The changing epidemiology, clinical syndrome and antibiotic resistance patterns of shigellosis in Vietnamese children

Thesis

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The Changing Epidemiology, Clinical Syndrome and Antibiotic Resistance Patterns of Shigellosis in Vietnamese Children

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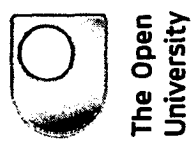
A thesis submitted in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy



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Abstract

Diarrhoeal diseases remain the second most common cause of death among children under-five globally, following closely behind pneumonia. In developing countries, rotavirus and *Shigella* spp. are thought to be the most important enteropathogens causing acute childhood diarrhoea. Systematic data on diarrhoeal diseases, particularly *Shigella* infection, in southern Viet Nam is lacking. An analysis of the historical trends in epidemiology, antibiotic susceptibility, and clinical features of *Shigella* infection and contemporary clinical studies of the response to antibiotic treatment in Vietnamese children were the central aims of this thesis.

By analysing *Shigella* strains isolated from children between 1995 and 2009 I documented a transition in the dominant *Shigella* species causing diarrhoea from *S.flexneri* to *S.sonnei*, a change previously linked with industrialisation and economic development. During the same period there was a sharp increase of nalidixic acid resistant *Shigella* strains from approximately 7% to 70%. In addition, the clinical presentation appeared to be more severe and the duration of hospital stays longer.

During the course of this thesis I also documented for the first time in Viet Nam *Shigella* spp. which harboured the plasmid-transferable drug resistance *bla*CTX-M genes. The occurrence of this plasmid-transferable gene may endanger the use of beta-lactams for this infection in the future. In a large randomised controlled trial, gatifloxacin, a new 8-methoxy-fluoroquinolone antibiotic proved comparable to ciprofloxacin for the treatment of acute dysentery including those caused by multi-resistant *Shigella*. Although gatifloxacin has been associated with dysglycaemic side-effects in adults, this adverse effect was not observed in this study.

Dedication

To My Children

"Learning is (Age) Unlimited"

Declaration

Other than the assistance outlined in the acknowledgements, the work described in this thesis is my own work and has not been submitted for a degree or other qualification to this or other university.

That evening, Martin Arrowsmith and Terry Wickett lolled in a clumsy boat, an extraordinary uncomfortable boat, far out on the water.

"I felt as if I were really beginning to work now," said Martin. "This new quinine stuff may prove pretty good. We'll plug along on it for two or three years, and maybe we'll get something permanent -- and probably we'll fail!"

"Arrowsmith"

By Sinclair Lewis (1885-1951)

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List of Abbreviations

AMC	Ampicillin plus Clavulanic acid
AMP	Ampicillin
CAZ	Ceftazidime
CHL	Chloramphenicol
CI	Confidence interval
CIP	Ciprofloxacin
CLA	Clavulanic acid
CLSI	Clinical and Laboratory Standards Institute
CRO	Ceftriaxone
CTX	Cefotaxime
CTX-M	Cefotaximase
DCT	Diarrhoea Clearance Time
DDST	Double disc synergy test
DHFR	Dihydro-folate reductase
DHPS	Dihydroopterate synthase
DTP	Dong Thap Provincial Hospital
EIEC	Enteroinvasive *E.coli*
ESBL	Extended-spectrum beta-lactamase
FCT	Fever Clearance Time
FEP	Cefepime
GAT	Gatifloxacin
GDP	Gross Domestic Product

HLA	Human Leukocyte Antigen
HPF	High power field
HTD	The Hospital for Tropical Diseases
HUS	Haemolytic-uremic syndrome
IL	Interleukin
ipa	Invasive plasmid antigen
IQ	Interquartile
IR	Incidence rate
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
mxi	Membrane expression of *ipa* proteins
NAL	Nalidixic Acid
OFX	Ofloxacin
OR	Odd ratio
ORS	Oral rehydration solution
OUCRU	Oxford University Clinical Research Unit
PAI	Pathogenicity islands
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophils
R factor	Resistance factor
RBC	Red blood cells
RCT	Randomised controlled trial
ShET	*Shigella* enterotoxin
SHV	Sulphydryl variable
spa	surface presentation of *ipa* antigens

SXT	Trimethoprim – Sulfamethoxazole
T3SS	Type III secretion system
TEM	Temoniera
TET	Tetracycline
TMP-SMZ	Trimethoprim-Sulfamethoxazole
WBC	White blood cells

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I am indebted to all patients and their parents who have participated in the studies included within this thesis. Without their participation, the thesis could not have been done.

I am grateful to Professor Nicholas John White whose knowledge, experience and enthusiasm in scientific research first motivated me to go forward in clinical research. I would like to express my sincere thanks to Professor Tran Tinh Hien, who has understood me in easy as well as difficult times, and always encouraged me in my work and with this thesis. I want to acknowledge and thank Dr. Constance Schultsz and Dr.Christiane Dolecek, my directors of study, who helped me with writing protocols, planning statistical analysis and drafting manuscripts and to Dr. Stephen Baker who became an invaluable mentor half-way through my PhD and who helped speed up my thesis writing spectacularly.

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I am also grateful to Dr. John Wain and Dr. Christopher Parry who had supported me when developing my first clinical trial on childhood dysentery.

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Thu Chinh, Nguyen Thi Thu Thao, Cao Thi Tam) and Infectious Diseases Ward at Dong Thap Provincial Hospital (Drs. Le Thi Phuong, Nguyen Thi Hong Tham, Mai Ngoc Lanh, Bui Li Mong), particularly Dr. Vo Thi Cuc Anh for enrolling patients, collecting samples and caring for the patients in my project.

The microbiology work in the studies included within this thesis (Chapter 3, 4, 5 and 6) were carried out in the laboratories at the Hospital for Tropical Diseases at Ho Chi Minh City by Nguyen Van Minh Hoang, Cao Thu Thuy, Pham Van Minh, Tran Thi Thu Nga, and at Dong Thap Provincial Hospital by Dr. Phan Van Be Bay. I am indebted to all of them. I also would like to express my gratitude to Dr. To Song Diep and Dr. Nguyen Van Vinh Chau for encouraging and giving me the warm working atmosphere at the hospital's microbiology laboratory. I want to express my special thanks to Mr. James Campbell, who taught me carefully "loop and Bunsen" during those days when I came back to the microbiology laboratory after more than 30 years. I am thankful to the molecular group of the Unit, particularly Le Thi Minh Vien who has guided me to do conjugation experiments and helped performing plasmid extraction; and Nguyen Thi Khanh Nhu who did the molecular characterisation of ESBL genes, both in Chapter 5. I thank The Wellcome Trust and the University of Oxford for support and for funding this work.

Last but not least, I thank Thu Nga, my wife, for the patience, support and dedication that she gave me during the years I have been working on this thesis.

Chapter One

Introduction

1.1 Introduction:

Diarrhoeal diseases are a major public health problem and the second leading cause of death in children under five years old (UNICEF/WHO 2009). According to the World Health Organization estimations, each year there are approximately two billion cases of diarrhoeal disease worldwide leading to 1.5 millions children deaths in the year 2004, in which 80% are under two years old (http://www.who.int/media centre/factsheets/fs330/en/print.html accessed July 10, 2010). A wide range of pathogens including viruses, bacteria, and parasites can cause diarrhoea. However, only a handful of organisms are responsible for most cases of acute diarrhoea. Rotavirus is the leading cause of acute diarrhoea both in developed and developing countries, and is responsible for about 40% of all hospital admissions due to diarrhoea among children under five worldwide (Weekly Epidemiological Report, vol. 83, no.47, 21 Nov. 2008). Parasites only contribute a relatively small part in the aetiology of acute diarrhoea, of which *Cryptosporidium* is most frequently isolated in stools of children with diarrhoea seen at health facilities, particularly among HIV-positive children.

Bacterial pathogens (including *Shigella*, enteropathogenic *E.coli*, *Campylobacter* and *Salmonella)* are the second most important group of pathogens causing acute diarrhoea. Of these *Shigella* spp. is the most frequently bacterial agent isolated from children treated at hospital with acute diarrhoea (Hien et al. 2008). A recent review by The World Health

Organisation experts estimated that the annual number of *Shigella* episodes and deaths in Asia to be 91 million and 414000 respectively (WHO 2005b).

