cells and buffy coat) will be aspirated into screw top cyrovial tubes for storage at -80$^{o}$C until required for host genetics or other analyses.

**4.13.4. Stool samples**

Aliquots of stool specimens (2 per subject) will be placed in 2ml cryovials and stored at -80$^{o}$C at OUCRU until required for further analysis. Access will be granted to other collaborators on request from all groups covered by the memorandum of understanding (MOU).

**4.13.5. Bacterial isolates**

All isolates will be stored at -80$^{o}$C (and freeze dried) at OUCRU for future studies.

**4.13.6. Nucleic acid from stool samples**

Nucleic acid extracted from all stool samples and bacterial isolates will be stored at -80$^{o}$C OUCRU for future studies.

**4.14. Reporting to the treating clinician**

The results of the microscopy, microbiological culture and antimicrobial susceptibility testing will be reported to the treating clinicians in a timely fashion as a diagnostic provision. Any isolated bacterial or parasitic pathogens known to cause diarrhoeal disease will be reported, along with sensitivity pattern within an acceptable time frame for culture, identification and sensitivity (as soon as microbiology staff at OUCRU are aware of the culture result). Those without a positive identification will be reported as “no bacterial or parasitic pathogen isolated”. In response to collaborative hospitals for prompt notice of any parasitic presence, we will use

provided facsimile facilities or phone call to inform treating clinicians in the earliest possible time. These results may contribute to the clinical care of the patient at the discretion of the treating doctor.

### 4.15. Routine data collection

Concurrently with the sampling from the disease syndromes; from each hospital study site we will request five years of routine hospital data as well as available retrospective data from the previous 5 years. This will be used to study the frequency and patterns of diarrhoea, jaundice, respiratory infections and central nervous system infections at each location. The data requested will be anonymous and include hospital registration number, admission date and time, age, sex, detailed address (province, district, ward), outcome at discharge and disease discharge code (ICD 10) of all patients (not only patients of studied syndromes) admitted to the selected hospital during the period of the last five years. Available data from the out-patient department will also be collected.

### 4.16. 72-hour follow up

The parents or guardians of the enrolled children will be called on the phone number they provide (out-patients) or question by a study nurse (in-patients) after 72 hours of standard of care treatment (currently 3 days of ciprofloxacin and zinc) and questioned on the 72 hour outcome. These are broadly defined as; recovered, improved, not improved, more severe, other and not known.

### 4.17. 28-day follow up

All patients enrolled in this study will be invited for follow up within 4 weeks of admission. The reason for follow up is to examine convalescent antibody level. Patients will be invited back to hospital and a 1ml EDTA blood sample and a rectal swab will be taken at both time points. Travel expenses will be supported.

### 4.18. Intussusception diagnosis

Patients who are enrolled to the study and subsequently diagnosed with intussusception will not be followed up for the 28 day visit. They will be included in the protocol analysis.

## 5. Analysis plan

### 5.1. Genome sequencing

*S. sonnei* (and other agents of dysenteric diarrhoea) isolates will be sequenced on Illumina analysers at the Wellcome Trust Sanger Institute in the United Kingdom. Resulting data will be combined with existing sequences of 210 Vietnamese *S. sonnei* isolates and the global collection, and analysed at OUCRU with the assistance of Dr Kathryn Holt and Professor Nicholas Thomson at Melbourne University and the Wellcome Trust Sanger Institute, respectively. Maximum likelihood phylogenies will be estimated from Single Nucleotide Polymorphisms (SNPs), and the BEAST software package will be utilised to define phylogeny, divergence dates, and geographical dispersal. Phylogenetic reconstruction and assembly of the accessory genome (e.g. plasmids, resistance genes and bacteriophage) will permit a calculation of the evolutionary rate, detect loci under selection, and define accessory genome structure.

**5.2. Mapping and analysis of routine admission data**

In collaboration with Professor Archie Clements at the University of Queensland, spatial analyses of *S. sonnei* across HCMC and in the surrounding provinces will be performed at OUCRU using established methods we developed and employed to study the spatial risk of typhoid fever in Nepal [48]. Residential GPS data from all participants will be visualised in qGIS and Google Earth software. These data will be stratified by isolation time and genome phylogeny to detect disease clusters and potential transmission networks. Spatial risk prediction for *S. sonnei* infections will be measured using a generalised additive modelling framework, using all concurrent non-diarrhoeal hospital admissions as a baseline population. Regions of high and low spatial risk of *S. sonnei* infections will be compared to our existing GIS datasets of potential predictors of diarrhoeal infections (e.g. population, water supply, flooding) through geostatistical regression methods.

**5.3. 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.

### 5.4. Host genetics studies

#### 5.4.1. 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.

#### 5.4.2. 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 (e.g. 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).

#### 5.4.3. 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 bloody and/or mucoid diarrhoea case and control cohort.

**5.4.4. 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.

**5.4.5. 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 new born 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.

#### 5.4.5.1. Inclusion criteria for control populations

1. Informed consent to collect, store and analyse samples for the purpose of genetic testing
2. Participant in one of the following approved studies; AN, FC, 07DX in Vietnam

#### 5.4.5.2. Exclusion criteria for control populations

1. Denial of consent to perform genetic testing on stored samples

## 6. Future investigations and sample shipment

Future investigations may take place on the stored specimens, either at OUCRU-VN or another collaborating institution in Vietnam or other global institutions.

### 6.1. Sample shipment and overseas investigations

Samples will be required to be sent overseas for laboratory analysis outside the scope and capabilities of OUCRU-VN to the collaborating institutions. Therefore, consent to send samples (nucleic acid, plasma, organism, stool samples) will be required at the time of consenting to the study. All site principal investigators will be informed on any material leaving OUCRU-VN and appropriate requests to the ethical and regulatory authorities will be made. For studies outside this protocol, additional IRB approval will be sought. The current locations identified for collaboration include; the Genome Institute of Singapore, the Wellcome Trust Sanger Institute UK, The University of California Irvine, the University of Melbourne Australia and the University of Umea Sweden.

### 6.2. Future investigations at OUCRU

Additional investigations may be performed on isolated nucleic acids and samples collected as part of this study. These samples will only be used to study the aetiology, the host response or the epidemiology of enteric pathogens and work will occur in the Laboratory of Dr Stephen Baker and will not be distributed to other investigators in Vietnam without permission from all collaborating institutions.

## 7. Protection of human subjects

### 7.1. Use of stored human specimens

With the consent of the participant or their representative, residual samples will be stored for future studies and, where laboratory capacity does not exist in Vietnam, samples will be sent

overseas for analysis. Additional investigations will only be performed with the approval of the appropriate ethical committees and the endorsement of the responsible investigators. All residual specimens will be stored at OUCRU in Vietnam for future studies related to infectious diseases. Participants will be asked to sign for consent for the storage of their specimens. Any proposed plans to use these specimens for secondary analyses will be submitted to the appropriate ethical committees. If intended analyses (pathogen nucleic acid and serological testing) cannot be performed within Vietnam samples will be sent to laboratories outside of Vietnam.

**7.2. Long term storage of data**

Data will be protected in the custody of OUCRU-VN under joint ownership of OUCRU-VN and the collaborating site. Data will be stored in password-protected computer servers, which will be located in locked rooms. Only investigators or their designee(s) will have access to the data, all data will be identified by an individual patient identification code and will be anonymous.

**7.3. Long term storage of specimens**

The sample specimens will be stored temporarily at the study sites and transferred to the central OUCRU reference laboratory for storage of an indefinite period after the completion of the study. Storage and shipments of specimens will be tracked using a central database using commercial specimen tracking software (Freezerworks). Stored specimens may be destroyed at the request of the participant/representative.

**7.4. Study withdrawal**

A subject may voluntarily withdraw from the study at any time. The investigators / treating clinicians may also, at their discretion, discontinue the subject from participating in this study at any time.

**7.5. Risks and benefits**

This is a minimal risk study because it does not involve any investigational new drugs or interventions. The collection of all biological samples in this study is clinically indicated and consistent with the local standard of care and good clinical practice. The volume of blood drawn will be up to 1 ml (HTD, CH2) or 2 ml (CH1) per visit greater than required by the standard of care at any of the study sites. The total amount of blood that will be collected is considered safe and of limited risk to the individuals. Stored samples will be used for research purposes relevant to the study aims. Accordingly, consent will be sought from patients to store all clinical

specimens. Benefits to the patient include a microbiological examination/diagnosis, which may not otherwise be performed.

### 7.6. Regulation and quality assurance

The study will be conducted in compliance with this protocol, relevant sections of the International Conference on Harmonization Good Clinical Practice (ICH GCP) guidelines and any applicable regulatory requirement(s). Quality assurance and quality control procedures will be implemented for all data collection, documentation, specimen collection and specimen handling.

### 7.7. Institutional review board/ethics committee

This protocol, informed consent document, relevant supporting information, and all types of patient recruitment or advertisement information will be submitted to the EC/IRB of each recruiting site for review and will not be initiated at that site until after approval. Any amendments will also need to be approved by the site IRB/IEC prior to implementing changes in the study.

### 7.8. Informed consent process

In addition to the procedures outlined above, the following will apply to this study

1. The informed consent for this study must be signed by the subject or representative before participation in the study.
2. A copy of the signed consent must be provided to the subjects.
3. Signed consents must remain in each subjects' study file, and be available for verification at any time.
4. Literate parents/guardians will document their provision of informed consent by signing and dating the informed consent forms.
5. Non-literate parents/legal guardians will undertake the informed consent process and asked to mark their informed consent forms (e.g., with an "X", thumb print, or other mark) in the presence of a literate third party impartial witness who will also sign the consent to attest to the accurate reading of the form and agreement of the participant/representative. Any other local EC/IRB requirements for obtaining informed consent from non-literate persons also will be followed.

6. Subjects over the age of 12 will be asked to give their verbal assent that they understand and agree to join the study. Their parent/guardian will sign the informed consent form if they also agree for the subject to join the study.

**7.9. Participant confidentiality**

1. All personal information will be stored securely at the study site in locked file cabinets or password protected devices in areas with access limited to study staff.
2. All laboratory specimens, reports, study data collection, process, and administrative forms will be identified by a coded number.
3. All records that contain names or other personal identifiers will be protected and access limited according to OUCRU policy.
4. Study databases will be secured with password-protected access systems and controlled distribution web-based security certificates.
5. No identifying information will be included in publications or presentations resulting from this work.

**7.10. Data handling and record keeping**

1. The investigator is responsible for maintaining all study records. The investigator is responsible for the timeliness, completeness and accuracy of the information in the CRFs and the clinical data management system.
2. The site staff will enter data to electronic devices, which will upload data securely to an Internet-based database.
3. Paper consent forms and records will be held by each independent site.
4. Laboratory staff will record specimens (and their aliquots), their storage location, their shipments using a central commercial database system (Freezerworks).
5. All necessary tools, instruction, and training will be provided to all site staff involved in data entry to ensure the correct and consistent completion of the eCRFs prior to the study start.

**7.11. Study records retention**

Any paper records will be transferred to the central site at OUCRU-VN and retained for a period of 3 years after the completion of the study. They will then be scanned and electronically archived indefinitely.

**8. Support**

OUCRU-VN will provide support for the study through the Sir Henry Dale fellowship funding of Dr Stephen Baker from the Royal Society and the Wellcome Trust of the United Kingdom.

## 9. Some definitions

### 9.1. Dysentery

Dysentery is bloody diarrhoea, i.e. any diarrhoeal episode in which the loose or watery stools contain visible red blood. Dysentery is most often caused by *Shigella* species (bacillary dysentery) or *Entamoeba histolytica* (amoebic dysentery).

### 9.2. Vomiting

Defined as the forceful expulsion of gastric contents at least once in a 24-h period.

### 9.3. Fever

Characterised as a temperature measured under the arm of >37.2$^{o}$C. Thresholds of 37.2 to 39$^{o}$C and > 39$^{o}$C will be considered moderate and high fevers, respectively.

### 9.4. Dehydration

Levels of dehydration will be assessed according to the recommendations of the World Health Organization Program for Control of Diarrhoeal Diseases. The Integrated Management of Childhood Illness (IMCI) guidelines have recommended four indicators used to classify a child with dehydration: the sensorium; the presence or absence of sunken eyes; whether a child drinks poorly or eagerly; and whether the skin pinch goes back very slowly or slowly. Therefore, A child with **severe dehydration (++)** will have at least two of the following four signs: sensorium is abnormally sleepy or lethargic, sunken eyes, drinking poorly or not at all, and a very slow skin pinch. A child with **moderate or some signs of dehydration (+)** will have two of the following: restlessness or irritability, sunken eyes, drinking eagerly or slow skin pinch. A child with either one or none of these signs is classified as having **no signs of dehydration (-).**

**References**


1 Thapar N, Sanderson I. Diarrhoea in children: an interface between developing and developed countries. 2004; **363**: 641–53.

2 WHO, UNICEF. Diarrhoea: Why children are still dying and what can be done. 2009.

3 Kosek M, Bern C, Guerrant RL. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bull World Health Organ* 2003; **81**: 197–204.

4 Parashar UD, Bresee JS, Glass RI. The global burden of diarrhoeal disease in children. *Bull World Health Organ* 2003; **81**: 236.

5 Shears P. Shigella infections. *Ann Trop Med Parasitol* 1996; **90**: 105–14.

6 Kotloff KL, Winickoff JP, Ivanoff B, *et al.* Global burden of Shigella infections: Implications for vaccine development and implementation of control strategies. *Bull World Health Organ* 1999; **77**: 651–66.

7 Zafar A, Hasan R, Nizami SQ, *et al.* Frequency of isolation of various subtypes and antimicrobial resistance of Shigella from urban slums of Karachi, Pakistan. *Int J Infect Dis* 2009; **13**: 668–72.

8 von Seidlein L, Kim DR, Ali M, *et al.* A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. *PLoS Med* 2006; **3**: e353.

9 Nygren B, Schilling K, Blanton E, Silk B, Cole D, Mintz E. Foodborne outbreaks of shigellosis in the USA, 1998-2008. *Epidemiol Infect* 2012; : 1–9.

10 De Schrijver K, Bertrand S, Gutiérrez Garitano I, Van den Branden D, Van Schaeren J. Outbreak of Shigella sonnei infections in the Orthodox Jewish community of Antwerp, Belgium, April to August 2008. *Eur Commun Dis Bull* 2011; **16**.

11 Guzman-Herrador B, Vold L, Comelli H, Macdonald E, Heier B, Wester A. Outbreak of Shigella sonnei infection in Norway linked to consumption of fresh basil, October 2011. *Eur Commun Dis Bull* 2011; **16**.

12 Bangtrakulnonth A, Vieira AR, Lo Fo Wong DM a, *et al.* Shigella from humans in Thailand during 1993 to 2006: spatial-time trends in species and serotype distribution. *Foodborne Pathog Dis* 2008; **5**: 773–84.

13 Vinh H, Nhu NTK, Nga TVT, *et al.* A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation. *BMC Infect Dis* 2009; **9**.

14 Holt KE, Thieu Nga T V., Thanh DP, *et al.* Tracking the establishment of local endemic

populations of an emergent enteric pathogen. *Proc Natl Acad Sci* 2013; **201308632**: 1–6.

15 Shigellosis: disease burden, epidemiology and case management. *Wkly Epidemiol Rec* 2005; **80**: 93–100.

16 Traa BS, Walker CLF, Munos M, Black RE. Antibiotics for the treatment of dysentery in children. *Int J Epidemiol* 2010; **39 Suppl 1**: i70-4.

17 Nguyen NTK, Ha V, Tran NVT, *et al.* The sudden dominance of blaCTX-M harbouring plasmids in Shigella spp. Circulating in Southern Vietnam. *PLoS Negl Trop Dis* 2010; **4**: e702.

18 Vinh H, Baker S, Campbell J, *et al.* Rapid emergence of third generation cephalosporin resistant Shigella spp. in Southern Vietnam. *J Med Microbiol* 2009; **58**: 281–3.

19 Keusch GT. Bacterial Infections of Humans. Boston, MA: Springer US, 2009.

20 DuPont HL, Levine MM, Hornick RB, Formal SB. Inoculum Size in Shigellosis and Implications for Expected Mode of Transmission. *J Infect Dis* 1989; **159**: 1126–8.

21 Lu L, Walker WA. Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. *Am J Clin Nutr* 2001; **73**: 1124–30.

22 Galán J, Alan Collmer. Type III Secretion Machines: Bacterial Devices for Protein Delivery into Host Cells. *Science (80- )* 1999; **284**: 1322–8.

23 Fernandez MI, Sansonetti PJ. Shigella interaction with intestinal epithelial cells determines the innate immune response in shigellosis. *Int J Med Microbiol* 2003; **293**: 55–67.

24 Ashkenazi S. Shigella infections in children: New insights. *Semin Pediatr Infect Dis* 2004; **15**: 246–52.

25 Germani Y, Sansonetti PJ. Replicating Vaccines. In: Dormitzer PR, Mandl CW, Rappuoli R, eds. Replicating Vaccines. Basel: Springer Basel, 2011: 99–117.

26 D. Legros. Guidelines for the control of shigellosis, including epidemics due to Shigella dysenteriae type 1. *World Heal Organ* 2005; : 1–70.

27 Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB. Clinical trials of Shigella vaccines: two steps forward and one step back on a long, hard road. *Nat Rev Microbiol* 2007; **5**: 540–53.

28 Isenbarger DW, Hien BT, Ha HT, *et al.* Prospective study of the incidence of diarrhoea and prevalence of bacterial pathogens in a cohort of Vietnamese children along the Red River. *Epidemiol Infect* 2001; **127**: 229–36.

29 Kotloff KL, Nataro JP, Losonsky GA, *et al.* Papers A modified Sliigella volunteer challenge model in which the inoculum is administered with bicarbonate buffer : clinical

experience and implications for ShZgella infectivity. 1995; **13**: 1488–94.

30 Mel DM, Terzin AL, Vuksic L. Studies on Vaccination against Bacillary Dysentery; 3. Effective Oral Immunization against Shigella flexneri 2a in a Field Trial. *Bull World Health Organ* 1965; **32**: 647–55.

31 Ferreccio C, Prado V, Ojeda A, *et al.* Epidemiologic patterns of acute diarrhea and endemic Shigella infections in children in a poor periurban setting in Santiago, Chile. *Am J Epidemiol* 1991; **134**: 614–27.

32 Noriega F, Liao F, Maneval D, Ren S, Formal S, Levine M. Strategy for cross-protection among Shigella flexneri serotypes. *Infect Immun* 1999; **67**: 782–8.

33 Riddle MS, Kaminski RW, Williams C, *et al.* Safety and immunogenicity of an intranasal Shigella flexneri 2a Invaplex 50 vaccine. *Vaccine* 2011; **29**: 7009–19.

34 Tribble D, Kaminski R, Cantrell J, *et al.* Safety and immunogenicity of a Shigella flexneri 2a Invaplex 50 intranasal vaccine in adult volunteers. *Vaccine* 2010; **28**: 6076–85.

35 Cohen D, Ashkenazi S, Green M, *et al.* Double-blind vaccine-controlled randomised efficacy trial of an investigational Shigella sonnei conjugate vaccine in young adults. *Lancet* 1997; **349**: 155–9.

36 Cohen D, Ashkenazi S, Green M, *et al.* Safety and immunogenicity of investigational Shigella conjugate vaccines in Israeli volunteers. *Infect Immun* 1996; **64**: 4074–7.

37 Orr N, Katz DE, Atsmon J, *et al.* Community-Based Safety, Immunogenicity, and Transmissibility Study of the Shigella sonnei WRSS1 Vaccine in Israeli Volunteers. *Infect Immun* 2005; **73**: 8027–32.

38 Kotloff KL, Simon JK, Pasetti MF, *et al.* Saftey and immunogenicity of CVD 1208S, a Live, Oral guaBA senset Shigella flexneri 2a vaccine grown on animal-free media. *Hum Vaccin* 2007; **3**: 268–75.

39 Katz DE, Coster TS, Wolf MK, *et al.* Two Studies Evaluating the Safety and Immunogenicity of a Live, Attenuated Shigella flexneri 2a Vaccine (SC602) and Excretion of Vaccine Organisms in North American Volunteers. *Infect Immun* 2004; **72**: 923–30.

40 Passwell JH, Ashkenzi S, Banet-Levi Y, *et al.* Age-related efficacy of Shigella O-specific polysaccharide conjugates in 1-4-year-old Israeli children. *Vaccine* 2010; **28**: 2231–5.

41 Passwell JH, Ashkenazi S, Harlev E, *et al.* Safety and immunogenicity of Shigella sonnei-CRM 9 and Shigella flexneri type 2a-rEPA succ conjugate vaccines in one- to four-year-old children. *Pediatr Infect Dis J* 2003; **22**: 701–6.

42 Levine MM. Enteric infections and the vaccines to counter them: future directions.

*Vaccine* 2006; **24**: 3865–73.

43 Barry EM, Pasetti MF, Sztein MB, Fasano A, Kotloff KL, Levine MM. Progress and pitfalls in Shigella vaccine research. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 245–55.

44 Kotloff KL, Taylor DN, Sztein MB, *et al.* Phase I Evaluation of deltavirG Shigella sonnei Live, Attenuated, Oral Vaccine Strain WRSS1 in Healthy Adults. *Infect Immun* 2002; **70**: 2016–21.

45 Bhan M, Mahalanabis D, Pierce N, Rollins N, Sack D, Santosham M. Treatment of Diarrhoea: A manual for physicians and other senior health workers. Geneva, 2005.

46 Vinh H, Anh VTC, Anh ND, *et al.* A multi-center randomized trial to assess the efficacy of gatifloxacin versus ciprofloxacin for the treatment of shigellosis in Vietnamese children. *PLoS Negl Trop Dis* 2011; **5**: e1264.

47 Dung TTN, Phat VV, Nga TVT, *et al.* The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting rotavirus A and norovirus. *J Virol Methods* 2013; **187**: 138–43.

48 Baker S, Holt KE, Clements ACA, *et al.* Combined high-resolution genotyping and geospatial analysis reveals modes of endemic urban typhoid fever transmission. *Open Biol* 2011; **1**.

## 9.2. Ethic Committee Approval

**Oxford Tropical Research Ethics Committee**

University of Oxford
Joint Research Office, Block 60
Churchill Hospital, Oxford OX3 7LE
Tel. +44 (0) 1865 (5)72346, fax +44 (0) 1865 (5)72224
E-mail: jacqueline.gerencser@admin.ox.ac.uk

Dr Stephen Baker
Oxford University Clinical Research Unit
Centre for Tropical Medicine
764 Vo Van Kiet
Quan 5, Ho Chi Minh City
Vietnam

30 June 2014

Dear Dr Baker

**Full Title of Study**: The Phylogeography and humoral immune responses of Shigella and other agents of dysentery in hospitalised children in Ho Chi Minh City, Vietnam.

**OXTREC Reference: 1045-13**

Thank you for your email of the 20 June 2014 and letter of the 18 June 2014, in which you have responded to the committees request for further clarifications and amendments, and included revised documents:

| Documents: | Version: | Date: |
|---|---|---|
| Protocol | V2.2 | 17/04/14 |
| Informed Consent Form | V2.2 | 17/04/14 |
| Local Approvals from the Children Hospital Number 1 | | 05/05/14 |
| Local Approvals from the Children Hospital Number 1 | | 07/01/14 |
| Peer Review by Dr Christopher Parry | | 27/08/13 |
| PI Signatory Page | | 22/08/13 |

I am therefore pleased as Chairman for OXTREC to give approval for this study.

Approval is given for the first five years.

Any subsequent changes to the application must be submitted to the Committee as an Amendment. This should include a letter to give the reasons for the proposed modifications and all modified documents with changes tracked.

Please submit a completed Annual Report form on every anniversary of this approval and a final End of Study Report.

Yours sincerely,

Mary Warrell

Dr Mary Warrell
OXTREC Chairman

Direct Line Tel: +44 (0)1865 (5)72224
Fax: +44 (0)1865 (5)72228 Email: jacqueline.gerencser@admin.ox.ac.uk
Web: www.admin.ox.ac.uk/rso/

CHILDREN'S HOSPITAL 1
**341 Su Van Hanh, Dist 10, HCMC, Vietnam**
**Tel : 84.8. 39271119- 39271156. Fax: 84.8.39270053**

May, 5th , 2014
To: Dr. Hoang Le Phuc
Gastroenterology Department
Children's Hospital 1 HCMC
Vietnam

Dear Dr. Hoang Le Phuc,

Full Title of Study: "The phylogeography and humoral immune responses of Shigella in children attending three hospitals in Ho Chi Minh City, Vietnam".

CH1 Study Code: CS/N1/14/06

Duration of Study: 02/2014-12/2017

Protocol and Informed consent form version 2.2 dated 17 April 2014

Participating site: Gastroenterology Department - Children's Hospital 1 HCMC

Principal Investigator: Hoang Le Phuc, MD

On behalf of the CH1's IRB (IORG0007285, FWA00009748), I am glad to give the approval for this study.

Sincerely,

Nguyen Thanh Hung, MD, Ph.D
Chairman of CH1's IRB
Director of Children's Hospital 1

SERVICE of HEALTH HCMC
**CHILDREN'S HOSPITAL 2**

SOCIALIST REPUBLIC of VIETNAM
*Independence – Freedom – Happiness*

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# THE ETHICS COMMITTEE'S APPROVAL

**The Ethics Committee of Children's Hospital 2**
14 Ly Tu Trong, District 1, Ho Chi Minh City, Vietnam

**PI's Name:**
Stephen Baker (OUCRU)
Lu Lan Vi (The Hospital for Tropical Diseases)
Nguyen Minh Ngoc (Children's Hospital 2)
**Study site:** Children's Hospital 2 – HCMC

**Study title: The phylogeography and humoral immune responses *Shigella* and other agents of dysentery in hospitalised children in HCMC, VN**
**Study code: 12EN**

**Study documents** both in English and Vietnamese:
- The protocol V2.0 09DEC13
- Informed Consent Form Child V2.1 09DEC13

**After :**
- reviewing all the documents listed above
- discussing between Committees' members

**The Ethics Committee of Children's Hospital 2 has approved for the above study to be conducted at Children's Hospital 2.**

**The Ethics Committee requirements:**
a. Study members must be trained by the Principle investigator on the protocol V2.0 09DEC13and Informed Consent Form Child V2.1 09DEC13
b. Principle investigator respond for fully relevant legal procedures in accordance with regulations.

We confirm that The Ethics Committee's decision has been made according to International Conference on Harmonisation (ICH) and Good Clinical Practice (GCP) guidelines as well as under Vietnamese regulations.

Ho Chi Minh City, Jan 07th, 2014
On behalf of Chairman of Ethics Committee

BỆNH VIỆN NHI ĐỒNG 2

Nguyen Thi Ho Diep, MD

SỞ Y TẾ TP HỒ CHÍ MINH
**BỆNH VIỆN BỆNH NHIỆT ĐỚI**

Số : 63 / HĐĐĐ - QĐ

**CỘNG HÒA XÃ HỘI CHỦ NGHĨA VIỆT NAM**
**Độc lập – Tự do – Hạnh phúc**

*TP Hồ Chí Minh, ngày 03 tháng 06 năm 2014*

# QUYẾT ĐỊNH

Về việc phê duyệt đề tài khoa học công nghệ cấp cơ sở

## GIÁM ĐỐC BỆNH VIỆN BỆNH NHIỆT ĐỚI

- Căn cứ quyết định số 117/QĐ-SYT ngày 21/01/2009 của Sở Y tế Thành phố Hồ Chí Minh về ban hành Quy chế tổ chức và hoạt động của Bệnh viện Bệnh Nhiệt đới.
- Căn cứ nội dung biên bản họp ngày 22/05/2014 của Hội đồng Đạo đức Bệnh viện Bệnh Nhiệt đới.
- Xét đề nghị của Chủ tịch Hội đồng Đạo đức Bệnh viện Bệnh Nhiệt đới.

## QUYẾT ĐỊNH

**Điều 1**: Nay phê duyệt đề tài cấp Cơ sở:

| | |
|---|---|
| Tên đề tài: | Liên quan địa lý về sự phát sinh chủng loài và đáp ứng miễn dịch dịch thể đối với chủng *Shigella* ở trẻ em điều trị tại 3 bệnh viện ở thành phố Hồ Chí Minh, Việt Nam<br>- Đề cương nghiên cứu phiên bản 2.2, ngày 17/04/2014.<br>- Phiếu chấp thuận tham gia nghiên cứu phiên bản 2.2, ngày 17/4/2014. |
| Chủ nhiệm: | BS. Lư Lan Vi<br>TS. Stephen Baker |
| Thời gian thực hiện: | 46 tháng (từ tháng 02/2014 đến 12/2017) |
| Mã số đề tài: | CS/ND/14/18 |

**Điều 2** : Hội đồng Đạo đức Bệnh viện Bệnh Nhiệt đới, Khoa/ Phòng có liên quan chịu trách nhiệm chỉ đạo, hỗ trợ và giám sát chủ nhiệm đề tài triển khai các nội dung nghiên cứu đăng ký trong đề cương nghiên cứu theo đúng các mục tiêu của đề cương nghiên cứu đã thông qua và định kỳ báo cáo kết quả về cơ quan quản lý đề tài. Chủ nhiệm đề tài có trách nhiệm thực hiện đúng quy định của nhà nước về Nghiên cứu khoa học.

**Điều 3**: Quyết định có hiệu lực kể từ ngày ký.

*Nơi nhận:*
- *Chủ nhiệm đề tài*
- *Lưu*

**GIÁM ĐỐC**

SỞ Y TẾ THÀNH PHỐ HỒ CHÍ MINH
BỆNH VIỆN BỆNH NHIỆT ĐỚI

**TS. NGUYỄN VĂN VĨNH CHÂU**

## 9.3. Patient Information Form

HOSPITAL

LOGO

**Participant Hospital Name**

Oxford University Clinical Research Unit

764 Vo Van Kiet, Quan 5, Ho Chi Minh City, Vietnam

oucru

**Information sheet for the study about "Bloody and/or mucoid diarrhoea in children attending three hospitals in Ho Chi Minh City"**
**OXTREC – 1045-13**

**Information about this study**
We are inviting parents/guardians of children attending this hospital for diarrhoea with mucus or blood in stool to participate in a research study called "Bloody and/or mucoid diarrhoea in children attending three hospitals in Ho Chi Minh City".

The aims of this research study are to identify the cause of your child's diarrhoea in an effort to treat your child with the correct medication. We are also interested in gathering information to understand how your child's blood responds to the infection, how diarrhoeal diseases spread across Ho Chi Minh City and how we can design a vaccine to prevent other children from getting sick in the future.

Part of this study will mean we will collect your child's blood to perform genetic studies. 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. Some people may consider tests like this 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.

This research is being done by the Oxford University Clinical Research Unit and the {Hospital Name} and has been approved by the ethical committee of both institutions. This document describes your rights, what will happen during the study, the benefits and risks so that you have all the appropriate information to make a decision about whether to enrol your child in the study. If there is any information that you do not understand, please ask one of the study staff. All of your questions will be answered.

**If I consent on behalf of my child, what will happen to my child and I in this study and what are the risks?**
At enrolment (today):
If you agree for your child to participate in the study, a study nurse will collect information about where you live and the child's health status. The nurse will also collect a 1-2 ml blood sample (about 10 drops) and a stool sample from your child. Treatment will not be delayed while you consider participating in the study. A study nurse will contact you in three days to ask how your child is doing.

**Study follow-up at 28 days after admission to hospital [Follow-up visit will be applied to HTD site ONLY]**

Patients will be invited back to HTD approximately 28 days after your child was initially admitted in order see how your child's blood remembers the disease. This visit will take approximately half an hour at HTD. A blood sample of 1-2 ml (about 10 drops) will be collected by a nurse. A rectal swab will also be collected. The child will receive a health check from a hospital doctor at this follow up visit. The nurse will give you a card and phone you a few days in advance to remind you of your follow-up appointment.

**Risks and benefits to being in the study**
The amount of blood we collect from your child at each visit is small. An 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. Collecting a stool sample is quick and painless. By being in the study, you will have access to experienced doctors in the study clinics who will examine your child at no cost at both initial and follow up visits and we will aim to find the cause of the infection so that your child receives the correct medication.

**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 study. 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 study or give it to anyone else. Any information obtained from any individual in connection with this study will be kept strictly confidential.

**Costs**
The Oxford University Clinical Research Unit is giving funding for this program. There will be no cost to you for participating in this study. The program does not pay for any specific treatment or hospital costs and you will be responsible for paying these as you usually would. {HTD only} You will be supported for the travel costs you incur by bringing your child to the clinic for the scheduled follow up visit.

**Voluntary participation**
If you do not want to be part of this study, it will not affect the care your child will receive in any way. Even if you do agree to become a study participant, you can withdraw from the study at any time (verbally) without affecting the care that you or your child will receive. If you decide at any time to take your child out of the program, no new information will be collected. However, information collected on your child up until that point will still be used.

**Obtaining additional information**
- You are encouraged to ask any questions related to this study during the time of participation. If you have any questions about this program, its procedures, risks and benefits, or alternatives please call Dr. {Dr. Name} at {Dr. Number}.
- If you have any questions about your child's rights as a subject in this study, you may want to talk to {Dr. Name}, or if you want to speak to someone outside of the program you may contact the Ethics Committee at the {Hospital Name} at {EC Phone Number}.

## 9.4. Informed Consent Form

**Consent form for the "Bloody and/or mucoid diarrhoea in Children Attending Three Hospitals" study, OXTREC – 1045-13 (to be signed by the participant's parent or guardian)**

- I have been fully informed of the possible risks and benefits of taking part in this study and agree that my child and I 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 study at any time without affecting the care I or my child will receive.

- I agree that specimens from my child (e.g. blood and stool samples) may be stored and that further tests on these samples may be undertaken in the future to further the understanding of infectious diseases in Vietnam, including tests done by researchers outside Vietnam.

- I agree that the district and ward of my house may be recorded in order to understand any relationship between location and disease.

Participant Number: 12EN - ___ ___ - ___ ___ ___ ___

**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 study to the participant named above and will provide her/him with a copy of this signed and dated informed consent form. If the subject is over 12 I confirm they have given verbal assent. The participant voluntarily agrees to be in the study.

_____ _____ _____
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

**Consent for genetic testing**
**OXTREC – 1045-13**
**(to be signed by the participant's parent or guardian)**

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

Participant Number: 12EN - ___ ___ - ___ ___ ___ ___

**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 study to the participant named above and will provide her/him with a copy of this signed and dated informed consent form. If the subject is over12 I confirm they have given verbal assent. The participant voluntarily agrees to be in the study.