Although distributed throughout the world, the prevalence of *Shigella* infection differs from between countries and within countries. It is estimated that the annual incidence rate (IR) of shigellosis (cases per 100,000 per year) is 6.5 in the United States, 3.3 in the United Kingdom, 5.6 in Australia, 1.8 in France, and 130 in Israel (Kotloff et al. 1999). In Viet Nam, between the year 1991 and 2001 the mean annual incidence rate of shigellosis was estimated to be 70/100.000 population, much higher than that of typhoid fever (mean IR 23/100.000 population) and cholera (mean IR 2.7/100.000 population) during the same period (Kelly-Hope et al. 2007).

Shigella infection manifests a wide range of clinical features from mild watery diarrhoea to dysentery with mucoid bloody stools accompanied by tenesmus. In some cases severe complications such as toxic megacolon, hyperleukocytosis, and haemolytic-uremic syndrome may lead to death. In addition, shigellosis is a protein-losing enteropathy which may promote malnutrition in children if left untreated.

The treatment of shigellosis includes using antibiotics, rehydration and nutrition. The use of antibiotics to treat shigellosis is increasingly complicated because of the spread of develop resistant *Shigellae*. Despite the long history since the discovery the causative agent, the development of a vaccine against shigellosis remains elusive because the immunity to *Shigella* infection is serogroup- and serotype-specific.

1.2 The agent:

Dysentery has been a well-characterized syndrome since antiquity. However the causative agent was only discovered in the year 1897 by Kiyoshi Shiga, a Japanese microbiologist, for whom the organism was ultimately named (Trofa et al. 1999). The original organism described by Dr. Shiga is now known as *S.dysenteriae* type 1. Over the 4 decades following Shiga's original description, three additional *Shigella* species were described. Organisms of the genus *Shigella* belong to the *Enterobacteriaceae* family. They are Gram negative rod-shaped, non-motile and non-lactose fermenting bacilli (Figure 1.1).

1.2.1 *Shigella* serogroups:

All four species (or sero-groups or subgroup) of the genus *Shigella* can cause illness in human and non-human primates. These are: Subgroup A; *Shigella dysenteriae* (*S.dysenteriae*) with 15 serotypes in which serotype 1 is the most virulent, Subgroup B; *S. flexneri* contains 6 serotypes, with many subtypes in each serotype, Subgroup C: *S.boydii* with 20 serotypes. Subgroup D: *S.sonnei* has a single serotype (WHO 2005a).

Serotyping is based on the O antigen component of lipo-polysaccharide present on the outer membrane of the cell wall. In clinical practice there are a small percentage of *Shigella* strains which cannot be typed by commercial antisera, but may be identified later on as new serotypes or subtypes using highly specific monoclonal antisera prepared by research laboratories or by molecular techniques.

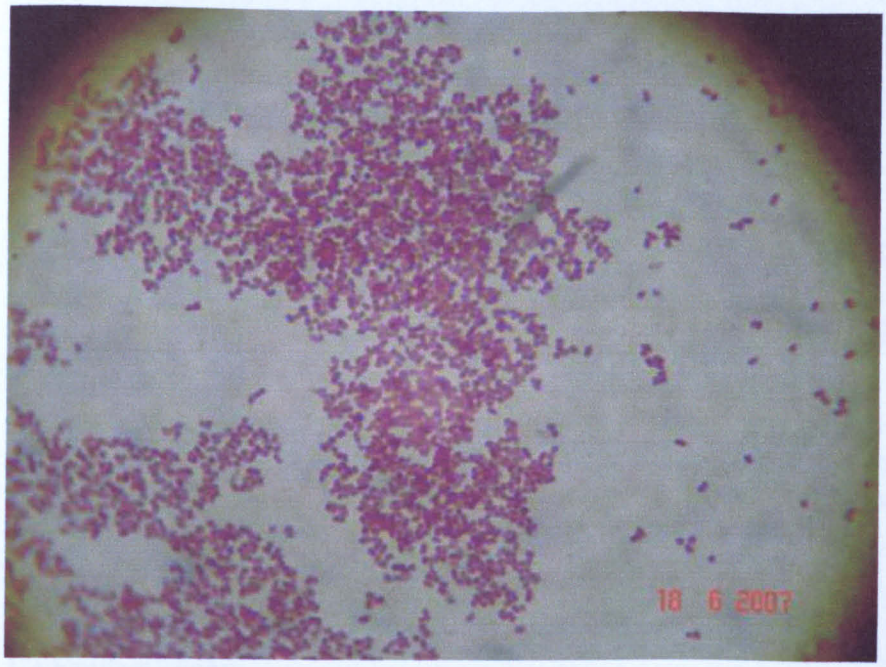

Figure 1.1 Image of a pure culture of *Shigella* spp. in Gram stain.

1.2.2 Genetic origin of *Shigella:*

The whole genome of all four species of the genus *Shigella* have been sequenced (Yang et al. 2005). Through comparative genomics, the sequence divergence between *S.flexneri* and *E.coli* K-12 is approximately 1.5% while between *S.flexneri* and *Salmonella enterica* is approximately 15%. Comparative genomics indicated that *Shigella* and *Enteroinvasive E.coli* (EIEC) evolved from multiple *E.coli* strains by convergent evolution (Lan and Reeves 2002). The *Shigella* bacteria have evolved from commensal *E.coli* to exhibit a pathogenic phenotype by the acquisition of a virulence plasmid and chromosomal pathogenicity islands as well as through the loss of other gene loci which are not functional intracellularly or impede virulence by genomic compensatory mechanism (Schroeder and Hilbi 2008) (Figure 1.2).

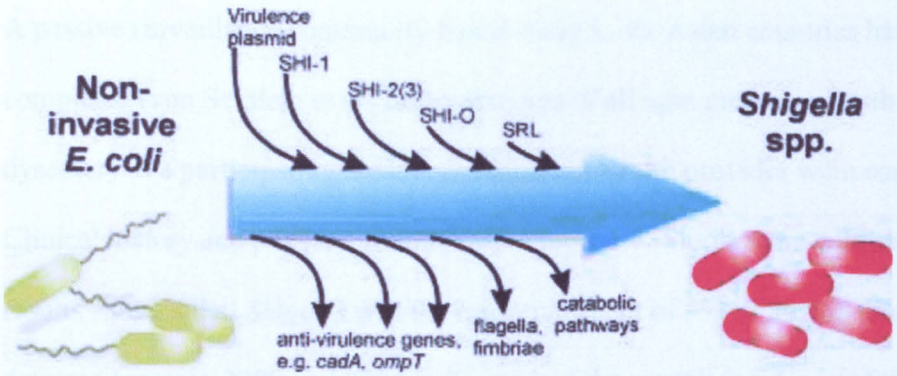

Figure 1.2 Evolution of *Shigella* spp. from non-invasive *E.coli* ancestors: acquisition of a large virulence plasmid and chromosomal pathogenicity islands plus loss of genetic foci, which are not functional intracellularly or impede virulence. (Reproduced from Schroeder and Hilbi 2008)

1.3 Epidemiology:

1.3.1 Disease burden:

The global burden of *Shigella* infection has been estimated by analyzing published data between 1966 to 1997, and gave estimates of 164.7 million of episodes of *Shigella* infection annually worldwide, of which 163.2 million were in developing countries (with 1.1 million deaths) and 1.5 million in industrialized countries. A total of 69% of all episodes and 61% of all deaths attributable to shigellosis involved children 5 years of age or younger (Kotloff et al. 1999). The figures from Kotloff et al's review may not reflect the current situation because they have been based upon retrospective studies of relatively old data. A newer analysis was based on more recent data of *Shigella* infection studies in low- and medium-Human Development Index (HDI) countries published between 1984 to 2005 (Ram et al. 2008). From this analysis, the annual incidence varied from 0-6/1000 person-year in Thailand to 107/1000 person-years in Egypt. However, because of the large gaps in data on the burden of *Shigella* infections for low human development index countries and, more specifically, for sub-Saharan Africa, the estimation may still not reflect the true global prevalence of the disease (Ram et al. 2008).