_____ _____ _____
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

### 9.5. Case Report Form

| **Screening** | | **SCR** |
|---|---|---|
| Study ID<br>12EN - [__\|__] - [__\|__\|__\|__] | | Initials<br>[__\|__\|__\|__\|__\|__] |
| 1. Site | ○ Children's Hospital 1<br>○ Children's Hospital 2<br>○ Hospital for Tropical Diseases | |
| 2. Screening date | [__\|__] / [__\|__] / [__\|__] *(day/month/year)* | |
| 3. Date of admission | [__\|__] / [__\|__] / [__\|__] *(day/month/year)* | |
| 4. Date of birth of the child | day [__\|__] month [__\|__] year [__\|__\|__\|__] | |
| 5. Gender | ○ Male | ○ Female |
| 6. Child admitted with diarrhoea disease | ○ Yes | ○ No |
| 7. Child has history of blood and/or mucus in their stool within the last 72 hours | ○ Yes | ○ No |
| 8. Does the child have suspected or confirmed intussusception? | ○ Yes | ○ No |
| 9. Informed consent provided | ○ Yes | ○ No |
| 10. I have discussed the purpose of the study, including the possible risks and benefits, with the parent or guardian. They have<br>○ Agreed to participate and have signed the consent form<br>○ Declined to participate. | | |
| a. Reason for declining:<br>○ Don't like the study<br>○ Don't have time<br>○ Afraid of harm to the child<br>○ Want to discuss or think about this first<br>○ Other, please specify [_____] | | |

*Completed by:* _____ *Date completed:* _____

| **Enrolment** | | | **ENROL** |
|---|---|---|---|
| Study ID<br>12EN - [__\|__] - [__\|__\|__\|__] | | | Initials<br>[__\|__\|__\|__\|__\|__] |
| **BASIC INFORMATION** | | | |
| 1. Date enrolment: | [__\|__]/[__\|__]/[__\|__] *(day/month/year)* | | |
| 2. Site<br>○ Children's Hospital 1 ○ Children's Hospital 2 ○ Hospital of Tropical Diseases | | | |
| 2a. If Children's Hospital 1, which ward?<br>○ Gastrointestinal ward ○ Outpatient ward | | | |
| 2b. If Children's Hospital 2, which ward?<br>○ Gastrointestinal ward ○ Service ward 2 ○ Outpatient ward | | | |
| 3. Address of child | | | |
| a. Ward | [_____] | | |
| b. District | [_____] | | |
| c. Province/ City | [_____] | | |
| 4. Contact person's name* | [_____] | | |
| 5. Contact person's phone number* | [_____] | | |
| 6. Contact person's name 2* | [_____] | | |
| 7. Contact person's phone number 2* | [_____] | | |
| **DISEASE HISTORY** | | | |
| 8. Number of days of diarrhoeal illness | [__\|__] | | |
| 9. Number of diarrhoeal episodes in last 24 hours | [__\|__] | | |
| 10. Faecal characteristic | ○ Bloody | ○ Mucoid | ○ Bloody and mucoid |
| 11. Vomiting | ○ Yes | ○ No | ○ Unknown |
| 12. Abdominal pain | ○ Yes | ○ No | ○ Unknown |
| 13. History of breast feeding | ○ Yes | ○ No | ○ Unknown |

** No data entry*

*Completed by:* _____ *Date completed:* _____

| **BASELINE** | | **BASE** |
|---|---|---|
| Study ID<br>12EN - [__\|__] - [__\|__\|__\|__] | | Initials<br>[__\|__\|__\|__\|__\|__] |
| 1. Date of examination | [__\|__]/[__\|__]/[__\|__] *(day/month/year)* | |
| 2. ICD10 at admission | [__\|__\|__].[__] | |
| 3. Condition at examination | ○ Well/alert ○ Restless/irritable<br>○ Glassy ○ Lethargic/unconscious<br>○ Other, please specify [_____] | |
| 4. Pulse | [__\|__\|__] beats per minute | |
| 5. Blood pressure | [__\|__\|__] / [__\|__\|__] mmHg | |
| 6. Temperature | [__\|__].[__] $^{o}$C | |
| 7. Respiratory rate | [__\|__\|__] breaths per minute | |
| 8. Weight | [__\|__].[__] kg | |
| 9. Height | [__\|__\|__] cm | |
| 10. Dehydration | ○No dehydration ○Some dehydration ○Severe dehydration | |
| 11. Rehydration | ○ Oral ○ IV ○ None | |
| 12. Antimicrobials prescribed? | ○ Yes ○ No | |
| a. Antimicrobial 1: | ○ Fluoroquinolone ○ 3$^{rd}$/4$^{th}$ generation Cephalosporin<br>○ Macrolide ○ Carbapenem ○Other | |
| b. Antimicrobial 2: | ○ Fluoroquinolone ○ 3$^{rd}$/4$^{th}$ generation Cephalosporin<br>○ Macrolide ○ Carbapenem ○Other | |
| c. Antimicrobial 3: | ○ Fluoroquinolone ○ 3$^{rd}$/4$^{th}$ generation Cephalosporin<br>○ Macrolide ○ Carbapenem ○Other | |
| 13. Zinc | ○ Yes ○ No | |
| 14. Probiotics | ○ Yes ○ No | |
| 15. Vitamin A | ○ Yes ○ No | |
| 16. Blood sample taken | ○ Yes ○ No | |
| a. Date of blood sampling | [__\|__] / [__\|__]/ [__\|__] *(day/month/year)* | |
| 17. Stool sample taken | ○ Yes ○ No | |
| a. Date of stool sampling | [__\|__] / [__\|__]/ [__\|__] *(day/month/year)* | |
| 18. Discharging date | [__\|__] / [__\|__]/ [__\|__] *(day/month/year)* | |

*Completed by:* _____ *Date completed:* _____

| **Laboratory in Hospital** | | **LAB_HOSP** |
|---|---|---|
| Study ID<br>12EN - [__\|__] - [__\|__\|__\|__] | | Initials<br>[__\|__\|__\|__\|__\|__] |
| **BLOOD TESTS AT STUDY SITE** | | |
| 1. White blood cell count | [__\|__\|__].[__] x$10^3$/µL | Not done ☐ |
| 2. %Neutrophil | [__\|__].[__] % | |
| 3. %Eosinophil | [__\|__].[__] % | |
| 4. %Lymphocyte | [__\|__].[__] % | |
| 5. %Monocyte | [__\|__].[__\|__] % | |
| 6. Red blood cell count | [__].[__] x $10^{12}$/L | |
| 7. Haemoglobin | [__\|__].[__] g/dL | |
| 8. Haematocrit | [__\|__].[__]% | |
| 9. Platelet count | [__\|__\|__] x$10^3$/µL | |
| 10. C reactive protein | [__\|__\|__] mg/L | Not done ☐ |
| 11. Procalcitonin | [__\|__].[__] mg/L | Not done ☐ |

*Completed by:* _____ *Date completed:* _____

| **Follow-up Day 3 (72 hours after enrol)** | | **FU72** |
|---|---|---|
| Study ID<br>12EN - [__\|__] - [__\|__\|__\|__] | | Initials<br>[__\|__\|__\|__\|__\|__] |
| **FOLLOW UP 72** | | |
| 1. Follow-up date | [__\|__]/[__\|__]/[__\|__] *(day/month/year)* | |
| 2. Can't contact to patient | [__] | |
| 3. Patient status | ○ Inpatient<br>○ Outpatient | |
| 4. Outcome of patient | ○ Recovered;<br>○ Diarrhoea improved (less episodes, no blood/mucus)<br>○ Diarrhoea not improved (same episodes/severity)<br>○ Infectious more severe (more episodes/ still blood or mucus)<br>○ Other, please specify [_____]<br>○ Unknown | |

*Completed by:* _____ *Date completed:* _____

### 9.6. Microbiology faecal result at OUCRU laboratory

| **Laboratory at OUCRU** | | **LAB-OUCRU** |
|---|---|---|
| Study ID<br>12EN - [__|__] - [__|__|__|__] | | Initials<br>[__|__|__|__|__|__] |
| **MICROSCOPY** | | |
| 1. Date of stool microscopy | [__|__]/[__|__]/[__|__] *(day/month/year)* | |
| 2. Microscopy for white blood cells | ○ ++++ ○+++ ○ ++ ○ + ○ Neg ○ Not done | |
| 3. Microscopy for red blood cells | ○ ++++ ○+++ ○ ++ ○ + ○ Neg ○ Not done | |
| 4. Microscopy for parasites | ○ *Giardia lamblia*<br>○ *Cryptosporidium*<br>○ Other<br>○ Not done | ○*Entamoeba histolytica*<br>○Helminth<br>○ Negative |
| a. Other identified parasite | [_____] | |
| **STOOL ISOLATE 1** | | |
| 5. Stool isolate 1 microbiological result | [_____]<br>○ Other ○ Negative ○ Not done | |
| 6. Stool isolate 1 microbiological result other | [_____] | |
| 7. Stool isolate 1 ID | 12EN-[__|__]-[__|__|__|__] A | |
| 8. Stool isolate 1 nalidixic acid disc diffusion | ○S ○I ○R ○Not done | |
| 9. Stool isolate 1 nalidixic acid zone size | [__|__|__].[__] >256 ☐ Not done ☐ | |
| 10. Stool isolate 1 ciprofloxacin disc diffusion | ○S ○I ○R ○Not done | |
| 11. Stool isolate 1 ciprofloxacin zone size | [__|__|__].[__] >256 ☐ Not done ☐ | |
| 12. Stool isolate 1 ceftriaxone disc diffusion | ○S ○I ○R ○Not done | |
| 13. Stool isolate 1 ceftriaxone zone size | [__|__|__].[__] >256 ☐ Not done ☐ | |
| 14. Stool isolate 1 ceftazidime disc diffusion | ○S ○I ○R ○Not done | |
| 15. Stool isolate 1 ceftazidime zone size | [__|__|__].[__] >256 ☐ Not done ☐ | |
| 16. Stool isolate 1 augmentin disc diffusion | ○S ○I ○R ○Not done | |
| 17. Stool isolate 1 augmentin zone size | [__|__|__].[__] >256 ☐ Not done ☐ | |
| 18. Stool isolate 1 ampicillin disc diffusion | ○S ○I ○R ○Not done | |

| | | | | |
|---|---|---|---|---|
| 19. Stool isolate 1 ampicillin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 20. Stool isolate 1 trimethoprim-sulfamethoxazole disc diffusion | ○S | ○I | ○R | ○Not done |
| 21. Stool isolate 1 trimethoprim-sulfamethoxazole zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 22. Stool isolate 1 azithromycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 23. Stool isolate 1 azithromycin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 24. Stool isolate 1 chloramphenicol disc diffusion | ○S | ○I | ○R | ○Not done |
| 25. Stool isolate 1 chloramphenicol zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 26. Stool isolate 1 imipenem disc diffusion | ○S | ○I | ○R | ○Not done |
| 27. Stool isolate 1 imipenem zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 28. Stool isolate 1 amikacin disc diffusion | ○S | ○I | ○R | ○Not done |
| 29. Stool isolate 1 amikacin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 30. Stool isolate 1 gentamycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 31. Stool isolate 1 gentamycin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 32. Stool isolate 1 erythromycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 33. Stool isolate 1 erythromycin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 34. Stool isolate 1 clindamycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 35. Stool isolate 1 clindamycin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 36. Stool isolate 1 ESBL result | ○ Positive | ○ Negative | ○ Not done | |
| **STOOL ISOLATE 2** | | | | |
| 37. Stool isolate 2 microbiological result | [_____] ○ Other | ○ Negative | ○ Not done | |
| 38. Stool isolate 2 microbiological result other | [_____] | | | |
| 39. Stool isolate 2 ID | 12EN-[__\|__]-[__\|__\|__\|__] B | | | |
| 40. Stool isolate 2 nalidixic acid disc diffusion | ○S | ○I | ○R | ○Not done |
| 41. Stool isolate 2 nalidixic acid zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 42. Stool isolate 2 ciprofloxacin disc diffusion | ○S | ○I | ○R | ○Not done |
| 43. Stool isolate 2 ciprofloxacin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |

| | | | | |
|---|---|---|---|---|
| 44. Stool isolate 2 ceftriaxone disc diffusion | ○S | ○I | ○R | ○Not done |
| 45. Stool isolate 2 ceftriaxone zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 46. Stool isolate 2 ceftazidime disc diffusion | ○S | ○I | ○R | ○Not done |
| 47. Stool isolate 2 ceftazidime zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 48. Stool isolate 2 augmentin disc diffusion | ○S | ○I | ○R | ○Not done |
| 49. Stool isolate 2 augmentin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 50. Stool isolate 2 ampicillin disc diffusion | ○S | ○I | ○R | ○Not done |
| 51. Stool isolate 2 ampicillin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 52. Stool isolate 2 trimethoprim-sulfamethoxazole disc diffusion | ○S | ○I | ○R | ○Not done |
| 53. Stool isolate 2 trimethoprim-sulfamethoxazole zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 54. Stool isolate 2 azithromycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 55. Stool isolate 2 azithromycin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 56. Stool isolate 2 chloramphenicol disc diffusion | ○S | ○I | ○R | ○Not done |
| 57. Stool isolate 2 chloramphenicol zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 58. Stool isolate 2 imipenem disc diffusion | ○S | ○I | ○R | ○Not done |
| 59. Stool isolate 2 imipenem zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 60. Stool isolate 2 amikacin disc diffusion | ○S | ○I | ○R | ○Not done |
| 61. Stool isolate 2 amikacin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 62. Stool isolate 2 gentamycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 63. Stool isolate 2 gentamycin zone size | [__\|__\|__].[__] | >256 ☐ | Not done ☐ | |
| 64. Stool isolate 2 erythromycin disc diffusion | ○S | ○I | ○R | ○Not done |
| **STOOL ISOLATE 3** | | | | |
| 65. Stool isolate 3 microbiological result | [_____] ○ Other ○ Negative ○ Not done | | | |
| 66. Stool isolate 3 microbiological result other | [_____] | | | |
| 67. Stool isolate 3 ID | 12EN-[__\|__]-[__\|__\|__\|__] C | | | |
| 68. Stool isolate 3 nalidixic acid disc diffusion | ○S | ○I | ○R | ○Not done |

| | | | | |
|---|---|---|---|---|
| 69. Stool isolate 3 nalidixic acid zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 70. Stool isolate 3 ciprofloxacin disc diffusion | ○S | ○I | ○R | ○Not done |
| 71. Stool isolate 3 ciprofloxacin zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 72. Stool isolate 3 ceftriaxone disc diffusion | ○S | ○I | ○R | ○Not done |
| 73. Stool isolate 3 ceftriaxone zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 74. Stool isolate 3 ceftazidime disc diffusion | ○S | ○I | ○R | ○Not done |
| 75. Stool isolate 3 ceftazidime zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 76. Stool isolate 3 augmentin disc diffusion | ○S | ○I | ○R | ○Not done |
| 77. Stool isolate 3 augmentin zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 78. Stool isolate 3 ampicillin disc diffusion | ○S | ○I | ○R | ○Not done |
| 79. Stool isolate 3 ampicillin zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 80. Stool isolate 3 trimethoprim-sulfamethoxazole disc diffusion | ○S | ○I | ○R | ○Not done |
| 81. Stool isolate 3 trimethoprim-sulfamethoxazole zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 82. Stool isolate 3 azithromycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 83. Stool isolate 3 azithromycin zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 84. Stool isolate 3 chloramphenicol disc diffusion | ○S | ○I | ○R | ○Not done |
| 85. Stool isolate 3 chloramphenicol zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 86. Stool isolate 3 imipenem disc diffusion | ○S | ○I | ○R | ○Not done |
| 87. Stool isolate 3 imipenem zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 88. Stool isolate 3 amikacin disc diffusion | ○S | ○I | ○R | ○Not done |
| 89. Stool isolate 3 amikacin zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 90. Stool isolate 3 gentamycin disc diffusion | ○S | ○I | ○R | ○Not done |
| 91. Stool isolate 3 gentamycin zone size | [__\|__\|__].[__] | | >256 ☐ | Not done ☐ |
| 92. Stool isolate 3 erythromycin disc diffusion | ○S | ○I | ○R | ○Not done |

*Completed by:* _____ *Date completed:* _____

## 9.7. Publications during DPhil candidature

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology

# Evaluation of Luminex xTAG Gastrointestinal Pathogen Panel Assay for Detection of Multiple Diarrheal Pathogens in Fecal Samples in Vietnam

**Vu Thuy Duong,[a,b] Voong Vinh Phat,[a] Ha Thanh Tuyen,[a] Tran Thi Ngoc Dung,[a] Pham Duc Trung,[a] Pham Van Minh,[a] Le Thi Phuong Tu,[a] James I. Campbell,[a,c] Hoang Le Phuc,[b] Ton Thi Thanh Ha,[b] Nguyen Minh Ngoc,[d] Nguyen Thi Thanh Huong,[d] Pham Thi Thanh Tam,[a] Dang Thao Huong,[a] Nguyen Van Xang,[e] Nguyen Dong,[e] Le Thi Phuong,[f] Nguyen Van Hung,[g] Bui Duc Phu,[h] Tran My Phuc,[a] Guy E. Thwaites,[a,c] Lu Lan Vi,[i] Maia A. Rabaa,[a,c] Corinne N. Thompson,[a,c] Stephen Baker[a,c,j]**

The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam[a]; Children's Hospital 1, Ho Chi Minh City, Vietnam[b]; Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, Oxford, United Kingdom[c]; Children's Hospital 2, Ho Chi Minh City, Vietnam[d]; Khanh Hoa General Hospital, Khanh Hoa, Vietnam[e]; Dong Thap General Hospital, Dong Thap, Vietnam[f]; Dak Lak General Hospital, Dak Lak, Vietnam[g]; Hue Central Hospital, Thua Thien Hue, Vietnam[h]; Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam[i]; The London School of Hygiene and Tropical Medicine, London, United Kingdom[j]

**Diarrheal disease is a complex syndrome that remains a leading cause of global childhood morbidity and mortality. The diagnosis of enteric pathogens in a timely and precise manner is important for making treatment decisions and informing public health policy, but accurate diagnosis is a major challenge in industrializing countries. Multiplex molecular diagnostic techniques may represent a significant improvement over classical approaches. We evaluated the Luminex xTAG gastrointestinal pathogen panel (GPP) assay for the detection of common enteric bacterial and viral pathogens in Vietnam. Microbiological culture and real-time PCR were used as gold standards. The tests were performed on 479 stool samples collected from people admitted to the hospital for diarrheal disease throughout Vietnam. Sensitivity and specificity were calculated for the xTAG GPP for the seven principal diarrheal etiologies. The sensitivity and specificity for the xTAG GPP were >88% for *Shigella* spp., *Campylobacter* spp., rotavirus, norovirus genotype 1/2 (GI/GII), and adenovirus compared to those of microbiological culture and/or real-time PCR. However, the specificity was low (~60%) for *Salmonella* species. Additionally, a number of important pathogens that are not identified in routine hospital procedures in this setting, such as *Cryptosporidium* spp. and *Clostridium difficile*, were detected with the GPP. The use of the Luminex xTAG GPP for the detection of enteric pathogens in settings, like Vietnam, would dramatically improve the diagnostic accuracy and capacity of hospital laboratories, allowing for timely and appropriate therapy decisions and a wider understanding of the epidemiology of pathogens associated with severe diarrheal disease in low-resource settings.**

Diarrheal disease remains a considerable public health challenge and is responsible for 0.8 million deaths and close to 90,000 disability-adjusted life years annually (1, 2), the majority of which occur in children <5 years of age in industrializing regions (3). Infectious diarrhea (caused by a pathogenic agent) can be caused by a number of different viruses, bacteria, and parasites. Rotavirus remains the most common cause of diarrhea in children <2 years of age (4, 5), although the introduction of rotavirus vaccines in many countries has led to a fall in incidence in locations where uptake has been significant (6, 7). Bacterial pathogens, such as *Shigella* spp., enterotoxigenic *Escherichia coli* (ETEC), *Campylobacter* spp., and *Salmonella* spp., norovirus, and the parasitic pathogens *Giardia* and *Cryptosporidium* spp. are also known to be present frequently in the stools of young children with diarrhea in resource-poor regions (4, 5, 8). As the etiology of diarrheal disease is diverse and complex, sensitive and specific diagnostics are required for prompt and accurate identification and treatment.

The detection of pathogens from stool specimens is challenging. In settings, such as Ho Chi Minh City (HCMC), Vietnam, hospital laboratories rarely perform routine microbiological culture, and if they do, they aim to isolate only a limited range of bacterial pathogens. Antimicrobial consumption in the community further reduces the sensitivity of microbiological culture for bacterial pathogens (9). Microscopy is used to detect occult white blood cells, red blood cells, and parasites, such as *Entamoeba histolytica*, *Cryptosporidium* spp., and *Giardia lamblia*; however, microscopic detection of parasites has poor sensitivity (10) and presents difficulties due to the requirement of a fresh specimen, the requirement for an experienced microscopist, and a lack of methodological standardization. In addition, real-time PCR systems are not used routinely in HCMC (or indeed across Vietnam) due to the cost, lack of equipment, and a paucity of trained staff. As such, a number of important pathogens, such as rotavirus, noro-

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Address correspondence to Corinne N. Thompson, cthompson@oucru.org.
V.T.D. and V.V.P. contributed equally to this work.
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virus, pathovars of *E. coli*, and *Clostridium difficile*, are not routinely detected by procedures currently in place. The lack of appropriate diagnostics hinders not only clinical treatment decisions but also a wider understanding of the epidemiology and the public health implications of severe pediatric diarrheal disease in locations, like HCMC.

Given the limitations of traditional diagnostics, a multipanel pathogen identification system that is sensitive, specific, and easy to operate represents a significant improvement on classical techniques. The Luminex xTAG gastrointestinal pathogen panel assay (GPP) (Luminex Molecular Diagnostics, Austin, TX, USA) is a qualitative multiplex test cleared by the U.S. Food and Drug Administration (FDA) that is able to identify 19 enteric pathogens in one reaction in 6 h; however, the assay has a prohibitive cost in Vietnam of approximately $75 per sample (as of December 2015). The Luminex platform has been shown to be highly sensitive and specific for a variety of pathogens in several clinical settings globally (11–13). In this study, we aimed to evaluate the sensitivity and specificity of the xTAG GPP platform against those of conventional diagnostic techniques on clinical samples isolated from patients hospitalized with diarrheal disease in Vietnam.

## MATERIALS AND METHODS

**Clinical specimens and study procedures.** Fecal specimens were collected from 479 patients hospitalized with diarrheal disease from three different studies across Vietnam during 2009 to 2014. The hospitals included in the study were Children's Hospital 1, Children's Hospital 2, and the Hospital of Tropical Diseases in HCMC. Provincial hospitals in Dong Thap, Dak Lak, Khanh Hoa, and Thua Thien Hue also participated as part of the Vietnam Initiative on Zoonotic Infections (VIZIONS) initiative (14). Patients were admitted at the discretion of the treating clinician and were excluded from each of the studies if they had suspected or confirmed intussusception, multiple complications unrelated to diarrheal disease, or suspected antimicrobial-induced diarrhea. Diarrhea was defined as three watery or loose stools within 24 h or one episode of bloody and/or mucoid diarrhea (15). After collection in a sterile container, fresh stool samples were stored at 4°C at the sites and transported to the central study microbiology laboratory within 24 h. The specimens were tested using microbiological culture and real-time PCR and then stored in 20% glycerol solution at −80°C.

This study consisted of three components. First, we selected 172 samples with known etiologies from microbiological culture for validation of the xTAG GPP system. Second, we chose an additional 307 specimens that were negative by conventional culture methods, microscopy, and real-time PCR to test with the xTAG GPP to detect pathogens that we may not have identified through conventional methodologies. Finally, we used all of the 479 stool tested samples to evaluate the sensitivity and specificity of the xTAG GPP for seven pathogens by using culture and real-time PCR as the gold standards.

**Microbial culture and molecular detection of bacterial pathogens.** Stool specimens were cultured on blood agar, MacConkey agar, xylose-lysine-deoxycholate agar, selenite broth, and *Campylobacter* media (Oxoid, Basingstoke, United Kingdom) to isolate *Shigella* spp., *Salmonella* spp., and *Campylobacter* species. Apart from *Campylobacter* plates, which were incubated microaerophilically at 42°C, all media were incubated at 37°C overnight and then examined for growth, as previously described (16). Colonies with a morphology indicative of a named pathogen were subcultured onto nutrient agar for purity, and bacterial identity was confirmed by either API 20E (bioMérieux, France) or serotyping, where applicable. Specific serotypes of *Shigella* spp. and *Salmonella* spp. were identified by slide agglutination with antigen-grouping sera and monovalent antisera, and *Campylobacter jejuni* was differentiated from *Campylobacter coli* by the hippurate hydrolysis test.

All stool specimens were also screened for the presence of *Shigella* spp. (target, *ipaH*), *Salmonella* spp., and *Campylobacter* spp. (*C. jejuni* target, *hipO*; *C. coli* target, *glyA*) by real-time PCR using the following cycling conditions: 95°C for 15 min, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s, as described previously (17, 18). *Salmonella* screening was performed using an in-house assay targeting the *invA* gene (forward primer, 5′-TCATCGCACCGTCAAARGA-3′; reverse primer, 5′-CGATTTGAARGCCGGTATTATT-3′; probe, 5′-FAM-ACGCTTCGCCGTTCRCGYGC-BHQ1-3′ FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1), under the following conditions: 95°C for 15 min, 45 cycles of 95°C for 5 s, and 60°C for 60 s (19).

**Molecular detection of rotavirus, norovirus GI/GII, and adenovirus.** For rotavirus and norovirus molecular testing, total RNA was extracted from fresh stool samples, reverse transcribed into cDNA, and used as the template to detect viruses by real-time PCR, as previously described (20). Rotavirus detection was performed by targeting the nonstructural protein 3 (NSP3). The norovirus primers and probes targeted the open reading frame 1 (ORF1)-ORF2 junction of norovirus genotype 1/2 (GI/GII) (21). PCR amplifications for rotavirus and norovirus GI/GII were performed using RNA Master hydrolysis probes (Roche Applied Sciences, United Kingdom) and optimized with 1.4 μl of activator on a LightCycler 480II (Roche Applied Sciences). Five microliters of RNA was mixed with a concentration of 20 μM for each primer and 10 μM probe, and thermal cycling was initiated at 61°C for 5 min for reverse transcription, 5 min at 95°C for amplification, and then by 45 cycles at 95°C for 5 s and 60°C for 45 s. Adenovirus real-time PCR amplification was performed using primers AdV-F (TCTTACAAAGTGCGC TTTACGC) and AdV-R (TTAAAGCTGGGRCCACGATC) and probe AdV-probe (Cy5-GACAACCGGGTKTTGGACATGGCCAG-BHQ3). Adenovirus DNA was mixed with a concentration of 20 μM for each primer and 10 μM probe and LightCycler 480 probes master (Roche Applied Sciences) and subjected a PCR cycle of 95°C for 5 min, followed by 45 cycles of 95°C for 5 s and 60°C for 1 min.

The assay was validated with positive controls using a previously described procedure (18).

**xTAG gastrointestinal pathogen panel testing.** The xTAG GPP assay is a multiplex assay that simultaneously detects *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *C. difficile* (toxins A and B), enterotoxigenic *E. coli* (ETEC) heat labile (LT) and heat stable (ST) enterotoxins, *E. coli* O157, Shiga toxin-producing *E. coli* (STEC) spp., *Vibrio cholerae*, *Yersinia enterocolitica*, fecal adenovirus 40/41, rotavirus A, norovirus GI/GII, *Giardia* spp., *E. histolytica*, and *Cryptosporidium* species. For nucleic acid extraction, 200 μl of each stool specimen was automatically extracted with 10 μl of xTAG MS2 (internal control) using the MagNA Pure-96 machine (Roche), according to the manufacturer's instructions. The xTAG GPP assay was performed according to the manufacturer's recommendations (Luminex Molecular Diagnostics, Austin, TX, USA).

**Statistical analysis.** The Luminex data were read by the TDAS software and imported and analyzed in Stata version 11 (College Station, TX, USA). Plots were made in R version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria) using the *ggplot2* package (22). Sensitivity and specificity were calculated separately using two gold standards: (i) microbiological culture and (ii) real-time PCR. As the specificity of the original Luminex cutoffs for *Salmonella* spp. was low (~60%), we attempted to redefine the cutoffs to improve the sensitivity and specificity for the *Salmonella* assay. To evaluate the capacity of probes 1 and 2 in discriminating between positive and negative *Salmonella* infection, the area under the receiver operating characteristic (ROC) curve was computed for univariate logistic regression models (containing either probe 1 or 2 alone as a predictor) and a multivariate logistic regression model (containing both probes 1 and 2 as predictors). The optimal cut points for probe 1, probe 2, or for probes 1 and 2 combined were estimated using the Youden index method (in which the sum of sensitivity and specificity of the logistic models were maximized) (23).

**Ethical approval.** Stool samples were collected from three studies, all of which were approved by the local ethical committees and Oxford Trop-

**TABLE 1** Number of positive Luminex xTAG GPP results on samples known to be positive by microbiological culture and real-time PCR

| Pathogen detected by method | No. detected by culture/real-time PCR | No. detected by GPP | % positive agreement |
|---|---|---|---|
| Culture | | | |
| *Shigella* | 40 | 40 | 100 |
| *Salmonella* | 40 | 38 | 95.0 |
| *Campylobacter* | 30 | 27 | 90.0 |
| Real-time PCR | | | |
| Rotavirus | 68 | 63 | 92.6 |
| Norovirus GI/GII | 58 | 54 | 93.1 |
| Adenovirus | 20 | 17 | 85.0 |
| *Shigella* | 44 | 44 | 100 |
| *Salmonella* | 48 | 45 | 93.8 |
| *Campylobacter* | 40 | 36 | 90.0 |

ical Research Ethics Committee (OxTREC) (no. 0109, 15-12, and 1045-13). Written informed consent was required for all patients or from parents/legal guardians if the patient was a child prior to enrollment into all three studies.

## RESULTS

**Baseline results.** Overall, 479 stool samples were collected from 92 adults (>15 years) and 387 children (≤15 years old) admitted to the hospital with diarrheal disease in Vietnam between 2009 and 2014. The median age of the adult patients was 50 years (interquartile range [IQR], 33 to 64 years), and 39% (36/92 patients) were male. In children, the median age was 16.5 months (IQR, 6.7 to 20 months), with 57% (221/387 patients) being male. A total of 105 samples (22%) were positive for a bacterial pathogen by microbiological culture, 268 samples (56%) were positive for a pathogen by real-time PCR, and 404 samples (84%) were positive for a pathogen by the xTAG GPP. From the 479 tested samples, the most commonly identified pathogens through the xTAG GPP platform were *Salmonella* ($n$ = 210 [44%]), rotavirus ($n$ = 121 [25%]), norovirus GII ($n$ = 89 [19%]), *Shigella* ($n$ = 91 [19%]), and *Cryptosporidium* ($n$ = 83 [17%]). Though less common, the xTAG GPP also detected *C. difficile* ($n$ = 45 [9%]), ETEC ($n$ = 40 [8%]), *Giardia* ($n$ = 15 [3%]), norovirus GI ($n$ = 15 [3%]), *E. coli* O157 ($n$ = 11 [2%]), Shiga toxin-producing *E. coli* (STEC) ($n$ = 8 [2%]), and *E. histolytica* ($n$ = 4 [1%]).

**Validation.** To initially validate the xTAG GPP, 40 stool samples positive for *Shigella* spp., 40 samples positive for *Salmonella* spp., and 30 samples positive for *Campylobacter* spp. by microbiological culture were retested using the xTAG GPP, as shown in Table 1. The proportion of positive agreement of xTAG GPP and microbiological culture was high, ranging from 90 to 100%. Furthermore, using stool samples, a previously detected viral pathogen by real-time PCR, the xTAG GPP, had similarly high agreement (90.5 to 93.0%) in 68 samples positive for rotavirus, 58 samples positive norovirus GI/GII, and 17 samples positive for adenovirus 40/41 infections.

**Negative-specimen evaluation.** A total of 307 fecal samples that were negative by microbiological culture and real-time PCR were tested by xTAG GPP. *Salmonella* was the most frequently detected pathogen, present in 45% (138/307) of the initially negative specimens. Further, rotavirus and *Shigella* spp. were identi-

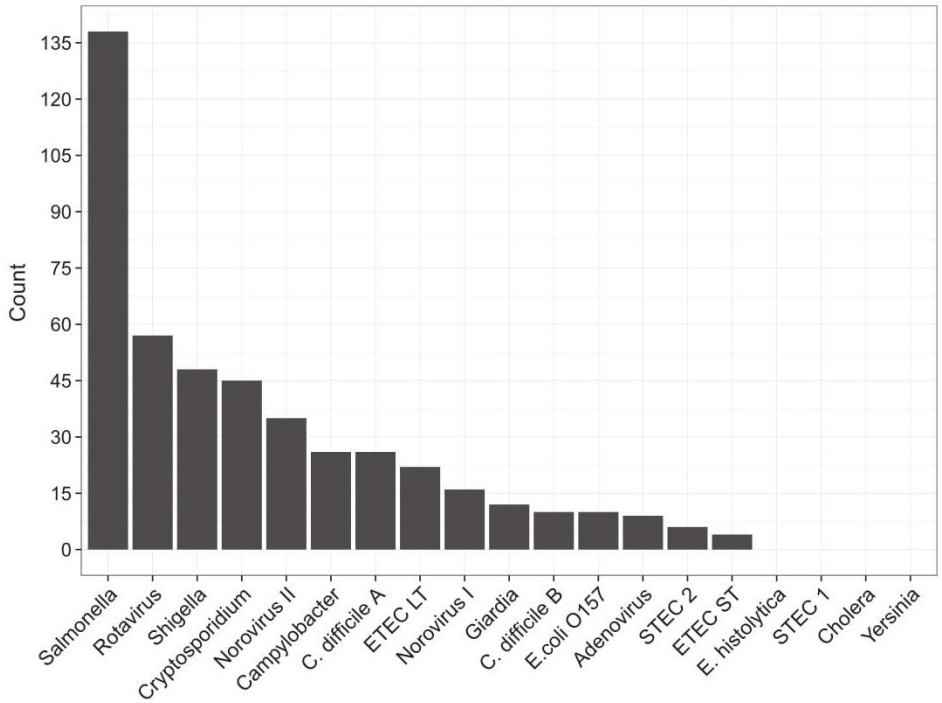


**FIG 1** Count of pathogens detected by the Luminex xTAG gastrointestinal pathogen panel in 307 specimens that tested negative by microbiological culture and real-time PCR.