A passive surveillance community-based study in six Asian countries has been recently completed (von Seidlein et al. 2006). Patients of all ages presenting with diarrhoea or dysentery at a participating health care centre or health provider were enrolled in the study. Clinical history and physical findings were recorded. Stools were culture for *Shigella*. The results showed that *Shigella* was the causative agent of 5% of 56,958 diarrhoea episodes detected between 2000 and 2004 in the region; the overall incidence of treated shigellosis was 2.1 episodes per 1,000 residents per year in all ages and 13.2/1,000/y (equal to 0.013 episodes of shigellosis per child-year) in children under 60 months old (von Seidlein et al. 2006). The researchers of this study also used real-time polymerase chain reaction (PCR), to amplify the gene encoding the invasion plasmid antigen H (*ipa*H), which they contend is a more sensitive diagnostic technique than classical culture method. PCR detected *ipa*H in 33% of a sample of culture-negative stool specimens, suggesting that the classical stool culture method may miss a lot of shigellosis cases. Hence the true prevalence of shigellosis may be much higher than previously thought.

Another longitudinal diarrhoeal disease study has been carried out in the Peruvian Amazon between 2002 and 2006. This was an active community-based surveillance, in which research health workers visited participating families 3-times a week to record the number, consistency, and characteristics of stool passed over the 24-hour period, and collected stool for culture whenever the definition of diarrhoea cases were met (Kosek et al. 2008). The incidence rate for shigellosis was 0.34 episodes per child-year, a 20-fold higher than the rate detected by passive surveillance in Asia reported by von Seidlein et al.
Shigellosis in these communities may have become milder during the study periods because there were no shigellosis-related deaths reported in these two community-based studies. The trend towards a more benign pattern of shigellosis may be due to the better nutritional status of children in economically emerging Asian countries, or earlier access to

health service and improvement of primary case management during the surveillance study period in Peru (Sansonetti 2006).

1.3.2 Transmission:

Shigella infection is by faecal-oral route with a low infectious dose: ingesting as few as 10 to 100 colony-forming-units of *S.dysenteriae* type 1 may result in disease in 10% to 20% of non-immune subjects (DuPont et al. 1989). Transmission is via direct person-to-person, contaminated food or water. Houseflies may transmit infection mechanically in some situations where control of houseflies reduces transmission of shigellosis (Cohen et al. 1991). *Shigella* transmission also takes place via oral-anal sexual contact particularly in men who have sex with men (Marcus et al. 2004).

In developing countries, epidemics of *S.dysenteriae* type 1, which produces Shiga toxin and typically carries R factors that encode resistance to multiple antibiotics, may occurred with considerable morbidity and mortality, particularly in extreme situations such as natural disasters or political upheaval leads to mass population displacement and crowding in refugees camps (Shears 1996). Endemic shigellosis is largely a paediatric disease, most cases occurred in children under 5 years old and the *S.flexneri* serotypes predominate (Kotloff et al. 1999; von Seidlein et al. 2006).

In developed countries, *S.sonnei* persists and causes sporadic diarrhoea and occasional outbreaks in epidemiological niches (such as mental institution and day-care centres) where personal hygiene may be suboptimal (Mohle-Boetani et al. 1995), or in population of men who have sex with men, in that case *S.flexneri* may be the dominant species.

Recently, outbreaks of shigellosis have occurred in industrialised countries and have been found to be linked with consumption of food imported from a developing country (Lewis et al. 2009). An international food-borne outbreak of *S.sonnei* in airlines passengers with

47 culture-confirmed shigellosis and 116 probable cases in 4 countries was related to consume raw carrot served on-board planes and supplied by one caterer. These outbreaks represent a new face of epidemiology of shigellosis in the era of modern air transportation.

1.3.3 Distribution of *Shigella* species:

The distribution of *Shigella* species varies with location of isolation and changes by time. *S.dysenteriae* type 1, for unknown reasons, is the only *Shigella* that can cause large outbreaks of infection in developing countries and which has been linked with a high mortality. Such major epidemics occurred in Central America in the late 1960s (Mendizabal-Morris et al. 1971) and South Asia (Pal 1984) in early 1980s and early 2000s in Central Africa (Kerneis et al. 2009; Paquet et al. 1995).

In Britain and Europe during the 1920s *S.flexneri* and *S.sonnei* were of equal prevalence, but the former gradually faded and after the Second World War *S.sonnei* accounted for the majority of the endemic Shigellosis in these areas. Similar trends have also been observed in the United States (Skirrow 1996). In Japan, where the prototype *Shigella* was first described, *S.dysenteriae* type 1 was the most common species in the latter half of 19th century and early 20th century. But after that most of the *Shigella* strains isolated in hospitalized patients in Tokyo were *S.flexneri*. The percentage of *S.sonnei* increased gradually from 10% in 1953 to 50% in 1963 (Hiroshi 1964) and now *S.sonnei* is the most prevalent indigenous *Shigella* species in Japan. Statistics in Korea showed that *S.sonnei* constituted 4.8% of all *Shigella* isolated in the 1961-1968 period, which increased to 23.6% in 1980-1987, and then became the most prevalent species in 1991-1997 period with 77.1% of all isolates (Je Chul Lee 2006).

While *S.sonnei* is the main sero-group of *Shigella* causing diarrhoeal diseases in industrialized countries, in developing countries *S. flexneri* remains the predominant species (Kotloff et al. 1999; Zafar et al. 2009). However, recently *S.flexneri* has been replaced by *S.sonnei* as the most important sero-group causing shigellosis in some "transitional" countries (e.g. Thailand, Turkey, Israel, von Seidlein et al. 2006, Bangtrakulnonth et al. 2008, Ceyhan et al. 1996, Ashkenazi et al. 1993). The reason for the shift in predominant *Shigella* species in these countries is not understood and although economic improvement is clearly an important factor (there is a significant correlation between the Gross Domestic Product (GDP) and the frequency of *S.sonnei* isolation) it is not clear how this affects the balance between the species. The predominance of *S.sonnei* over *S.flexneri* probably reflects the expanding economies in these countries (Ram et al. 2008). However there has been no study aimed at providing the answer of the change of dominant *Shigella* sero-group in the presence of rising GDP. One possibility is that economic improvement leads to better access to health services thus bringing more patients with relatively mild diseases caused by *S.sonnei* to the hospital.

1.4 Pathogenicity of *Shigella:*

The cellular and molecular pathogenicity of *Shigella* infections has been studied in depth in the past decades using *S.flexneri* infection in animal models or *in vitro* cell assay systems (Sansonetti 1992; Schroeder and Hilbi 2008). Two important mechanisms in the pathogenesis of *Shigella* infection are (i) the production of enterotoxins and (ii) tissue invasiveness by the organism. Tissue invasiveness was recognised for a long time and attracted more attention because it caused inflammation in the human colon and terminal ileum which resulted in typical dysentery picture with bloody diarrhoea and tenesmus. The production of enterotoxins was first described thirty years ago (Keusch et al. 1972).

1.4.1 Production of enterotoxins:

Clinicians recognised that many patients with shigellosis manifest only watery diarrhoea, and even dysenteric patients passing scanty mucoid-bloody stools usually recount a period of watery diarrhoea without blood in the early stage of their illness. This observation plus experiments in non-human primate and human volunteer models led to the discovery and characterisation of *Shigella* enterotoxins (Levine 2007). Two enterotoxins (*Shigella* enterotoxin 1 (ShET1) and *Shigella* enterotoxin 2 (ShET2)) which are secreted by *Shigella* contribute to the pathogenesis of the disease. Deletion of *set* and *sen* genes encoding *Shigella* enterotoxin 1 and 2 leads to an attenuated but immunogenic *Shigella* vaccine candidate strain (Kotloff et al. 2004). ShET1 is a chromosomally encoded toxin of 55 kDa that is essentially expressed by *S.flexneri* 2a, and ShET2 is a plasmid-encoded enterotoxin of 63 kDa which can be secreted by various *Shigella* species. These enterotoxins are thought to be responsible for the early watery diarrhoeal phase at the onset of shigellosis (Fasano et al. 1997). Another well described toxin is the Shiga toxin, also known as verotoxin, verocytotoxin or Shiga-like toxins, and which are produced by several enteric pathogens, most importantly *S. dysenteriae* (serotype 1 only) and Entero-haemorrhagic *E.coli* (EHEC).

Shiga toxin is a cytotoxic toxin and is responsible for the development of vascular lesions seen in the severe complications of shigellosis such as haemorrhagic colitis and the haemolytic-uremic syndrome (HUS) (Cherla et al. 2003).

1.4.2 Epithelium invasion:

The most easily visible lesions found in gross pathology of shigellosis are the ulcerations in the terminal ileum and colon. This reflects the mucosal invasiveness of the pathogen, which is a multi-step process (Figure 1.3).