**TABLE 2** Sensitivity and specificity of Luminex xTAG GPP in comparison to real-time PCR and microbiological culture[a]

| Organism detected | xTAG GPP compared to real-time PCR | | | | | xTAG GPP compared to culture | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | GPP result | No. with real-time PCR result of: | | Sensitivity (% [95% CI]) | Specificity (% [95% CI]) | GPP result | No. with culture result of: | | Sensitivity (% [95% CI]) | Specificity (% [95% CI]) |
| | | Pos | Neg | | | | Pos | Neg | | |
| *Shigella* | Pos | 86 | 6 | 95.6 (89.0–98.8) | 98.5 (96.7–99.4) | Pos | 40 | 51 | 100 (91.2–100) | 88.4 (85.0–91.2) |
| | Neg | 4 | 383 | | | Neg | 0 | 388 | | |
| *Salmonella* | Pos | 84 | 128 | 90.3 (82.4–95.5) | 66.8 (61.9–71.5) | Pos | 38 | 172 | 95.0 (83.1–99.4) | 60.8 (56.1–65.4) |
| | Neg | 9 | 258 | | | Neg | 2 | 267 | | |
| *Campylobacter* | Pos | 59 | 3 | 90.8 (81.0–96.5) | 99.3 (97.9–99.8) | Pos | 27 | 35 | 90.0 (73.5–97.9) | 92.2 (89.3–94.5) |
| | Neg | 6 | 411 | | | Neg | 3 | 414 | | |
| *Adenovirus* | Pos | 23 | 5 | 92.0 (74.0–99.0) | 98.9 (97.4–99.6) | | | | | |
| | Neg | 2 | 449 | | | | | | | |
| Norovirus GI | Pos | 15 | 5 | 88.2 (63.6–98.5) | 98.9 (97.5–99.6) | | | | | |
| | Neg | 2 | 457 | | | | | | | |
| Norovirus GII | Pos | 85 | 4 | 96.6 (90.4–99.3) | 99.0 (97.4–99.7) | | | | | |
| | Neg | 3 | 387 | | | | | | | |
| Rotavirus | Pos | 117 | 4 | 92.9 (86.9–96.7) | 98.9 (97.1–99.7) | | | | | |
| | Neg | 9 | 349 | | | | | | | |

[a] GPP, gastrointestinal pathogen panel; Pos, positive; Neg, negative; 95% CI, confidence interval.

fied in 19% (57/307) and 16% (48/307) of the negative specimens, respectively. Additionally, several significant enteric pathogens that are not well studied (or even previously described in Vietnam) due to a lack of diagnostic capacity were also identified, including *Cryptosporidium* (15% [45/307 specimens]), *C. difficile* (12% [36/307 specimens]), and pathogenic *E. coli* (14% [42/307 specimens]), as shown in Fig. 1.

**Performance evaluation on clinical specimens.** Table 2 shows the performance of xTAG GPP in comparison to both a positive microbiological culture and a real-time PCR positive gold standard for seven enteric pathogens, including *Shigella* spp., *Campylobacter* spp., and *Salmonella* spp., and also rotavirus, norovirus GI/GII, and adenovirus. The xTAG GPP had high sensitivity for all pathogens, ranging from 88.2% to 100% using both culture and real-time PCR as gold standards. The specificity of xTAG GPP was also moderately high for most of the detected bacterial and viral pathogens, ranging from 88.4% to 99.3%, with the notable exception of *Salmonella* species. A total of 128 and 172 stool specimens were negative for *Salmonella* spp. by real-time PCR and culture, respectively, but were positive by the xTAG GPP assay, resulting in specificities of the xTAG GPP assay of 60.8% and 66.8% for *Salmonella* in comparison to real-time PCR and culture, respectively. However, when we attempted to redefine the cutoff values to improve the specificity, we found no adjusted cutoff rules that significantly improved the sum of the sensitivity and specificity for probes 1 and 2 combined compared to that of the originally defined cutoffs, according to the manufacturer's instructions.

**Coinfection.** Of all evaluated stool samples with a detected pathogen, 58% (233/404 samples) had more than one enteric pathogen identified by the xTAG GPP assay. The majority of coinfections had two pathogens detected, although the number of detected pathogens was as high as seven in one sample. As shown in Fig. 2, *Cryptosporidium* spp. and ETEC were the most common pathogens detected in stool samples, with other pathogens, including *E. histolytica*, *Salmonella* spp., rotavirus, and norovirus with *Cryptosporidium* spp. and STEC, *C. difficile*, and adenovirus with ETEC. Additionally, norovirus and rotavirus were commonly identified in the same sample. Notably, patients with *Campylobacter* infections tended to have negative correlations with most other pathogens, suggesting a higher prevalence of monoinfection.

## DISCUSSION

Diarrheal disease is the second most common cause of death in children <5 years of age globally (3). The need for rapid and reliable diagnostics is important for treatment decisions and is even more pressing given the alarming trends of antimicrobial resistance in many of the most common bacterial enteric pathogens (24), which may in part be due to the unregulated and excessive use of antimicrobials worldwide. In this study, we demonstrate that the multiplex molecular Luminex xTAG GPP detection system is both highly sensitive and specific compared to both microbiological culture and real-time PCR for the majority of the surveyed pathogens. Additionally, the xTAG GPP system allowed for identification of a number of important pathogens, including ETEC and *Cryptosporidium* spp., which are not detected through routine procedures in hospitals in Vietnam or across other parts of Southeast Asia.

The sensitivity and specificity of the xTAG GPP were >88% for all compared pathogens (three bacteria and four viruses) using both culture and real-time PCR; however, a notable exception was *Salmonella*. Our sensitivity and specificity findings are similar to, if

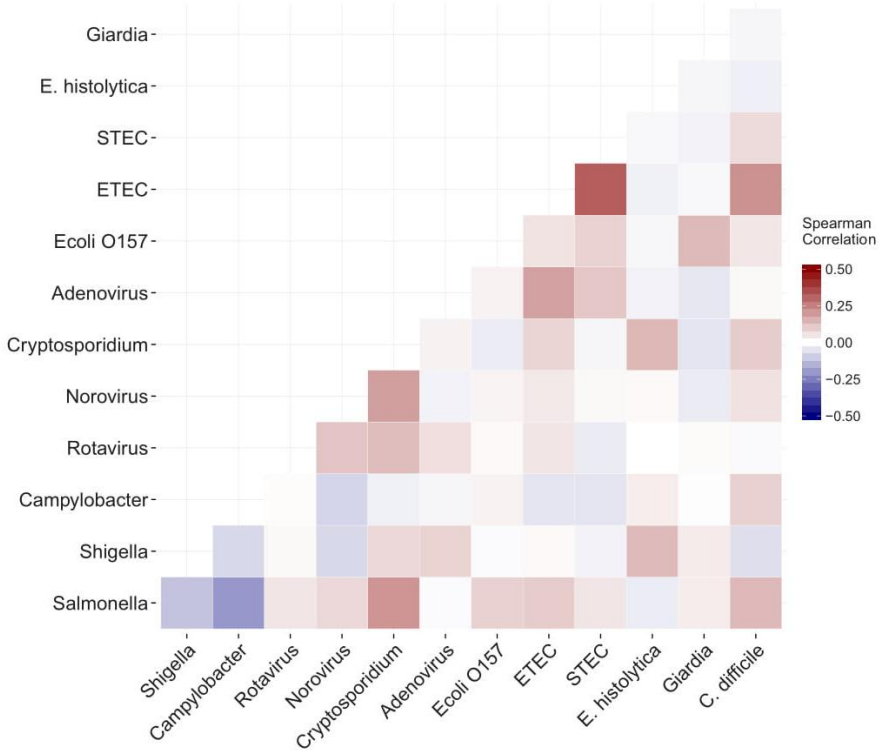


**FIG 2** Correlation matrix of coinfections from 479 diarrheal stool samples. Common pathogens detected with the Luminex xTAG gastrointestinal pathogen panel are listed in a matrix format along the *x* and *y* axes. The color within each square represents the Spearman correlation coefficient for each pairwise coinfection. The darker the red, the more positive the correlation, and the darker the blue, the more negative the correlation. Note that the range of the color scale spans −0.5 to 0.5.

somewhat lower than, those of other studies conducted in the North America, Europe, and China (11–13, 25). The low specificity (~60%) of *Salmonella* detection in our study has not commonly been reported in other studies, although Wessels et al. (26) found a higher rate of false-positive *Salmonella* detection in a small study from the Netherlands. This may be due to a high rate of asymptomatic carriage with *Salmonella* in Vietnamese children (8) or cross-reactivity with an alternative component of the gastrointestinal microbiota present in this population. The low specificity of the xTAG for detecting *Salmonella* likely led to an artificially inflated rate of overall coinfection in our samples (58% of positive samples) compared to that in work conducted elsewhere (13, 25, 27, 28). However, the rates of pairwise coinfections, such as *C. difficile* and *Cryptosporidium* spp., are similar to those found in a multicenter Italian study (29). An obvious issue raised by the high rate of coinfection and the use of the xTAG GPP in general is pathogenicity. Platts-Mills and colleagues (5) recently found that *Cryptosporidium* spp., STEC, and *Shigella* spp. were more commonly associated with severe diarrhea than other pathogens through a birth cohort that sampled healthy and diarrheal stools. In order to investigate relative pathogenicity in the future, use of the xTAG GPP platform in combination with a quantitative technique (30) may provide more insight into the relationship between the presence of a pathogen and clinical disease.

Through the use of the xTAG GPP platform, we were able to identify a far wider range of pathogens than those normally screened in hospitals and in research studies in Vietnam. *Cryptosporidium* for example, was the fifth most common pathogen detected in our sample set (17%), although almost nothing is known about the epidemiology of this parasite in Vietnam. Globally, *Cryptosporidium* is known to be common in young children with moderate-to-severe diarrhea and can commonly be identified at a very high rate (15 to 25%) in children with persistent diarrhea (4, 31). Because the intestinal damage due to infection with *Cryptosporidium* can result severe long-term sequelae (32), specific studies on this parasite and its epidemiology are warranted. Additionally, 9% of the patients in our cross-sectional sample had *C. difficile* detected in their stool. *C. difficile* is known to be much less common in children than adults, and asymptomatic infection with toxigenic strains is common (33–35) and may represent a reservoir for infection (36), although additional investigation into the epidemiology and clinical significance of *C. difficile* infection in children in this setting is warranted. The xTAG GPP assay allows for the detection of such important pathogens and may aid not only in treatment decisions but also in the generation of hypotheses for further epidemiological and etiological studies.

While the benefits of the xTAG GPP system are numerous, a significant drawback for use in a country, such as Vietnam, is the cost. The price of a single xTAG GPP reaction is currently prohibitive for Vietnam, although recent work from the United King-

dom has demonstrated that routine use of the multiplex diagnostic led to massive savings at the hospital level due to rapid identification of noninfectious diarrheal disease and subsequent reduction of the need for single-patient rooms that far exceeded the cost of implementing the xTAG GPP itself (37). Whether or not such economic analyses are applicable to Vietnam is questionable, and future such analyses tailored to industrializing regions are recommended. An additional drawback is the lack of an organism for antimicrobial susceptibility testing. Given the rampant antimicrobial resistance found in pathogens, such as *Shigella* spp., in Vietnam (8), this is a significant limitation.

Our study has several limitations. First, the microbiological culture was performed at different laboratories across Vietnam and may have introduced variability into our results. Second, we were not able to identify some pathogens, such as *V. cholerae* or *Yersinia*, so we were unable to evaluate the performance of the xTAG GPP against these pathogens, which may be more relevant in other settings across the region. Notwithstanding these limitations, our results shed valuable insight into the accuracy of the xTAG GPP system and its utility in an industrializing setting.

In conclusion, the Luminex xTAG GPP diagnostic platform is highly sensitive and specific for common bacterial and viral enteric pathogens in Vietnam, with the notable exception of *Salmonella* species. Furthermore, the xTAG GPP platform allowed for detection of a number of important pathogens that are not routinely identified through current hospital laboratory procedures in Vietnam. While there are a number of benefits to the xTAG GPP platform, cost and lack of an organism for antimicrobial susceptibility testing may represent barriers to widespread use in low-income regions. However, the use of such a diagnostic in locations, like HCMC, Vietnam, would dramatically improve the ability of clinicians to appropriately treat gastrointestinal infections in a timely manner, provide evidence for a more prudent use of antimicrobials, and allow for an unprecedented level of epidemiological granularity to aid in prevention and control efforts for diarrheal disease.

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## REFERENCES

1. **Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, Rudan I, Campbell H, Cibulskis R, Li M, Mathers C, Black RE, Child Health Epidemiology Reference Group of WHO and UNICEF.** 2012. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet **379:**2151–2161. http://dx.doi.org/10.1016/S0140-6736(12)60560-1.
2. **Murray CJL, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V, Abraham J, Ackerman I, Aggarwal R, Ahn SY, Ali MK, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Bahalim AN, Barker-Collo S, Barrero LH, Bartels DH, Basáñez MG, Baxter A, Bell ML, Benjamin EJ, Bennett D, Bernabé E, Bhalla K, Bhandari B, Bikbov B, Bin Abdulhak A, Birbeck G, Black JA, Blencowe H, Blore JD, Blyth F, Bolliger I, Bonaventure A, Boufous S, Bourne R, Boussinesq M, Braithwaite T, Brayne C, Bridgett L, Brooker S, et al.** 2012. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet **380:**2197–2223. http://dx.doi.org/10.1016/S0140-6736(12)61689-4.
3. **Walker CLF, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, O'Brien KL, Campbell H, Black RE.** 2013. Global burden of childhood pneumonia and diarrhoea. Lancet **381:**1405–1416. http://dx.doi.org/10.1016/S0140-6736(13)60222-6.
4. **Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque ASGS, Zaidi AKM, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acácio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM.** 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet **382:**209–222. http://dx.doi.org/10.1016/S0140-6736(13)60844-2.
5. **Platts-Mills JA, Babji S, Bodhidatta L, Gratz J, Haque R, Havt A, McCormick BJ, McGrath M, Olortegui MP, Samie A, Shakoor S, Mondal D, Lima IF, Hariraju D, Rayamajhi BB, Qureshi S, Kabir F, Yori PP, Mufamadi B, Amour C, Carreon JD, Richard SA, Lang D, Bessong P, Mduma E, Ahmed T, Lima AA, Mason CJ, Zaidi AK, Bhutta ZA, Kosek M, Guerrant RL, Gottlieb M, Miller M, Kang G, Houpt ER, MAL-ED Network Investigators.** 2015. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). Lancet Glob Heal **3:**e564–e575. http://dx.doi.org/10.1016/S2214-109X(15)00151-5.
6. **Tate JE, Patel MM, Cortese MM, Lopman BA, Gentsch JR, Fleming J, Steele AD, Parashar UD.** 2012. Remaining issues and challenges for rotavirus vaccine in preventing global childhood diarrheal morbidity and mortality. Expert Rev Vaccines **11:**211–220. http://dx.doi.org/10.1586/erv.11.184.
7. **Patel MM, Steele D, Gentsch JR, Wecker J, Glass RI, Parashar UD.** 2011. Real-world impact of rotavirus vaccination. Pediatr Infect Dis J **30**(1 Suppl):S1–S5. http://dx.doi.org/10.1097/INF.0b013e3181fefa1f.
8. **Thompson CN, Phan MV, Hoang NV, Minh PV, Vinh NT, Thuy CT, Nga TT, Rabaa MA, Duy PT, Dung TT, Phat VV, Nga TV, Tu le TP, Tuyen HT, Yoshihara K, Jenkins C, Duong VT, Phuc HL, Tuyet PT, Ngoc NM, Vinh H, Chinh NT, Thuong TC, Tuan HM, Hien TT, Campbell JI, Chau NV, Thwaites G, Baker S.** 2015. A prospective multi-center observational study of children hospitalized with diarrhea in Ho Chi Minh City, Vietnam. Am J Trop Med Hyg **95:**1045–1052.
9. **Nga DTT, Chuc NTK, Hoa NPQ, Hoa NPQ, Nguyen NTT, Loan HT, Toan TK, Phuc HD, Horby P, Van Yen N, Van Kinh N, Wertheim HFL.** 2014. Antibiotic sales in rural and urban pharmacies in northern Vietnam: an observational study. BMC Pharmacol Toxicol **15:**6. http://dx.doi.org/10.1186/2050-6511-15-6.
10. **Elsafi SH, Al-Maqati TN, Hussein MI, Adam AA, Hassan MMA, Al Zahrani EM.** 2013. Comparison of microscopy, rapid immunoassay, and molecular techniques for the detection of *Giardia lamblia* and *Cryptospo-*

*ridium parvum*. Parasitol Res **112:**1641–1646. http://dx.doi.org/10.1007/s00436-013-3319-1.

11. **Claas EC, Burnham CAD, Mazzulli T, Templeton K, Topin F.** 2013. Performance of the xTAG gastrointestinal pathogen panel, a multiplex molecular assay for simultaneous detection of bacterial, viral, and parasitic causes of infectious gastroenteritis. J Microbiol Biotechnol **23:**1041–1045. http://dx.doi.org/10.4014/jmb.1212.12042.
12. **Navidad JF, Griswold DJ, Gradus MS, Bhattacharyya S.** 2013. Evaluation of Luminex xTAG gastrointestinal pathogen analyte-specific reagents for high-throughput, simultaneous detection of bacteria, viruses, and parasites of clinical and public health importance. J Clin Microbiol **51:**3018–3024. http://dx.doi.org/10.1128/JCM.00896-13.
13. **Deng J, Luo X, Wang R, Jiang L, Ding X, Hao W, Peng Y, Jiang C, Yu N, Che X.** 2015. A comparison of Luminex xTAG gastrointestinal pathogen panel (xTAG GPP) and routine tests for the detection of enteropathogens circulating in southern China. Diagn Microbiol Infect Dis **83:**325–330.
14. **Rabaa MA, Tue NT, Phuc TM, Carrique-Mas J, Saylors K, Cotten M, Bryant JE, Dang H, Nghia T, Cuong Van N, Pham HA, Berto A, Phat VV, Thi T, Dung N, Bao LH, Hoa NT, Wertheim H, Nadjm B, Monagin C, Doorn Van HR, Rahman M, Phan M, Tra V, Campbell JI, Boni MF, Thi P, Tam T, Hoek Van Der L, Simmonds P, Rambaut A, Toan TK, Chau NVV, Hien TT, Wolfe N, Farrar JJ, Thwaites G, Kellam P, Woolhouse MEJ, Baker S.** 2015. The Vietnam Initiative on Zoonotic Infections (VIZIONS): a strategic approach to studying emerging zoonotic infectious diseases. Ecohealth **12:**726–735. http://dx.doi.org/10.1007/s10393-015-1061-0.
15. **World Health Organization.** 2005. Treatment of diarrhoea: a manual for physicians and other senior health workers. World Health Organization, Geneva, Switzerland. http://apps.who.int/iris/bitstream/10665/43209/1/9241593180.pdf.
16. **My PVT, Thompson C, Phuc Le H, Tuyet PTN, Vinh H, Hoang NVM, Minh Van P, Vinh NT, Thuy CT, Nga TTT, Hau NTT, Campbell J, Chinh NT, Thuong TC, Tuan HM, Farrar J, Baker S.** 2013. Endemic norovirus infections in children, Ho Chi Minh City, Vietnam, 2009–2010. Emerg Infect Dis **19:**29–32. http://dx.doi.org/10.3201/eid1901.120044.
17. **Vu DT, Sethabutr O, Von Seidlein L, Tran VT, Do GC, Bui TC, Le HT, Lee H, Houng HS, Hale TL, Clemens JD, Mason C, Dang DT.** 2004. Detection of *Shigella* by a PCR assay targeting the *ipaH* gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. J Clin Microbiol **42:**2031–2035. http://dx.doi.org/10.1128/JCM.42.5.2031-2035.2004.
18. **LaGier MJ, Joseph LA, Passaretti TV, Musser KA, Cirino NM.** 2004. A real-time multiplexed PCR assay for rapid detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli*. Mol Cell Probes **18:**275–282. http://dx.doi.org/10.1016/j.mcp.2004.04.002.
19. **Anders KL, Thompson CN, Thuy Van NT, Nguyet NM, Tu LTP, Dung TTN, Phat VV, Van NTH, Hieu NT, Tham NTH, Ha PTT, Lien LB, Chau NVV, Baker S, Simmons CP.** 2015. The epidemiology and aetiology of diarrhoeal disease in infancy in southern Vietnam: a birth cohort study. Int J Infect Dis **35:**3–10. http://dx.doi.org/10.1016/j.ijid.2015.03.013.
20. **Dung TTN, Phat VV, Nga TVT, My PVT, Duy PT, Campbell JI, Thuy CT, Hoang NVM, Van Minh P, Le Phuc H, Tuyet PTN, Vinh H, Kien DTH, Huy HLA, Vinh NT, Nga TTT, Hau NTT, Chinh NT, Thuong TC, Tuan HM, Simmons C, Farrar JJ, Baker S.** 2013. The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting rotavirus A and norovirus. J Virol Methods **187:**138–143. http://dx.doi.org/10.1016/j.jviromet.2012.09.021.
21. **Trujillo AA, McCaustland KA, Zheng D-P, Hadley LA, Vaughn G, Adams SM, Ando T, Glass RI, Monroe SS.** 2006. Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. J Clin Microbiol **44:**1405–1412. http://dx.doi.org/10.1128/JCM.44.4.1405-1412.2006.
22. **Wickham H.** 2009. ggplot2: elegant graphics for data analysis. Springer, New York, NY.
23. **Youden WJ.** 1950. Index for rating diagnostic tests. Cancer **3:**32–35. http://dx.doi.org/10.1002/1097-0142(1950)3:1<32::AID-CNCR2820030106>3.0.CO;2-3.
24. **World Health Organization.** 2014. Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva, Switzerland. http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1.
25. **Khare R, Espy MMJ, Cebelinksi E, Boxrud D, Sloan LM, Cunningham S, Pritt B, Patel R, Binnicker M.** 2014. Comparative evaluation of two commercial multiplex panels for detection of gastrointestinal pathogens by use of clinical stool specimens. J Clin Microbiol **52:**3667–3673. http://dx.doi.org/10.1128/JCM.01637-14.
26. **Wessels E, Rusman LG, van Bussel MJAWM, Claas ECJ.** 2014. Added value of multiplex Luminex gastrointestinal pathogen panel (xTAG GPP) testing in the diagnosis of infectious gastroenteritis. Clin Microbiol Infect **20:**O182–O187. http://dx.doi.org/10.1111/1469-0691.12364.
27. **Mengelle C, Mansuy JM, Prere MF, Grouteau E, Claudet I, Kamar N, Huynh A, Plat G, Benard M, Marty N, Valentin A, Berry A, Izopet J.** 2013. Simultaneous detection of gastrointestinal pathogens with a multiplex Luminex-based molecular assay in stool samples from diarrhoeic patients. Clin Microbiol Infect **19:**E458–E465. http://dx.doi.org/10.1111/1469-0691.12255.
28. **Perry MD, Corden SA, Howe RA.** 2014. Evaluation of the Luminex xTAG gastrointestinal pathogen panel and the Savyon diagnostics gastrointestinal infection panel for the detection of enteric pathogens in clinical samples. J Med Microbiol **63:**1419–1426. http://dx.doi.org/10.1099/jmm.0.074773-0.
29. **Vocale C, Rimoldi SG, Pagani C, Grande R, Pedna F, Arghittu M, Lunghi G, Maraschini A, Gismondo MR, Landini MP, Torresani E, Topin F, Sambri V.** 2015. Comparative evaluation of the new xTAG GPP multiplex assay in the laboratory diagnosis of acute gastroenteritis. Clinical assessment and potential application from a multicentric Italian study. Int J Infect Dis **34:**33–37.
30. **Liu J, Kabir F, Manneh J, Lertsethtakarn P, Begum S, Gratz J, Becker SM, Operario DJ, Taniuchi M, Janaki L, Platts-Mills JA, Haverstick DM, Kabir M, Sobuz SU, Nakjarung K, Sakpaisal P, Silapong S, Bodhidatta L, Qureshi S, Kalam A, Saidi Q, Swai N, Mujaga B, Maro A, Kwambana B, Dione M, Antonio M, Kibiki G, Mason CJ, Haque R, Iqbal N, Zaidi AKM, Houpt ER.** 2014. Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study. Lancet Infect Dis **14:**716–724. http://dx.doi.org/10.1016/S1473-3099(14)70808-4.
31. **Checkley W, White AC, Jr, Jaganath D, Arrowood MJ, Chalmers RM, Chen X-M, Fayer R, Griffiths JK, Guerrant RL, Hedstrom L, Huston CD, Kotloff KL, Kang G, Mead JR, Miller M, Petri WA, Priest JW, Roos DS, Striepen B, Thompson RCA, Ward HD, Van Voorhis WA, Xiao L, Zhu G, Houpt ER.** 2015. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. Lancet Infect Dis **15:**85–94. http://dx.doi.org/10.1016/S1473-3099(14)70772-8.
32. **Guerrant DI, Moore SR, Lima A, Patrick PD, Schorling JB, Guerrant RL.** 1999. Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four-seven years later in a poor urban community in northeast Brazil. Am J Trop Med Hyg **61:**707–713.
33. **Bryant K, McDonald LC.** 2009. *Clostridium difficile* infections in children. Pediatr Infect Dis J **28:**145–146. http://dx.doi.org/10.1097/INF.0b013e318198c984.
34. **Larson HE, Barclay FE, Honour P, Hill ID.** 1982. Epidemiology of *Clostridium difficile* in infants. J Infect Dis **146:**727–733. http://dx.doi.org/10.1093/infdis/146.6.727.
35. **Al-Jumaili IJ, Shibley M, Lishman AH, Record CO.** 1984. Incidence and origin of *Clostridium difficile* in neonates. J Clin Microbiol **19:**77–78.
36. **Hecker MT, Riggs MM, Hoyen CK, Lancioni C, Donskey CJ.** 2008. Recurrent infection with epidemic *Clostridium difficile* in a peripartum woman whose infant was asymptomatically colonized with the same strain. Clin Infect Dis **46:**956–957. http://dx.doi.org/10.1086/527568.
37. **Goldenberg SD, Bacelar M, Brazier P, Bisnauthsing K, Edgeworth JD.** 2014. A cost benefit analysis of the Luminex xTAG gastrointestinal pathogen panel for detection of infectious gastroenteritis in hospitalised patients. J Infect **44:**504–511.

*Clinical Infectious Diseases*

MAJOR ARTICLE

# No Clinical Benefit of Empirical Antimicrobial Therapy for Pediatric Diarrhea in a High-Usage, High-Resistance Setting

**Vu Thuy Duong,[1,2] Ha Thanh Tuyen,[1] Pham Van Minh,[1] James I. Campbell,[1] Hoang Le Phuc,[2] Tran Do Hoang Nhu,[1] Le Thi Phuong Tu,[1,3] Tran Thi Hong Chau,[1] Le Thi Quynh Nhi,[1,4] Nguyen Thanh Hung,[2] Nguyen Minh Ngoc,[5] Nguyen Thi Thanh Huong,[5] Lu Lan Vi,[6] Corinne N. Thompson,[7] Guy E. Thwaites,[1,7] Ruklanthi de Alwis,[1,7] and Stephen Baker[1,7,8]**

[1]The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University Clinical Research Unit and [2]Children's Hospital 1, Ho Chi Minh City, Vietnam; [3]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom; [4]University of Medicine and Pharmacy at Ho Chi Minh City, [5]Children's Hospital 2, and [6]The Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam; and [7]Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University and [8]The Department of Medicine, University of Cambridge, United Kingdom

***Background.*** Pediatric diarrheal disease presents a major public health burden in low- to middle-income countries. The clinical benefits of empirical antimicrobial treatment for diarrhea are unclear in settings that lack reliable diagnostics and have high antimicrobial resistance (AMR).

***Methods.*** We conducted a prospective multicenter cross-sectional study of pediatric patients hospitalized with diarrhea containing blood and/or mucus in Ho Chi Minh City, Vietnam. Clinical parameters, including disease outcome and treatment, were measured. *Shigella*, nontyphoidal *Salmonella* (NTS), and *Campylobacter* were isolated from fecal samples, and their antimicrobial susceptibility profiles were determined. Statistical analyses, comprising log-rank tests and accelerated failure time models, were performed to assess the effect of antimicrobials on disease outcome.

***Results.*** Among 3166 recruited participants (median age 10 months; interquartile range, 6.5–16.7 months), one-third (1096 of 3166) had bloody diarrhea, and 25% (793 of 3166) were culture positive for *Shigella*, NTS, or *Campylobacter*. More than 85% of patients (2697 of 3166) were treated with antimicrobials; fluoroquinolones were the most commonly administered antimicrobials. AMR was highly prevalent among the isolated bacteria, including resistance against fluoroquinolones and third-generation cephalosporins. Antimicrobial treatment and multidrug resistance status of the infecting pathogens were found to have no significant effect on outcome. Antimicrobial treatment was significantly associated with an increase in the duration of hospitalization with particular groups of diarrheal diseases.

***Conclusions.*** In a setting with high antimicrobial usage and high AMR, our results imply a lack of clinical benefit for treating diarrhea with antimicrobials; adequately powered randomized controlled trials are required to assess the role of antimicrobials for diarrhea.

**Keywords.** nontyphoidal *Salmonella*; *Campylobacter*; *Shigella*; pediatric diarrhea; antimicrobial resistance; multidrug resistance; fluoroquinolones; disease outcome.

Diarrhea remains the second most significant cause of morbidity and mortality in children aged <5 years worldwide [1]. In 2010, the global burden of diarrhea was estimated to be 1.73 billion episodes, of which 36 million were characterized as moderate or severe; 26% (9.3 million) of the severe episodes were estimated to arise in Southeast Asia [1]. Among the bacterial pathogens causing diarrhea in children *Campylobacter* spp., nontyphoidal *Salmonella* (NTS), *Shigella* spp., *Escherichia coli,* and *Yersinia enterocolitica* are the most commonly identified [2, 3]. *Campylobacter*, NTS, and *Shigella* are major contributors to the global morbidity of diarrhea and account for an estimated 7.5, 7.1, and 4.8 million disability-adjusted life years, respectively; the majority of these occur in low- to middle-income countries (LMICs) [4].

The prevalence of all-cause diarrhea in children aged <5 years in Vietnam is estimated to be 7%–11% (Multiple Indicator Cluster Survey in 2000, 2006, and 2011 [5]) and accounts for as much as 12% of all-cause deaths in this age group [6]. A large burden of diarrheal disease, combined with the lack of financial and diagnostic resources in Vietnam, means that the causative agents of most diarrheal episodes are never determined. As a consequence, antimicrobials are empirically administered to children with diarrhea based solely on clinical presentation. A previous study found that antimicrobials were prescribed in 38% of acute watery diarrhea episodes in Vietnamese children found to be associated with a viral pathogen and in 60% of cases with unknown etiology [7]. The excessive use of antimicrobials in animals and humans in Southeast Asia has led to

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Correspondence: S. Baker, The Hospital for Tropical Diseases, 764 Vo Van Kiet, Quan 5, Ho Chi Minh City, Vietnam (sbaker@oucru.org).

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the current antimicrobial resistance (AMR) crisis in the region, with increasing resistance against many first-line antimicrobials, including fluoroquinolones and third-generation cephalosporins, in many gram-negative pathogens across the region [8]. Therefore, a better understanding of the bacterial agents of diarrhea, their corresponding AMR profile, the impact of antimicrobial treatment on clinical outcome, and the effects of empirical antimicrobials is required.

Little has been reported regarding the disease burden and clinical management of hospitalized pediatric diarrheal diseases in Vietnam. Although NTS, *Campylobacter,* and *Shigella* have been identified as major bacterial causes of diarrhea in Vietnamese children [7, 9–11], the epidemiology, AMR profiles, treatment, and the associated outcome of these bacteria in pediatric diarrhea have not been not well described. The Vietnamese healthcare system currently follows the World Health Organization (WHO) guidelines for treatment of pediatric diarrhea. These guidelines recommend the use of low-osmolarity oral rehydration solution, zinc, and ciprofloxacin or 1 of the 3 alternatives (pivmecillinam, azithromycin, or ceftriaxone) for all patients with bloody diarrhea, irrespective of age [12, 13]. However, as the prevalence of AMR in enteric pathogens increases across the region, it is uncertain how existing guidelines correspond to circulating AMR profiles, antimicrobial treatment practices, and patient outcomes in children hospitalized with diarrhea. Therefore, we conducted a prospective multicenter cross-sectional study in Ho Chi Minh City to improve the understanding of bacteria-associated diarrhea in Vietnamese children and to assess the duration of hospital stay in diarrheal patients infected with bacterial pathogens and receiving antimicrobials.

## MATERIALS AND METHODS

### Ethics

Ethical approval for this study was provided by the ethics committees of all 3 participating local hospitals and the University of Oxford Tropical Research Ethics Committee (OxTREC No. 1045-13). Written consent from parents or legal guardians of all participants was obtained before enrollment.

### Study Design and Enrollment

This study was a prospective, observational, multicenter cross-sectional study to evaluate the etiology, epidemiology, and outcomes in children (aged <16 years) hospitalized for diarrhea. Study participants were recruited from 3 tertiary hospitals (Children's Hospital 1, Children's Hospital 2, and the Hospital for Tropical Diseases) in Ho Chi Minh City, Vietnam, from May 2014 to April 2016.

Children hospitalized with diarrhea, defined as ≥3 passages of loose stools within 24 hours [12] with ≥1 loose stool containing blood and/or mucus, were recruited into the study. Based on characteristics of the diarrheal stools and the duration of illness, participants were classified into 3 groups: acute nonbloody diarrhea (diarrhea with mucus, <14 days), acute bloody diarrhea (diarrhea with blood, <14 days), and persistent diarrhea (diarrhea with mucus and/or blood, ≥14 days). Children were not eligible if they had suspected or confirmed intussusception at the time of enrollment. After enrollment, a short questionnaire was completed, and a fecal sample was collected and processed within 24 hours. All enrolled patients were provided with the routine standard-of-care practices at each hospital. Treatment and proxy outcomes, including patient recovery status at 3 days after enrollment and duration of hospitalization, were recorded by clinical staff at study sites. Patient status was recorded as "recovered" if the patient had <3 passages of loose stools in the past 24 hours or "improved" if the patient had fewer episodes of diarrhea and/or less mucus and/or blood compared with status at enrollment.

### Microbiological Methods

Fecal specimens were inoculated onto MacConkey agar (MC agar; Oxoid) and xylose-lysine-deoxycholate agar (Oxoid) and into selenite broth (Oxoid) and incubated at 37°C for 18–24 hours. *Salmonella* and *Shigella* were detected based on their characteristic appearance on xylose-lysine-deoxycholate and MC agar and confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker) and API20E (bioMerieux), following the manufacturer's guidelines. *Campylobacter* was identified using *Campylobacter* selective agar (Oxoid) under microaerophilic conditions, followed by Gram staining and microscopy.