(i) In the initial phase of infection, *S.flexneri* does not invade the epithelial barrier from the apical side but instead triggers its uptake by the microfold cell (M cells) and is transcytosed across the epithelium.

(ii) After transcytosis, *S.flexneri* is released into an intraepithelial pocket and is engulfed by resident macrophages. *Shigella multiplies* within infected macrophages then induces macrophage apoptosis which releases the organism into the sub-mucosa space. Macrophage cell death is accompanied by the release of interleukin-1β (IL-1 β) and IL-18. Both cytokines are mediators of acute and massive inflammatory response.

(iii) Once released from the dying macrophage *S.flexneri* is able to invade other epithelial cells from baso-lateral side, and replicates in the cytoplasm. Cytoplasmic *S.flexneri* then spreads to adjacent epithelial cell (via a novel host actin-based propulsion mechanism) without exposure to the extra-cellular components of host immune defence.

(iv) Infected epithelial cell secretes the chemokine IL-8. IL-8 mediates recruitment of polymorphonuclear neutrophil leukocytes (PMN) to the site of infection. The infiltrating PMN leukocytes kill *S.flexneri* but also destroy the integrity of the epithelial lining, thus enable more luminal bacteria to reach the sub-mucosa directly (Schroeder and Hilbi 2008). The invasiveness of *Shigellae* occurs at the terminal ileum and colon and clearly explains the dysenteric phase of the disease.

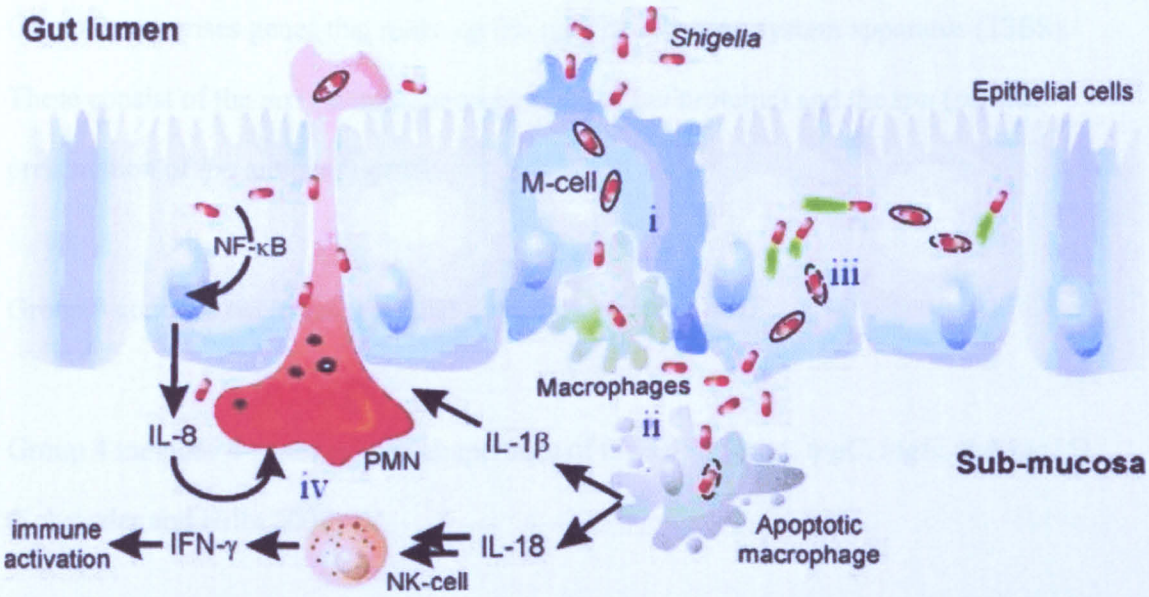

Figure 1.3 Cellular pathogenesis of *Shigella* spp.: (i) *S. flexneri* triggers its uptake by the M cell and is transcytosed across the epithelium layer; (ii) After transcytosis, *S.flexneri* is released out off the M cell and is engulfed by resident macrophages; *Shigellae* multiplies within the macrophage then induces macrophage apoptosis releasing the organism into the sub-mucosa space plus proinflammatory cytokines; (iii) Free bacteria invade other epithelial cell from the baso-lateral side, multiply and spread to adjacent cells; (iv) Proinflammatory cytokines activates the innate immune response and attracts PMN to cause inflammation and tissue destruction facilitating the invasion of more bacteria. Finally, PMNs kill *Shigella*, thus contributing to the resolution of the infection (Reproduced from Schroeder and Hilbi, 2008)

1.4.3 Molecular basis of *Shigella* pathogenicity:

The genetic determinants of virulence in *Shigella* are located on the "pathogenicity islands" (PAI) found in bacterial chromosome and on a large virulence associated plasmid of 200kb molecular weight found in all *Shigella* species. Genes in the large virulence plasmid which control the production of proteins secreted by *Shigella* can be divided into 4 functional groups.

Group 1 consists of genes encoded for invasion plasmid antigens IpaA to IpaD that act as effectors and translocator proteins directly responsible for inducing cytoskeletal rearrangements, membrane ruffling and pathogen uptake which is essential for host cell invasion and intracellular survival.

Group 2 comprises genes that make up the type III secretion system apparatus (T3SS). These consist of the *mxi* (membrane expression of *ipa* proteins) and the *spa* (surface presentation of *ipa* antigens) genes.

Group 3 contains two transcriptional activators *virB* and *mixE*.

Group 4 includes 4 genes encode chaperones of the T3SS (*ipgA*, *ipgC*, *ipgE*, and *spa15*) (Schroeder and Hilbi 2008).

1.5 Clinical features:

Man is the only natural host for *Shigella* spp. although higher non-human primate can develop diarrhoeal illness after ingestion of the bacteria.

1.5.1 Clinical presentations:

Shigella infection may present a wide range of illness in human from asymptomatic infection to frank dysentery syndrome with acute watery diarrhoeal illness in between (Stoll et al. 1982). The clinical picture of *Shigella* infection varies with the bacteria inoculation size, host status and *Shigella* species: low inoculum size can cause a mild disease, bigger inoculum size may lead to frank dysentery (DuPont et al. 1989); overwhelming dysentery is usually seen in *S.dysenteriae* type 1 infection while *S.sonnei* infection causes a milder illness.

In volunteer challenge studies, the classical bacillary dysentery caused by *Shigella* commences with fever, malaise, abdominal cramps and watery diarrhoea for about 18-24 hours. These symptoms are followed by scanty stools which contain mucus and blood and by tenesmus, which is painful straining at defecation (Kotloff et al. 1995a).

In hospitalised children, few patients presented with the initial stools are bloody (the majority of them present initially with watery diarrhoea), while more than 50% of cases never develop bloody stools (Ashkenazi 2004). In community surveillance studies the proportion of *Shigella* infection presented with overt dysentery with mucus and blood in stool contributes only 11% to 20% of all *Shigella*-associated diarrhoeal cases, the majority of cases had no fever or blood in stools (Abu-Elyazeed et al. 2004; Kosek et al. 2008; von Seidlein et al. 2006). While the presence of mucoid bloody diarrhoea may be indicative of shigellosis, the absence of this symptom cannot preclude the diagnosis (Abu-Elyazeed et al. 2004). So using the term "bloody diarrhoea" to denote "*Shigella* infection" or "shigellosis" may lead to an under-estimate of the true prevalence of the infection because in the majority of cases blood is not visible in stools.

1.5.2 Complications:

Severe *Shigella* infection may lead to a number of systemic complications:
Severe dehydration and acidosis although rare in shigellosis are a very important complication.

Toxic megacolon (Christianson 1987) and leukemoid reaction (Butler et al. 1984) have been reported in Bangladeshi children with high fatality rate but have been rarely reported from other areas.

Haemolytic-uremic syndrome (Bhimma et al. 1997) is a major complication of infections due to *S.dysenteriae* type 1 (and also of Shiga-toxin producing *E.coli*) which lead to renal failure and death if renal replacement therapy not applied early.

Bacteraemia is rare in previously healthy patients but may be seen more frequent in malnourished or immunodeficiency children and is accompanied by a high case fatality rate (Struelens et al. 1985).

Seizure may be seen in up to 27% of all children with shigellosis (Hiranrattana et al. 2005; Lahat et al. 1984) and mostly have a benign course (Ashkenazi et al. 1987); encephalopathy occurs rarely but is associated with increased morbidity and mortality (Chisti et al. 2009).