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid) for *Salmonella* and *Shigella,* and on blood agar containing 5% sheep blood for *Campylobacter* and interpreted using Clinical and Laboratory Standards Institute guidelines [14] (Supplementary Table S1). Multidrug resistance (MDR) was defined as nonsusceptibility to ≥1 agent in ≥3 antimicrobial categories listed in Supplementary Table S1. Microbiology results were reported to the collaborating hospitals within 3 days of sampling.

### Statistical Analysis

Data were analyzed using Stata (version 11; StataCorp) and R (versions 3.2.2; R Foundation for Statistical Computing) software. Figures were constructed with R software, using the ggplot2 [15] and prodlim packages. Descriptive comparisons between groups were conducted using nonparametric tests, including the Fisher exact test for categorical variables and the Mann-Whitney *U* test for continuous data. Statistical comparisons between >2 groups were conducted using the $\chi^2$ test and the Kruskal-Wallis test for categorical and continuous variables, respectively. Kaplan-Meier curves for length of hospital stay were compared between groups, using log-rank tests. The growth status of participating patients was assessed using the WHO global database on growth and nutrition [16], the Institute for Clinical Systems Improvement's guidelines on preventing and managing

obesity in children and adolescents [17], and the macro package for Stata software (version 11) developed by WHO.

An accelerated failure time (AFT) regression model (incorporating all study patients hospitalized for ≥1 day) was constructed in 3 steps. First, the best AFT distribution fit was identified for the dependent variable, that is, length of hospital stay. Second, 11 demographic variables (eg, sex and age), clinical symptoms of disease severity, treatment types, and MDR were tested by means of univariate analysis, using a log-normal distribution. The 11 variables were chosen because of their potential to affect duration of hospital stay. Third, a multivariate log-normal model was constructed using a stepwise backward elimination method, where variables were removed based on the likelihood ratio test ($P < .05$).

## RESULTS

### General Characteristics of Patients Hospitalized With Diarrhea

Between May 2014 and April 2016, a total of 3166 hospitalized children meeting the study criteria were recruited at the 3 study hospitals. The majority of patients were male (1945 of 3166; 61.4%), with ages ranging from 1 month to 15 years (median age, 10 months; interquartile range [IQR], 6.5–16.7 months). Patients were hospitalized for a median of 5 days (IQR, 3–7 days) with 88.7% (2808 of 3166) of patients showing improvement or resolving symptoms within 3 days of enrollment.

Patients were segregated into 3 diarrheal types: acute nonbloody (1775 of 3166; 56.1%), acute bloody (1096 of 3166; 34.6%), or persistent (295 of 3166; 9.3%) (Table 1). Owing to the clinical complexity of persistent diarrhea, statistical comparisons were conducted between the 2 acute diarrhea groups only. Patients with nonbloody diarrhea were more likely to experience vomiting than those with bloody diarrhea (66.8% vs 38.7%; $P = .001$) and dehydration (15.0% vs 2.6%, respectively; $P = .001$). A significantly greater proportion of patients with bloody diarrhea experienced abdominal pain (26.4% vs 19.3% of those with nonbloody diarrhea; $P = .001$). Systemic C-reactive protein (CRP) concentrations were significantly higher in patients with bloody than in those with nonbloody diarrhea (21.0 [IQR, 6.7–40.2] vs 7.0 [4.0–24.0] mg/L; $P < .001$).

We cultured the 3 key bacterial diarrheal pathogens in Vietnam (ie, NTS, *Campylobacter,* and *Shigella*), isolating 816 pathogens from 804 patients (11 coinfections) and stratified clinical manifestations and treatment data by these organisms. At least 1 of these bacteria was isolated from the fecal specimens of 44.3% with bloody diarrhea (485 of 1096) and 17.9% (318 of 1775) with nonbloody diarrhea ($P < .001$) (Table 1). Overall, NTS was the most frequently isolated of the 3 bacterial pathogens from the diarrheal children, accounting for 15.1% (478 of 3166) of all diarrheal cases, followed by *Campylobacter* and *Shigella*. *Shigella* infections were more common in older children (median age, 3 years) and were associated with more severe symptoms. However, children with *Shigella* infections recovered more rapidly than those infected with NTS or *Campylobacter* (Supplementary Table S2).

### Antimicrobial Usage for Treatment of Hospitalized Diarrheal Diseases

We also recorded the treatment regimens of the enrolled patients, which included oral rehydration solution, intravenous rehydration, zinc, probiotics, and antimicrobials. More than 90% of patients were administered oral rehydration solution, and >80% were given zinc supplementation. The use of antimicrobials within this population was high, with 85.2% of patients (2697 of 3166) receiving empirical antimicrobial treatment after admission to the hospital and before a bacterial culture result was obtained. Fluoroquinolones were the most commonly used class of antimicrobials (1799 of 2697; 66.7%). Differences in standard-of-care treatment were observed between patients with bloody versus nonbloody diarrhea (Table 1); antimicrobials were more regularly administered to patients with bloody diarrhea than to those with nonbloody diarrhea ($P < .001$). Antimicrobials, specifically fluoroquinolones, were commonly (>70%) prescribed before an etiological diagnosis in those eventually found to be infected with *Campylobacter*, *Salmonella,* or *Shigella* (Supplementary Table S2).

### Antimicrobial Susceptibility

*Campylobacter*, *Salmonella,* and *Shigella* isolates displayed a high prevalence of nonsusceptibility against many of the screened antimicrobials (Figure 1). Notably, a high proportion of *Campylobacter,* NTS, and *Shigella* exhibited nonsusceptibility against ciprofloxacin: 94.2% (242 of 257), 58.4% (279 of 478), and 70.4% (57 of 81), respectively. In addition, 56.8% of *Shigella* (46 of 81) and 13.8% of NTS (66 of 478) isolated were nonsusceptible to the third-generation cephalosporins, ceftriaxone and ceftazidime. A smaller fraction of the *Campylobacter,* NTS, and *Shigella* isolates also exhibited resistance against azithromycin: 9.7% (25 of 257), 17.4% (83 of 478), and 22.2% (18 of 81), respectively. The majority of the organisms (65.4%; 531 of 816 isolates) were categorized as MDR. The prevalence of MDR was highest within the *Campylobacter* isolates (218 of 257; 84.8%), with 3 isolates also exhibiting nonsusceptibly to imipenem. The MDR prevalence in NTS and *Shigella* was 53.9% (258 of 478) and 67.9% (55 of 81), respectively (Figure 1).

### Diarrheal Disease Outcome

We assessed the effect of antimicrobial treatment on 2 proxy disease outcome measures, clinical outcome (ie, improved/recovered) at 3 days after enrollment and the duration of hospital stay. More than 80% of patients showed improvement or had recovered at 3 days after enrollment, regardless of antimicrobial treatment (Supplementary Figure S1). However, those given an antimicrobial, specifically a fluoroquinolone, had a longer hospital stay than those not receiving an antimicrobial ($P < .001$ and $P = .01$, respectively) (Figure 2). Notably, the duration of hospital stay did not differ significantly between those receiving

**Table 1. Demographic and Clinical Manifestations of Pediatric Patients Admitted With Diarrhea in Ho Chi Minh City[a]**

| Characteristics | Patients, No. (%) | | | |
|---|---|---|---|---|
| | Nonbloody Diarrhea (n = 1775)[b] | Bloody Diarrhea (n = 1096) | Persistent Diarrhea (n = 295) | *P* Value[c] |
| Sociodemographic | | | | |
| Male sex | 1135 (63.9) | 633 (57.8) | 177 (60.0) | .001 |
| Age, median [IQR], mo | 11.5 [7.6–18.5] | 9.0 [5.9–15.5] | 5.5 [4.0–8.2] | <.001 |
| Growth[d] | | | | |
| Obese or overweight | 184 (11.0) | 83 (8.3) | 24 (8.7) | .02 |
| Wasted or severely wasted | 176 (10.5) | 107 (10.6) | 25 (9.1) | .69 |
| Clinical symptoms | | | | |
| Episodes per day, median [IQR], No. | 7 [5–10] | 7 [5–10] | 7 [5–10] | ... |
| Moderate or severe dehydration[e] | 267 (15.0) | 28 (2.6) | 9 (3.1) | .001 |
| Abdominal pain | 343 (19.3) | 289 (26.4) | 50 (17.0) | .001 |
| Fever (≥37.5°C) at enrollment | 1137 (64.1) | 615 (56.1) | 83 (28.2) | .001 |
| Vomiting | 1185 (66.8) | 424 (38.7) | 101 (34.4) | .001 |
| Hematology | | | | |
| Neutrophil count, median [IQR], $10^3/\mu L$ | 4.4 [2.6–7.0] | 4.1 [2.4–6.7] | 2.5 [1.6–3.9] | .01 |
| CRP, median [IQR], mg/L | 7.0 [4.0–24.0] | 21.0 [6.7–40.2] | 5.0 [3.3–7.0] | <.001 |
| Stool culture positive | | | | .7 |
| *Campylobacter* | 100 (5.6) | 155 (14.1) | 2 (0.7) | <.001 |
| *Salmonella* | 179 (10.1) | 289 (26.4) | 10 (3.4) | <.001 |
| *Shigella* | 39 (2.2) | 41 (3.7) | 1 (0.3) | .02 |
| Treatment | | | | |
| Low-osmolarity oral rehydration solution | 1740 (98.0) | 956 (87.2) | 260 (88.1) | .8 |
| Intravenous rehydration | 278 (15.7) | 48 (4.4) | 15 (5.1) | .001 |
| Antimicrobials | 1392 (78.4) | 1079 (98.4) | 226 (76.6) | .001 |
| Fluoroquinolones[f] | 739 (53.1) | 920 (85.3) | 140 (61.9) | .001 |
| Zinc | 1586 (89.4) | 962 (87.8) | 276 (93.6) | .20 |
| Probiotics | 1395 (78.6) | 627 (57.2) | 200 (67.8) | .001 |
| Outcome | | | | |
| Hospital stay, median [IQR], d | 5 [3–6] | 4 [3–6] | 9 [4–10] | .003 |
| Improved or recovered after 3 d[g] | 1624 (91.5) | 961 (87.7) | 223 (75.6) | .008 |

Abbreviations: CRP, C-reactive protein; IQR, interquartile range.

[a]Data represent No. (%) unless otherwise specified.

[b]All children with nonbloody diarrhea had mucus in stools.

[c]*P* values represent comparisons between nonbloody and bloody diarrhea using Fisher exact test for categorical data or Mann-Whitney *U* test for continuous data.

[d]Obese: weight for length *z* score >3 standard deviations [SDs] in children aged <24 months; body mass index (BMI) for age *z* score >3 SDs in children aged ≥24 months. Overweight: weight for length *z* score >2 SDs in children aged <24 months; BMI for age *z* score >2 SDs in children aged ≥24 months. Wasted: weight for length *z* score ≤2 SDs in children aged <24 months; BMI for age *z* score ≤2 SDs in children aged ≥24 months. Severely wasted: weight for length *z* score ≤3 SDs in children aged <24 months; BMI for age *z* score ≤3 SDs in children aged ≥24 months [18].

[e]Dehydration classified as described by Basaleem and Amin [19].

[f]Percentage of those receiving antimicrobials. Fluoroquinolones included ciprofloxacin and norfloxacin.

[g]Condition was described as "recovered" if patient had <3 passages of loose stool in the past 24 hours or "improved" if patient had fewer episodes of diarrhea and less mucus and/or blood than at enrollment.

and those not receiving an antimicrobial among patients with bloody diarrhea (Figure 2C). However, antimicrobial treatment in those with nonbloody diarrhea was significantly associated with a longer hospital stay (median [IQR] hospital stay for antimicrobial vs no antimicrobial use, 5 [3–7] vs 4 [3–5] days; $P < .001$; (Figure 2C). Similarly, antimicrobial treatment in patients with low CRP levels (≤5 mg/L) was significantly associated with an increased hospital stay, compared with patients with high CRP levels (>5 mg/L) ($P < .001$; Figure 2D).

We then stratified all patients by antimicrobial treatment, and within those treated with antimicrobials we compared disease outcome (ie, duration of hospitalization) between those infected with MDR or non-MDR organisms and between those infected with fluoroquinolone-susceptible or nonsusceptible organisms. In patients empirically treated with any antimicrobial or a fluoroquinolone, >90% (526 of 564) and 70% (361 of 515) were infected with an MDR or a fluoroquinolone nonsusceptible organism, respectively. However, regardless of the MDR status and fluoroquinolone nonsusceptibility of the infecting organisms, no differences in the duration of hospitalization were observed among patients treated with antimicrobials (Figure 3). Comparable findings were observed for recovery status at 3 days after enrollment (Supplementary Figure S1).

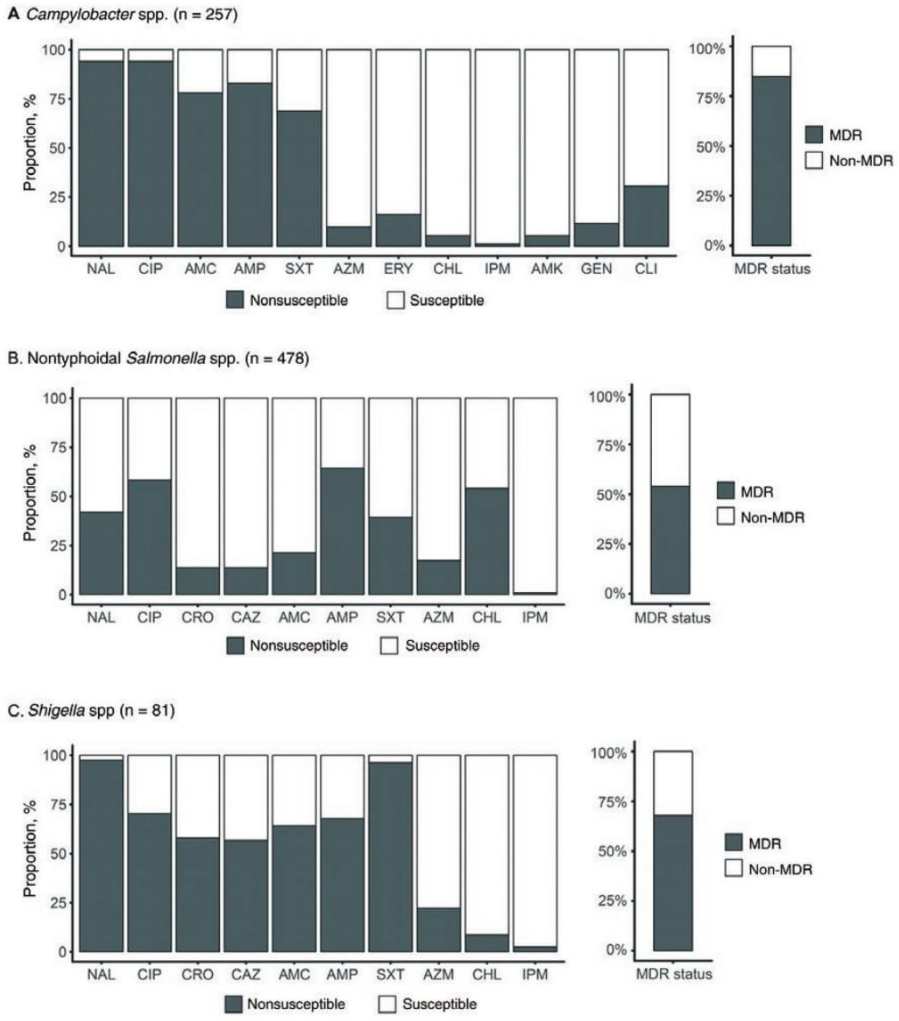


**Figure 1.** The antimicrobial resistance profiles for isolated *Campylobacter* spp. (*A*), nontyphoidal *Salmonella* spp. (*B*), and *Shigella* spp. (*C*), showing antimicrobial susceptibility and multidrug resistance (MDR), defined as nonsusceptibility to ≥1 agent in ≥3 antimicrobial categories). Bar graphs shows proportion of organisms exhibiting nonsusceptibility (dark gray) to nalidixic acid (NAL), ciprofloxacin (CIP), ceftriaxone (CRO), ceftazidime (CAZ), amoxicillin-clavulanic acid (AMC), ampicillin (AMP), trimethoprim-sulfamethoxazole (SXT), azithromycin (AZM), chloramphenicol (CHL), amikacin (AMK), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), and IPM ( ).

An AFT multiple regression model was considered to be an appropriate method for describing how each of the adjusted variables multiplicatively alters the duration of hospitalization. Therefore, an AFT was constructed to investigate the effect of antimicrobials on the duration of hospital stay, adjusting for age, disease severity, and other prescribed treatments. After adjustment for age, diarrhea presentation, and dehydration, antimicrobials were associated with a significant increase in the duration of hospital stay of diarrheal patients by a time ratio of 1.32 [1.24–1.41]. Finally, infection with an MDR organism was found to not significantly prolong hospitalization ($P = .55$; Table 2).

## DISCUSSION

Moderate-to-severe diarrhea has a significant healthcare burden in Vietnamese children [20]. Although previous observational studies of diarrhea in Vietnam have described some of the epidemiological features, the bacterial causes, and their associated AMR profiles, these studies have focused chiefly on children with acute watery diarrhea [7]. Little is known about the epidemiology and clinical management of bloody and/or mucoid diarrhea in Vietnam. Therefore, we aimed to address this paucity of data by enrolling >3000 of children hospitalized with diarrheal. This large sample size not only enabled the isolation of >800 enteric pathogens, but it also provided data regarding antimicrobial usage in medical practice and outcome. Furthermore, this study investigated the clinical role of AMR in a relevant population empirically prescribed antimicrobials at presentation to the hospital.