Some patients infected with *S. flexneri* and who are genetically predisposed (Human Leukocyte Antigen (HLA) B27) can develop Reiter's syndrome (pains in their joints, irritation of the eyes, and painful urination) that can lead to a difficult to treat chronic arthritis (Calin and Fries 1976).

Despite formerly thought that Shiga toxin cause all the neurological derangement, a recent study showed that Shiga toxin production is not essential for the developing of neurological manifestations of shigellosis (Ashkenazi et al. 1990).

1.5.3 Special cases:

Neonatal shigellosis is rare possibly because of the presence of maternal derived factors which pass through the placenta or are transferred during breastfeeding. Initial clinical presentation in neonates may be mild (low-grade fever, mild watery diarrhoea), but complications occur more frequent than in older children (septicaemia, toxic megacolon, meningitis, colonic perforation) (Haltalin 1967; Viner et al. 2001).

Shigellosis in patients with acquired immunodeficiency syndrome (AIDS) may be recurrent and relapsing, and more often is associated with bacteremia requiring prolonged antibiotic treatment (Kristjansson et al. 1994).

1.5.4 Negative impact of *Shigella* infection on child nutrition:

Shigellosis exerts a long term impact on the growth of children through its negative effect on nutrition leading to malnutrition. Two factors may contribute to this negative effect:

(i) *Shigella* dysentery is associated with profound anorexia leading to decreased food intake,

(ii) In *Shigella* infection, the patients lose considerable amounts of protein in the gut owing to epithelial cell death, ulceration, and the transudation of serum into the intestinal lumen (Bennish et al. 1993). In Bangladesh, where shigellosis is hyper-endemic, this is an important factor contributing to the high prevalence of children malnutrition.

The mortality rate from *Shigella* infection, which was as high as 10%-20% in the 1960s, has been decreased to 0% to 21% in most recent hospital-based studies, and to 0% to 2.6% in community-based studies (Ram et al. 2008). It seems shigellosis has a more benign course in recent years (Sansonetti 2006). The contributing factors for this may be improvements in the nutritional status of children, the improvement of the access to health services and better case management in health facilities. Risk factors for death included very young age, poor nutritional status and central nervous system manifestations such as altered consciousness or seizures (Ram et al. 2008).

1.6 Diagnosis:

1.6.1 Clinical diagnosis:

Typical cases with acute high fever, frequent small-volume bloody diarrhoea with cramp and tenesmus are easy to diagnosis but contribute to less than 50% of hospitalised patients. The majority of cases presents with watery diarrhoea and are difficult to distinguish with diarrhoea caused by viral aetiology (such as rotavirus or norovirus) unless microbiological investigations are conducted.

1.6.2 Bacteriological diagnosis:

The diagnosis of *Shigella* infection is based on stool culture on appropriate media. Selective media, such as MacConkey, xylose-lysine-deoxycholate (XLD), Hektoen enteric (HE), or *Salmonella-Shigella* agar, are usually used for isolation from clinical specimens. Ideally clinical samples should be streaked on plates within two hours of the sample being taken. The isolation of *Shigellae* from clinical stool samples not only confirms the diagnosis of Shigellosis but also helps to determine the antibiotic susceptibility of *Shigellae* which is important for the treatment and control of the infection (WHO 2005c). However because *Shigellae* are relatively fragile organisms, the positive culture rate from clinical specimens is not high.

1.6.3 Molecular diagnosis:

The polymerase-chain reaction (PCR) techniques has been used to detect specific pathogenic genes coding for the invasion plasmid antigen H (*ipa*H) which present in all *Shigellae* and also in enteroinvasive *E.coli* (EIEC) (Islam et al. 1998; Sethabutr et al. 1993). The real time PCR recently detected the presence of *ipa*H gene sequence in 33% of culture-negative specimens (Vu et al. 2004). When the PCR technique was considered as gold-standard, the culture technique has 72% sensitivity and 100% specificity (Islam et al.

1998). Unfortunately PCR technique requires sophisticated equipment and is expensive and not usually available in the settings where *Shigella* infection is prevalent. Moreover PCR techniques could not give information on the sensitivity to antibiotics of the *Shigella* strains to guide treatment. Therefore the techniques may be suitable for incidence studies at the field but are not valuable for clinical diagnosis in hospital.

1.6.4 Rapid test:

Recently a simple immunochromatographic dipstick based on the detection of *S.flexneri* 2a lipopolysaccharide (LPS) using serotype 2a-specific monoclonal antibodies coupled to gold particles has given promising results with the specificity and sensitivity of 99.2% and 91.5% respectively when testing on clinical specimens (Nato et al. 2007a).

1.7 Treatment:

The treatment of shigellosis comprises of (i) providing water and electrolytes rehydration, (ii) good nutrition, and (iii) administering appropriate antibiotics.

1.7.1 Rehydration:

Rehydration is usually through oral rehydration therapy because in most cases the patients have none or mild-to-moderate dehydration (Khan et al. 1999). The use of reduced-osmolarity oral rehydration solution (ORS) recommended by WHO is sufficient for the majority of cases (WHO 2005c). Intravenous rehydration is indicated in rare cases with severe dehydration or profound vomiting.

1.7.2 Regular feeding:

This is an important practice to prevent acute hypoglycaemia and long-term malnutrition. High-protein diet can help during convalescence from shigellosis in children (Kabir et al. 1993).

1.7.3 Antibiotics:

Antibiotics are helpful for the treatment of shigellosis. Although diarrhoea caused by *S.sonnei* is generally mild and self-limited in healthy adults in industrialised countries, infection with any *Shigella* species can be lethal to children in developing countries. This is particularly true in the very young, malnourished or immuno-compromised patients. Without effective antibiotic treatment, mortality due to *Shigella* infection, especially from infection with *S.dysenteriae* type 1 may exceed 10%, particularly among the young and the elderly (Bennish and Wojtyniak 1991). Antibiotics shorten the duration of symptoms, prevent severe complications and eradicate *Shigellae* from the stool more quickly hence prevent the spread of the disease in the community (Salam and Bennish 1991). The choice of antibiotics to use as first line against *Shigella* infection should be based on the local antibiotic sensitivity patterns and should be updated regularly. The World Health Organisation guidelines have recommended ciprofloxacin as the drug of choice for all patients with bloody diarrhoea, irrespective of their age. Although quinolones have been reported to cause arthropathy in young laboratory animals, the risk of joint damage in children appears to be minimal and is clearly outweighed by the value of these drugs for treatment of this potentially life-threatening disease. Pivmecillinam (amdinocillin pivoxil), ceftriaxone and azithromycin are considered as alternatives for treatment of multi-resistant strains of *Shigella* in all age groups (WHO 2005a). Unfortunately, strains of *Shigella* developed resistance to fluoroquinolones and other antibiotics have been reported (Taneja et al. 2005).

1.7.4 Other antimicrobial agents:

Because of increasing concerns about emergence of drug resistance and side-effects, other kind of antimicrobial agents have been investigated. Rifaximin, a non-absorbed rifamycin derivative, has been shown to be effective in preventing shigellosis in a volunteers challenge study (Taylor et al. 2006), but is not approved for the treatment of diarrhoea caused by invasive enteric pathogens such as *Shigella* spp., *Campylobacter jejuni*, and *Salmonella* spp.. Sodium butyrate, the salt of a short-chain fatty-acid which can increase the production of endogenous antimicrobial peptides may help resolve *Shigella* infection in laboratory animals without using antibiotics (Raqib et al. 2006). There are no human clinical trials to evaluate this drug in the treatment of *Shigella* infection. A diet with green banana, which is rich in amylase-resistant starch that stimulates colonic production of short-chain fatty-acid, has been reported improving the clinical severity of childhood shigellosis treated with ciprofloxacin (Rabbani et al. 2009).

1.8 Antibiotic resistance:

1.8.1 Occurrence of antibiotic resistance:

Resistance to antibiotics has been recognized in *Shigellae* very early in the era of antimicrobial therapy. In Japan shortly after sulphonamide, chloramphenicol and tetracycline were introduced into clinical practice, strains of *Shigella* which were resistant to these antibiotics developed rapidly from 0.7% in 1956 to 38.7% in 1963 (Hiroshi 1964). Japanese scientists carried out experiments to show that resistance to multiple antibiotics spreads easily in the *Shigellae* community and to other Gram negative bacteria by a "resistance factor" (R factor) now known as transferable plasmid (Akiba et al. 1960; Watanabe 1963).