AMR in pathogenic bacteria, including those associated with diarrhea, is a global public health problem [21, 22]. Data

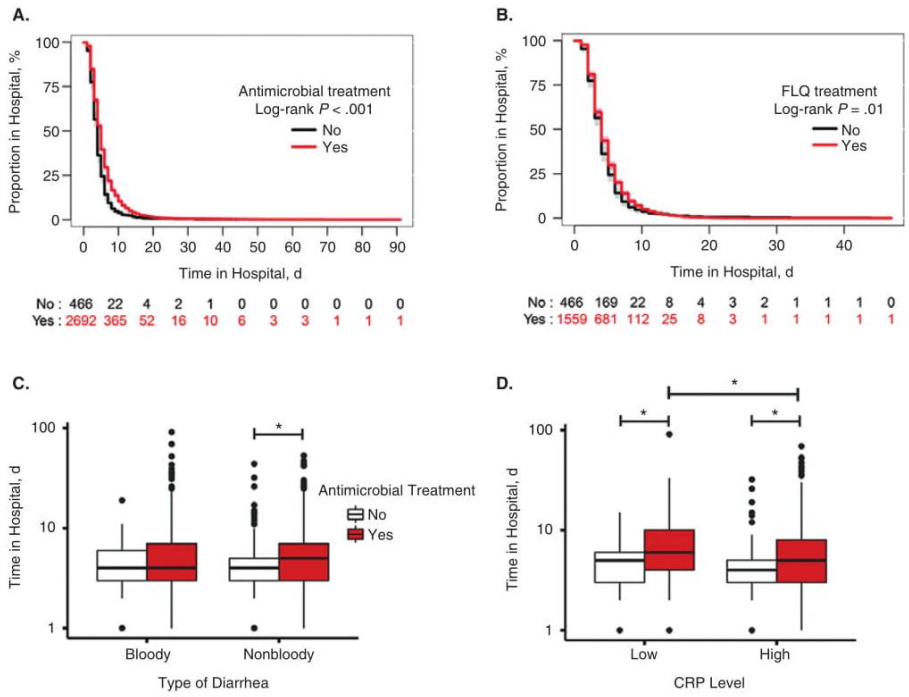


**Figure 2.** Effect of antimicrobial treatment on clinical outcome. *A, B,* Kaplan-Meier curves show days in the hospital for diarrheal children treated with antimicrobials (*A*) or specifically, fluoroquinolones (FLQs) (*B*). *C, D,* Effect of antimicrobial usage on the length of hospital stay by different diarrheal types (*C*) and blood C-reactive protein (CRP) concentration (5 mg/L cutoff) (*D*). Statistical comparisons for categorical variables were conducted using the Kruskal-Wallis test, where *.05 < *P* < .01, and *** *P* < .001. Log-rank tests were used to compare Kaplan-Meier curves for length of hospital stay between groups.

from Vietnam highlight the increasing trend in diarrheagenic bacteria of AMR to the current first-line antimicrobials, such as fluoroquinolones and third-generation cephalosporins. Moreover, despite recent increases in fluoroquinolone resistance in Asia and beyond, current guidelines still endorse the use of this class of antimicrobials to treat bloody diarrhea [23–25]. Many of the organisms isolated during this investigation were also nonsusceptible to other (nonfluoroquinolone) antimicrobials, including some "last resort" choices, such as imipenem.

In comparison to estimates from other industrializing countries, we observed a similar or elevated prevalence of NTS exhibiting nonsusceptibility to third-generation cephalosporins and increased nonsusceptibility to both ciprofloxacin and azithromycin [26, 27]. An extraordinarily high prevalence (~90%) of fluoroquinolone-resistant *Campylobacter* has been recently observed in other industrializing countries [28, 29]. The situation in Vietnam seems to be exacerbated by the nonsusceptibility of *Campylobacter* isolates to macrolides. The *Shigella* isolated here also had a high MDR rate; emerging MDR *Shigella* isolates with resistance to fluoroquinolones and extended-spectrum cephalosporins are now commonly reported across Asia [10, 30, 31].

The treatment of diarrhea with antimicrobials is a complex issue. Apart from the limited capability of most LMICs to confirm etiological agents associated with disease and the current complication of increasing AMR, there are conflicting data regarding the clinical efficacy of antimicrobials in reducing symptoms [33]. In line with WHO recommendations, we observed that >85% of patients hospitalized with diarrhea containing mucus and/or blood were prescribed an antimicrobial, most commonly fluoroquinolones (ciprofloxacin/norfloxacin). Our current analysis found that antimicrobial use during hospitalized diarrhea did not add benefit to supportive therapy only (ie, rehydration and zinc supplementation).

The recovery of patients regardless of antimicrobial treatment or AMR status when treated with a first-line antimicrobial for bloody diarrhea treatment (eg, ciprofloxacin) may be explained by either the differing in vivo effects of antimicrobials or the self-limiting nature of the infections. In addition, in diarrhea with less pronounced inflammation (indicated by the absence of blood and/or a low CRP level), the use of antimicrobials was associated with a prolonged hospital stay. These observations support previous findings in studies of NTS and *Campylobacter,* where antimicrobial treatment did not provide a clinical advantage and sometimes even caused harm (compared with placebo) in decreasing the duration of symptoms [34, 35]. The routine antimicrobial treatment may also affect the transmission of diarrhea-causing bacteria by increasing fecal carriage and consequently spreading AMR organisms [34].

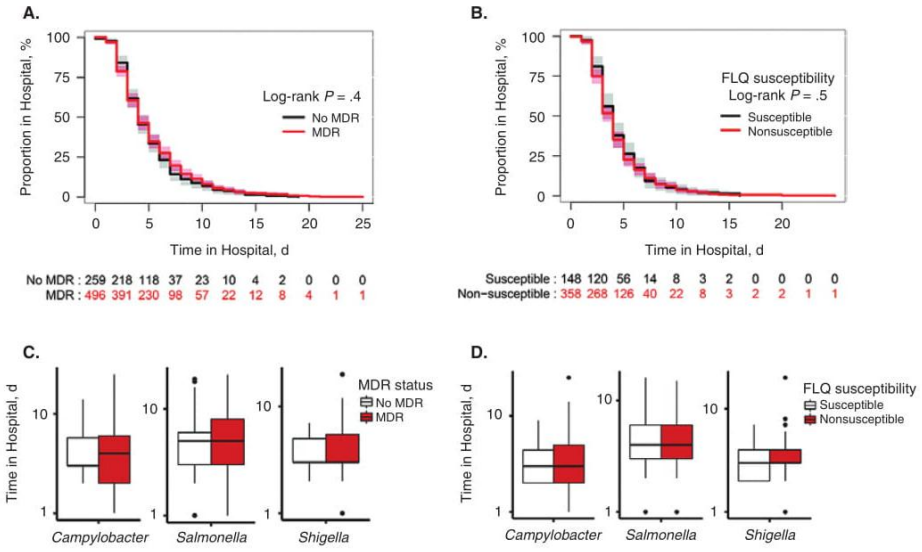


**Figure 3.** Effect of antimicrobial resistance on clinical outcome. *A, B,* Kaplan-Meier curves for length of hospital stay in diarrheal children treated with either antimicrobials or fluoroquinolones (FLQs) and stratified by multidrug resistance (MDR) (*A*) or FLQs resistance (*B*) profile of the isolated bacteria. *C, D,* Effect of MDR (*C*) and FLQ resistance (*D*) on the length of hospital stay in diarrheal children infected with *Campylobacter,* nontyphoidal *Salmonella,* and *Shigella,* while being treated with antimicrobials or FLQs, respectively. Statistical comparisons for categorical variables were conducted using the Kruskal-Wallis test. Log-rank tests were used to compare Kaplan-Meier curves for length of hospital stay between groups.

The main limitation in the present study is that this investigation was observational, with patients receiving standard-of-care treatment, which made it difficult to assess whether our observations were biased by more severe cases being prescribed antimicrobials. Furthermore, the duration of hospital stay and recovery of patients at 3 days after enrollment were proxy measures of clinical

**Table 2. Univariate and Multivariate Analysis of Diarrheal Symptoms and Treatment on Diarrheal Disease Outcome (Length of Hospital Stay) Using an Accelerated Failure Time (Log-Normal) Model**

| | Univariate Model | | | Final Multivariate Model[a] | | |
|---|---|---|---|---|---|---|
| Variable | β Value | TR (95% CI)[b] | *P* Value | β Value | TR (95% CI)[b] | *P* Value |
| Age group | | | | | | |
| 0–6 mo | 1 | 1 | … | 1 | 1 | … |
| 7–12 mo | −.075 | 0.93 (.88–.98) | .01 | −.106 | 0.90 (.85–.95) | <.001 |
| 13–60 mo | −.188 | 0.83 (.78–.88) | <.001 | −.231 | 0.79 (.75–.84) | <.001 |
| >60 mo | −.381 | 0.68 (.59–.79) | <.001 | −.445 | 0.64 (.56–.74) | <.001 |
| Sex | | | | | | |
| Female | 1 | 1 | … | 1 | 1 | … |
| Male | .017 | 1.02 (.97–1.07) | .44 | … | … | … |
| Diarrhea type | | | | | | |
| Nonbloody | 1 | 1 | … | 1 | 1 | … |
| Bloody | −.059 | 0.94 (.90–.98) | .01 | −.122 | 0.89 (.84–.93) | <.001 |
| Fever | −.047 | 0.95 (.88–1.03) | .26 | … | … | … |
| Dehydration | .193 | 1.21 (1.13–1.31) | <.001 | .179 | 1.20 (1.11–1.29) | <.001 |
| Abdominal pain | −.049 | 0.95 (.90–1.01) | .10 | … | … | … |
| Probiotic treatment | .067 | 1.07 (1.02–1.12) | .005 | … | … | … |
| Zinc treatment | .043 | 1.04 (.97–1.12) | .23 | … | … | … |
| Rehydration | .081 | 1.08 (.99–1.18) | .07 | … | … | … |
| Antimicrobial use | .229 | 1.26 (1.18–1.34) | <.001 | .279 | 1.32 (1.24–1.41) | <.001 |
| MDR | −.027 | 0.97 (.89–1.06) | .55 | … | … | … |

Abbreviations: CI, confidence interval; MDR, multidrug resistance; TR, time ratio.

[a]Adjusted β and TR values from the multivariable log-normal analysis.

[b]TRs >1 indicate an extended hospital stay; TRs <1, a decreased stay.

outcome, and we cannot discount that some children may have been discharged before the cessation of symptoms.

In conclusion, bacteria associated with pediatric diarrhea in southern Vietnam displayed an extensive AMR profile, thereby emphasizing the significance of etiological diagnosis of diarrhea in LMICs. Our key finding was that antimicrobial treatment was not associated with a reduction in diarrheal symptoms and even prolonged hospital stay in some groups. Therefore, we urge that adequately powered randomized controlled trials be conducted to better assess the potential benefits of antimicrobial therapy for treatment of diarrhea. These data will become essential for controlling antimicrobial usage during the present AMR crisis.

## Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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## References

1. Walker CL, Rudan I, Liu L, et al. Global burden of childhood pneumonia and diarrhoea. Lancet **2013**; 381:1405–16.
2. Scallan E, Mahon BE, Hoekstra RM, Griffin PM. Estimates of illnesses, hospitalizations, and deaths caused by major bacterial enteric pathogens in young children in the United States. Pediatr Infect Dis J **2012**; 32:217–21.
3. Fletcher SM, McLaws M-L, Ellis JT. Prevalence of gastrointestinal pathogens in developed and developing countries: systematic review and meta-analysis. J Public Health Res **2013**; 2:9.
4. Murray CJL, Vos T, Lozano R, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet **2012**; 380:2197–223.
5. Lee HY, Van Huy N, Choi S. Determinants of early childhood morbidity and proper treatment responses in Vietnam: results from the Multiple Indicator Cluster Surveys, 2000–2011. Glob Health Action **2016**; 9:1–8.
6. WHO and UN partners. Vietnam: WHO statistical profile, **2015**.
7. Thompson CN, Phan MV, Hoang NV, et al. A prospective multi-center observational study of children hospitalized with diarrhea in Ho Chi Minh City, Vietnam. Am J Trop Med Hyg **2015**; 92:1045–52.
8. World Health Organization. Antimicrobial resistance: global report on surveillance 2014. Bull World Health Organ **2014**; 61:383–94.
9. Thompson CN, Phan VT, Le TP, et al. Epidemiological features and risk factors of *Salmonella* gastroenteritis in children resident in Ho Chi Minh City, Vietnam. Epidemiol Infect **2013**; 141:1604–13.
10. Vinh H, Nhu NT, Nga TV, et al. A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation. BMC Infect Dis **2009**; 9:204.
11. Harrison JW, Dung TT, Siddiqui F, et al. Identification of possible virulence marker from *Campylobacter jejuni* isolates. Emerg Infect Dis **2014**; 20:1026–9.
12. World Health Organization. The treatment of diarrhoea. **2005**.
13. Legros D. Guidelines for the control of shigellosis, including epidemics due to *Shigella dysenteriae* type 1. World Health **2005**; 1–70.
14. Clinical and Laboratory Standards Institute. M100S performance standards for antimicrobial susceptibility testing. **2016**.
15. Wickham H. Hadley Wickham. Media **2009**; 35:211. Available at: http://had.co.nz/ggplot2/book.
16. de Onis M, Onyango AW, Borghi E, Garza C, Yang H. Comparison of the World Health Organization (WHO) Child Growth Standards and the National Center for Health Statistics/WHO international growth reference: implications for child health programmes. Public Health Nutr **2007**; 9:942–7.
17. Fitch A, Fox C, Bauerly K, et al. Prevention and management of obesity for children and adolescents. Inst Clin Syst Improv **2013**:10.
18. de Onis BM, Blössner M. WHO global database on child growth and malnutrition. World Heal Organ **2016**; 25:228–34.
19. Basaleem HO, Amin RM. Integrated management of childhood illness in Lahej, Yemen: a qualitative analysis from the perspective of health providers. East Mediterr Health J **2011**; 17:101–8.
20. Anders KL, Thompson CN, Thuy NT, et al. The epidemiology and aetiology of diarrhoeal disease in infancy in southern Vietnam: a birth cohort study. Int J Infect Dis **2015**; 35:3–10.
21. Collignon PC, Conly JM, Andremont A, McEwen SA, Aidara-Kane A; members of the World Health Organization Advisory Group B. World Health Organization ranking of antimicrobials according to their importance in human medicine: a critical step for developing risk management strategies to control antimicrobial resistance from food animal production. Clin Infect Dis **2016**; 63:1087–93.
22. Jean SS, Hsueh PR. High burden of antimicrobial resistance in Asia. Int J Antimicrob Agents **2011**; 37:291–5.
23. Meng CY, Smith BL, Bodhidatta L, et al. Etiology of diarrhea in young children and patterns of antibiotic resistance in Cambodia. Pediatr Infect Dis J **2011**; 30:331–5.
24. Pathania M. Clinical study of acute childhood diarrhoea caused by bacterial enteropathogens. J Clin Diagnostic Res **2014**; 1–5.
25. Zhang H, Pan F, Zhao X, et al. Distribution and antimicrobial resistance of enteric pathogens in Chinese paediatric diarrhoea: a multicentre retrospective study, 2008–2013. Epidemiol Infect **2015**; 1–8.
26. Langendorf C, Le Hello S, Moumouni A, et al. Enteric bacterial pathogens in children with diarrhea in Niger: diversity and antimicrobial resistance. PLoS One **2015**; 10:e0120275.
27. Li Y, Xie X, Xu X, et al. Nontyphoidal salmonella infection in children with acute gastroenteritis: prevalence, serotypes, and antimicrobial resistance in Shanghai, China. Foodborne Pathog Dis **2014**; 11:200–6.
28. Ghosh R, Uppal B, Aggarwal P, Chakravarti A, Jha AK. Increasing antimicrobial resistance of *Campylobacter jejuni* isolated from paediatric diarrhea cases in a tertiary care hospital of New Delhi, India. J Clin Diagn Res **2013**; 7:247–9.
29. Pollett S, Rocha C, Zerpa R, et al. *Campylobacter* antimicrobial resistance in Peru: a ten-year observational study. BMC Infect Dis **2012**; 12:1.
30. Von Seidlein L, Deok RK, Ali M, et al. A multicentre study of *Shigella* diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. PLoS Med **2006**; 3:1556–69.
31. The HC, Rabaa MA, Thanh DP, et al. South Asia as a reservoir for the global spread of ciprofloxacin resistant *Shigella sonnei*. PLoS Med **2015**; 1–12.
32. Fischer Walker CL, Fontaine O, Young MW, Black RE. Zinc and low osmolarity oral rehydration salts for diarrhoea: a renewed call to action. Bull World Health Organ **2009**; 87:780–6.
33. Traa BS, Fischer Walker CL, Munos M, Black RE. Antibiotics for the treatment of dysentery in children. Int J Epidemiol **2010**; 39:70–4.
34. Onwuezobe IA, Oshun PO, Odigwe CC. Antimicrobials for treating symptomatic non-typhoidal *Salmonella* infection. Cochrane Libr **2012**; 11:1–50.
35. Ternhag A, Asikainen T, Giesecke J, Ekdahl K. A meta-analysis on the effects of antibiotic treatment on duration of symptoms caused by infection with *Campylobacter* species. Clin Infect Dis **2007**; 44:696–700.