Ampicillin and trimethoprim-sulfamethoxazole (co-trimoxazole) were then used and continue to be effective in many industrialized countries. Unfortunately, resistance to these low-cost oral antibiotics developed quickly in both tropical and industrialized countries (Salam and Bennish 1991; Sivapalasingam et al. 2006).

Nalidixic acid, a drug of the first generation of quinolone family, was then introduced for the treatment of shigellosis. At first, the clinical response to Nalidixic acid treatment was impressive, but resistance developed very quickly. The story of Central Africa best illustrates the typical scenario of antibiotic resistance. An epidemic of shigellosis started in south-eastern Zaire in November 1979, and quickly spread to neighbouring areas of Rwanda, Burundi, and the United Republic of Tanzania. The initial *S.dysenteriae* type 1 isolates were resistant to ampicillin, chloramphenicol and tetracycline.

In July 1981 co-trimoxazole replaced tetracycline as the first-line treatment, and by September of that year trimethoprim resistant strains were isolated. In November 1981, Nalidixic acid was introduced as first-line therapy for Shigellosis, which reduced the case fatality rate. Predictably, by April of 1982, the first *S. dysenteriae* type 1 strains resistant to Nalidixic acid were reported (Shears 1996).

Fluoroquinolones, the second generation quinolones including ciprofloxacin, norfloxacin, ofloxacin, have wider antibacterial spectrum and better intracellular distribution, and proved highly effective in vitro against *Shigella.* Controlled clinical trials have supported the clinical efficacy of these fluoroquinolones for the treatment of drug-resistant shigellosis (Gotuzzo *et al.* 1989; Vinh *et al.* 2000; ZimBaSA_Dysentery_Study_Group 2002).

Since 2005 the World Health Organization recommended ciprofloxacin as the drug of choice for the treatment of shigellosis in both adult and children (Anonymous 2005). Unfortunately, ciprofloxacin-resistant *S. dysenteriae* type 1 strains were detected at the start of the 21st Century (Naheed et al. 2004; Taneja et al. 2005).

Ceftriaxone, the injectable third generation cephalosporin antibiotics, has been used to treat severe shigellosis with good results (Eidlitz-Marcus et al. 1993; Varsano et al. 1991). Cefixime, the oral third generation cephalosporin drug however failed in the treatment for shigellosis in adults when used in standard dosage (400mg/dayX 5 days) (Salam et al. 1995), but still had some efficacy in children (Martin et al. 2000). Strains of *Shigella* which are resistant to third generation cephalosporin have been recognized recently (Fortineau et al. 2001; Radice et al. 2001) and have spread widely (Vinh et al. 2009a).

Azithromycin is a second generation macrolide. It inhibits growth of *Shigella in vitro* and has high intracellular concentration (40 times the extracellular concentration). In a malaria control campaign, azithromycin prophylaxis prevented epidemic dysentery caused by *S.dysenteriae* (Shanks et al. 1996). Later on Azithromycin proved effective in the treatment of moderate to severe shigellosis caused by multidrug-resistant strains (W. A. Khan et al. 1997). Azithromycin resistant *S.sonnei* has been identified recently (Boumghar-Bourtchai et al. 2008).

1.8.2 Mechanisms of resistance:

Similar to other Gram negative bacteria, the mechanisms of antibiotic resistance of *Shigella* include: alteration/modification of the target site; modification/degradation of the antibiotic molecule; reduced drug uptake, and the energy-dependent efflux leading to decreased intracellular antibiotic concentration. Resistance to commonly used antibiotics

(sulphonamide, trimethoprim, beta-lactams, tetracycline, and streptomycin) can be transferred from *Shigella* spp. to *E.coli* and vice versa by transferable plasmids (Tanaka et al. 1983). This may help explain the rapid emergence of drug resistant *Shigella* strains resulting in dysentery outbreaks (Shears 1996).

Trimethoprim-Sulfamethoxazole resistance in *Shigellae* was first detected in 1980 (Finlayson 1980). Trimethoprim inhibits bacterial dihydro-folate reductase (DHFR) enzyme, sulfamethoxazole blocks dihydroopterate synthase (DHPS) enzyme. Synergy between TMP and SMZ is based on their blocking sequential steps in the pathway of folic acid which is essential for nucleic acid synthesis. The most common mechanism of resistance to TMP is acquisition of an additional variant DHFR enzyme which has reduced affinity to TMP. The most common variant is DHFR I. Similar to TMP resistance, the most common mechanism for SMZ resistance in Gram negative bacteria including *Shigella* is the acquisition of a plasmid encoding altered DHPS with reduced affinity for SMZ.

Tetracycline resistance was first recognized in *S.dysenteriae* in 1953 and demonstrated to be plasmid-transferable (Roberts 1996). The resistance gene *tet* is carried on a transposon or on a plasmid. Streptomycine and aminoglycoside resistance are encoded by *aad*A1a and *aad*A2 genes usually located in a Class 1 integron (Navia et al. 2004).

Nalidixic acid resistance is usually mediated by a mutation at position 83 and 87 in *gyrA* or position 80 in *parC* genes (Chu et al. 1998; Pu et al. 2009). There have been reports of fluoroquinolone resistance mediated by double mutations (Ser83→Leu, Asp87→Asn or Gly) in the *gyrA* gene and a single mutation (Ser80→Ile) in *parC* gene (Talukder et al. 2006).

Ampicillin is the most commonly used antibiotic of the beta-lactam class for the treatment of *Shigella* infection. Beta-lactams target penicillin-binding proteins (PBPs), which carry out functions essential for the bacterial cell-wall. The binding of beta-lactams to PSBs in susceptible species results in bactericidal activity. The resistance to beta-lactams may arise in *Shigella* as a result of mutations which reduce affinity of PBPs to antibiotics. But the most important mechanism of resistance to this class of antibiotics is the production of beta-lactamases. Beta-lactamases are a diverse group of enzymes which hydrolyzed the beta-lactam ring, inactivating the drug. Classification of beta-lactamases has been based on the functional characteristics (group 1, 2, 3, 4) and molecular structure (class A, B, C, D) of the enzymes. The majority of beta-lactamases belong to the TEM (Temoniera), SHV (Sulphydryl variable), OXA (oxacillinase), and extended-spectrum beta-lactamase (ESBLs). The genes encoding these enzymes may be located on the chromosomes, on plasmids, or on transposons.

Resistance to third generation cephalosporin is primarily through the production of ESBLs. Most ESBLs produced by *Shigella* belong to the CTX-M group, very few are TEM and extremely rare are those from the SHV group.

Macrolide resistance is mediated by a plasmid-born *mph*(A) gene encoding a macrolide 2'-phosphotransferase that inactivates macrolides (Boumghar-Bourtchai et al. 2008; Phuc Nguyen et al. 2009).

1.8.3 Randomised controlled trials in the treatment of drug-resistant shigellosis:

Antibiotic resistance is a well-known phenomenon in *Shigella* spp., new and effective antibiotics are needed to replace old ones in the treatment of shigellosis caused by resistant strains. A good antimicrobial drug should be effective microbiologically to local strains

(low MIC, good intracellular penetration), easy to use (administered orally is preferable to intravenous administration), less side-effects particularly in children, and be available at an affordable price. Clinical trials have been carried out in the hope of finding such appropriate antibiotics.

The International Centre for Diarrhoeal Diseases Research, Bangladesh has been one of the most innovative institutions in the treatment of diarrhoea and have carried out a large series of randomised controlled trials looking for effective antimicrobial agents for the treatment of shigellosis (Alam et al. 1994; Bennish et al. 1990; W. A. Khan et al. 1997; Salam and Bennish 1988; Salam et al. 1995). Studies from other places in the world have also contributed to the guidance on effective antibiotics for the treatment of shigellosis (Basualdo and Arbo 2003; Martin *et al.* 2000; Vinh *et al.* 2000; ZimBaSA_Dysentery_Study_Group 2002).

A systemic review of randomised controlled trials comparing antibiotics of different class for the therapy for *Shigella* dysentery has been recently published (Christopher et al. 2009). The results of this systematic review provide evidence that the most commonly used antibiotics are potentially effective against *Shigella* dysentery, provided the local species and strains of *Shigella* are susceptible. Regular, periodic antibiotic-susceptibility testing of isolates is essential to guide local empiric therapy for *Shigella* dysentery.

Another paper reviewed all literature reporting the effect of ciprofloxacin, ceftriaxone and pivmecillinam for the treatment of dysentery in children in the developing countries has also been recently released. The authors concluded "The antibiotics recommended by the WHO—ciprofloxacin, ceftriaxone and pivmecillinam—are effective in reducing the clinical and bacteriological signs and symptoms of dysentery and thus can be expected to decrease diarrhoea mortality attributable to dysentery" (Traa et al. 2010).

1.9 Prevention:

The source of *Shigella* is primarily infected humans and the transmission faecal-oral route. Hence prevention of *Shigella* infection should firstly comprise better individual hygiene and improvement in community sanitation. Frequent hand washing with soap before food preparation, before meals, after defecation and changing baby diapers, will reduce the spread of the disease, particularly in outbreaks (Pal 2004). In some regions, the control of house-flies may be helpful for the prevention of shigellosis.

Safe water supply and storage as well as proper disposal of human excreta are of utmost important in the prevention of shigellosis and other diarrhoeal diseases. While improving environment sanitation and personal hygiene cannot be achieved in a short period of time, an effective and cheap vaccine is obviously a high priority.

1.9.1 Vaccine:

A vaccine for the prevention of shigellosis was set as a high priority by the World Health Organisation almost two decades ago (Anonymous 1987).

1.9.1.1 Protective immune response:

Shigella infection confers protective immunity, although its mechanisms are not fully elucidated. Epidemiologically, shigellosis peaks during the first five years of life and subsequently declines suggesting that immunity occurs following repeated exposures to *Shigella* during childhood. The natural immunity to *Shigella* infection is species and serotype-specific. A study in Chilean children showed that the primary *Shigella* infection conferred 72% protective efficacy against re-infection with a homologous serotype (Ferreccio et al. 1991). Serum and stool levels of antibodies to *Shigella* O-antigen LPS and

Ipa proteins have been detected following natural infection and infection in volunteers (Cam et al. 1993).

Considering that the protective immunity against *Shigella* infection is species and serotype-specific, the protective capacity of any *Shigella* vaccine candidate will depend greatly on the representation of species and serotypes incorporated in the vaccine and the epidemiological importance of these respective serotypes in the area. Because the prevalence of *Shigella* species and serotypes in an area may change by time, it is prudent to monitor the disease burden and the prevalence of representative of *Shigella* species and serotypes in any area where such vaccine candidates are to be studied.

The highest incidence, morbidity, and mortality of shigellosis are in children less than 5 years old. There a good vaccine must elicit sufficient protective immunity in this age group.

1.9.1.2 Current approaches to *Shigella* vaccines:

In addition to the preparation of sub-cellular vaccines (such as ribosomal-based vaccine composed of O-antigen and ribosome of *S.flexneri* 2a which has been shown to be immunogenic and provide efficacy in mice (Shim et al. 2007), however there still needs to demonstration of the protective capacity in humans), two major approaches have been investigated recently with encouraging results: live, attenuated vaccine and inactivated vaccine.

- Live, attenuated *Shigella* vaccine: An oral live-attenuated vaccine which mimics the natural infection without causing symptoms should be an ideal candidate given that it elicits good immunity and potentially has fewer associated adverse events. Three vaccine candidates using this approach have been tested recently in various phase of clinical trials.

SC602 is an *S.flexneri* 2a vaccine constructed at the Institute Pasteur Paris, by deleting the *icsA* and *iuc* genes. SC602 is fully invasive for tissue culture cell, but intercellular spread is negated. Studies in North American volunteers have demonstrated that SC602 can evoke protection against the most severe symptoms of shigellosis in a stringent human challenge model of disease (Katz et al. 2004). But clinical studies in children in Bangladesh showed that the candidate vaccine is apparently overly attenuated for the target population in an endemic region.

SC599 *S.dysenteriae* type 1 vaccine strain was constructed by creating deletions in several genes including *icsA*, *ent*, *fep* and *stxA*-negative: HgR. Phase I and II clinical trials in St. George's Vaccine Institute in London and Cochin-Vaccinology Centre (France) showed that it was highly attenuated, well tolerated and capable of inducing mucosal IgA ASC response and significant serum antibody response (Launay et al. 2009).

WRSS1 is an *S.sonnei* vaccine strain in which the *icsA* gene has been deleted. The vaccine candidate elicited a dose-dependent anti-LPS IgA response (Orr et al. 2005). Second generation of the live vaccine strain has been constructed recently by further deleting the genes encoded for ShET2-1 enterotoxin which showed lower level of reactogenicity without hampering the robust immune responses achieved with previous live vaccines (Barnoy et al. 2010; Ranallo et al. 2007).

CVD series of *Shigella* vaccines: live, attenuated vaccine candidates have been constructed at University of Maryland Center for Vaccine Development (CVD). CVD 1023, an *S.flexneri* 2a vaccine (with deletion of *aroA* and *icsA*) gave unacceptable reactogenicity, CVD 1207 (with deletions in *guaBA*, *set*, *sen* and *icsA*) gave immunogenic response but could have been hyperattenuated. CVD 1208 (deletion in *guaBA* and *sen* and *set*) was

considered a highly attractive candidate that reflects the desired balance clinical acceptability and robust immunogenicity (Kotloff et al. 2007).

Multivalent *Shigella* vaccines: A global *Shigella* vaccine must protect against 16 serotypes and sub-serotypes, namely *S.dysenteriae* type 1, *S.sonnei* and all 14 classical *S.flexneri* types and sub-serotypes (Levine et al. 2007). Such a vaccine would be impractical and expensive. An alternative approach proposed by researchers at CVD is to include *S.dysenteriae* type 1, *S.sonnei* and *S.flexneri* 2a, *S.flexneri* 3a and *S.flexneri* 6 in a multivalent vaccine, because these 3 *S.flexneri* serotypes have O-antigen group determinants that are shared by the remaining 11 *S.flexneri* serotypes and sub-serotypes (Noriega et al. 1999).

- *Shigella* conjugate vaccine: In subjects immune to *Shigella* a small amounts of serum IgG transudate onto the gut surface where they can neutralized or inactivate inocula of wild-type *Shigella* organisms (Robbins et al. 1992). Based on this concept researchers created parenteral conjugate vaccines that consist of O-polysaccharides derived from the LPS of relevant *Shigella* serotypes covalently linked to a carrier protein (*Pseudomonas aeruginosa* exotoxin A (PsA) or CRM9-mutant diphtheria toxin). In a randomised, controlled, double-blind Phase III efficacy trial involving several hundred Israeli soldiers, a single dose of the *S.sonnei* conjugate vaccine conferred 74% protection against *S.sonnei* diarrhoea during outbreaks on army bases (Cohen et al. 1997). The efficacy of the *S.sonnei*-CRM9 and *S.flexneri* 2a-rEPA vaccine in 1-4-year-old children was related to the level of conjugate-induced IgG anti-O-antigen antibody (Passwell et al. 2003).

Alternative strategies with inactivated vaccines: A new generation of *Shigella* conjugate vaccine, synthetic carbohydrate-protein conjugate vaccine, based on synthetic

oligosaccharides conjugated with proteins, offers promise for enhanced immunogenicity and diminished production costs (Phalipon et al. 2009). Formalin-inactivated whole-cell *Shigella* spp. retains the ability to induce protective immune response in mice and therefore may be an effective vaccine candidate approach (Osorio et al. 2007).

Despite many attractive candidate vaccines having entered clinical trials, none of *Shigella* vaccines is yet available for use. There is clearly a long way to go and much work to be done before a *Shigella* vaccine will help protect the vast majority of young children in developing countries as well as travelers from industrialized countries from shigellosis.

1.10 Studies on *Shigella* infection in Viet Nam:

As an important cause of diarrhoeal diseases in Viet Nam, *Shigella* infection has been studied as early as the 1950s (Le Gac et al. 1954), and continued to the 1960s but mainly in patients belong to foreign military forces working in Viet Nam (Grant et al. 1969; Vaichulis et al. 1967). From the 1990s, more and more studies were carried out in Viet Nam dealing with epidemiology and immune responses to *Shigella* infection (Cam et al. 1992; Cam et al. 1993; Li et al. 1993; Lindberg et al. 1991).

In Viet Nam shigellosis (as well as cholera and typhoid fever) are reportable diseases by the national health system. The median reported incidence rate for shigellosis for the whole country in the period 1991-2001 was 41/100,000/year (Kelly-Hope et al. 2007). Because microbiological facility is limited in most provinces, the report of shigellosis cases was usually based on treated episodes of acute dysentery; so that the number will undoubtedly be an underestimate.

From the turn of the 21st century more studies on the incidence rate of *Shigella* infection were carried out at the community and at the hospital level. At the community level, *Campylobacter*, enterotoxigenic *E.coli* and *Shigella* were most commonly isolated from diarrhoea stools of children living along the Red River Delta in northern Viet Nam (Isenbarger et al. 2001). Recently, the data of a prospective population-based study in six Asian countries showed that *Shigella* incidence rate was 4.9/1000/year in Viet Nam, higher than that of Thailand but lower than those of Indonesia, Pakistan, China and Bangladesh (von Seidlein et al. 2006). A study in children hospitalised because of acute diarrhoea in Ha Noi revealed that *Shigella* was the most important bacteria (along with rotavirus, the most important virus) associated with acute diarrhoea (Hien et al. 2008; Isenbarger et al. 2001; von Seidlein et al. 2006). Studies investigating the willingness of people to purchase or use a *Shigella* vaccine were also published (DeRoeck et al. 2005; Kaljee et al. 2004). The epidemiology of *Shigella* infection in southern Viet Nam has been changing dramatically: the dominant *Shigella* species circulating in southern Viet Nam changed from *S.flexneri* in the 1990s to *S.sonnei* at the first decade of the 21st century (Vinh et al. 2009a; Vinh et al. 2009b).

The occurrence of multiple drug resistant *Shigella* strains were increasingly recognized: 62% of 63 *Shigella* strains in Ho Chi Minh City were resistant to multiple antibiotics (Vinh et al. 2000); antibiotics susceptibility of *Shigella* strains and other enteric pathogens in Viet Nam was comparable with those in Thailand (Isenbarger et al. 2002). The proportion of nalidixic acid resistant *Shigella* strains was 2% in mid 1990s (Vinh et al. 2000) has increased to 68% in 2008 (Vinh et al. 2009b). Recently, the rapid occurrence of ceftriaxone-resistant *Shigella* strains was reported for the first time in Viet Nam (Vinh et al. 2009a). The molecular nature of the third-generation cephalosporine resistant genes were characterised and the transferrable plasmids which carried these genes were also described (Nhu et al. 2010).

1.11 Conclusion:

It is more than 100 years from the days Dr. Shiga and the first identification of the microbe that causes severe dysentery. However in 2010 shigellosis remains a very real public health issue globally. The true burden of disease is difficult to assess because the fragility of the causative organism and a lack of microbiological facilities in many parts of the world. An effective vaccine is as elusive as ever for the prevention of the disease and there is the continued emergence and spread of drug resistance.

More work needs to be done to better document the importance of *Shigella*, the wide range of clinical pictures of the disease, the changing distribution of *Shigella* species and the extent and importance of antibiotic resistance. My thesis focuses on these issues in a country still troubled by shigellosis despite its impressive developmental and economic gains.

1.12 Focus, aims and structure of the thesis:

This thesis focuses upon the clinical features, epidemiology and treatment of *Shigella* infection in Vietnamese children and aims to address four questions:

- What is the magnitude of *Shigella* infections as a causative agent of childhood acute diarrhoea in southern Viet Nam? Is clinical picture specific enough to differentiate acute diarrhoea caused by *Shigella* from that caused by rotavirus?

- Are there changes in epidemiology, antibiotic susceptibility and clinical aspects of childhood shigellosis in southern Viet Nam over the last 15 years?

- What is the molecular characteristic of ESBL genes in *Shigella* population in Viet Nam? What plasmids are responsible for the transmission of ESBL genes?

- Is gatifloxacin as effective as ciprofloxacin in the treatment of *Shigella* infection in children?

Chapter Two

Materials and Methods

2.1 Introduction:

This chapter describes the setting of the work undertaken, the region, the hospitals and the study wards. Clinical and laboratory methods are also described, although further relevant details are provided in subsequent chapters. Statistical tests and statistic software used for data collection and analysis are included in this chapter.

The studies included in this thesis were carried out at The Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Dong Thap Provincial Hospital (DTP) in Dong Thap Province in the Mekong Delta, and the Oxford University Clinical Research Unit (OUCRU), Ho Chi Minh City, Viet Nam.

2.1.1 Geography of Viet Nam:

Viet Nam, officially the Socialist Republic of Viet Nam (SRV), is an S-shaped country located in Southeast Asia. It is bordered by China to the north, Laos to the northwest, Cambodia to the southwest, and the Tonkin Gulf and the South China Sea, referred to as East Sea, to the east and the Gulf of Thailand to the south.

The total area of Viet Nam is 331,210 sq km of which land 310,070 sq km and water 21,140 sq km. The climate in Viet Nam is tropical in the south and temperate in the north. The average annual temperature is generally higher in the plains than in the mountains and plateau, and higher in the south than in the north. Temperature in the southern plains (Ho Chi Minh City and the Mekong Delta) varies less, and is between 21 and 38°C over the course of a year. The temperature variations in the mountains and

plateaus and in the north are more dramatic, and temperature may vary from 5°C in December and January to 37 °C in July and August. The annual average amount of rainfall is 1960 mm. Bac Quang in the north has the highest rainfall with an annual average of 4760 mm. The lowest average rainfall is Phan Rang in Central Viet Nam with only 650 mm per year.

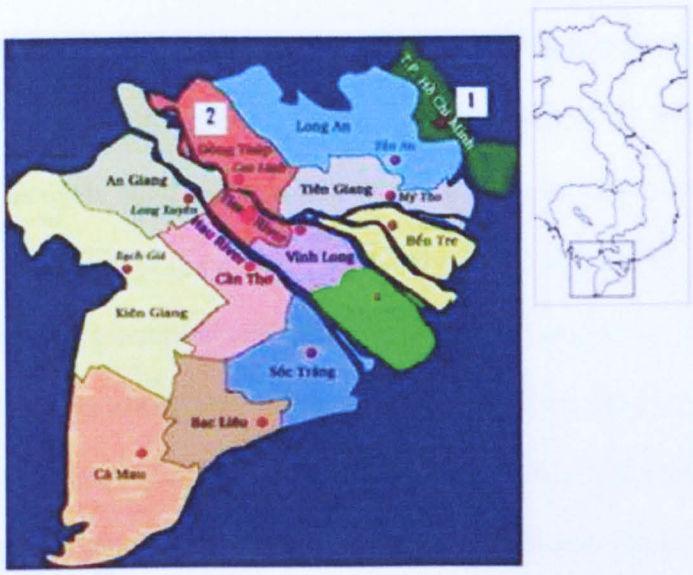

Figure 2.1 Map of Viet Nam (right upper black and white map) and study sites in southern Viet Nam (left colour map): (1) Ho Chi Minh City and (2) Dong Thap Province with adjacent provinces in the Mekong Delta.

2.1.2 Health Care in Viet Nam:

Viet Nam has a population of over 86 million making it the 13th most populous country in the world. Children aged 0-14 years comprise 26.1% of the population. The population growth rate was 1.1% in 2009. The birth rate was 17.73 births/1000 population (2009) and the death rate 5.98 deaths/1,000 population (2009). (General_Statistics_Office 2010). Literacy rates are estimated to be 90.3% (2002) (www.cia.gov/library/publications/the-world-factbook/geos/vm.html, accessed 20-4-2010).

The Gross Domestic Product *per capita* was US$1064 (2009), with an annual growth rate or 6.31% (2008) (General_Statistics_Office 2010) and per capita purchasing power parity of approximately $3,300 (2006).

The Viet Nam health system has been established at four levels across the country: Commune, District, Provincial and National levels. The overall health system performance in Viet Nam was ranked 160 over 191 by the World Health Organization (WHO 2000, Annex Table 1). Infant mortality rate (IMR) was 22.26 deaths/1,000 live births, life expectancy at birth total population: 77.71 years (2009). Although Viet Nam has made rapid progress in improving its water supply situation over the past decades, many parts of the country, especially those areas heavily populated with ethnic minority groups, often rural and remote and with the poorest communities have been left behind. Progress towards providing access to sanitation and hygiene has been especially slow. A recent survey on sanitation revealed that 52% of rural populations have access to some sort of sanitation facilities; however, only 18 per cent of them have access to latrines that meet acceptable hygienic standards. The same survey indicates that only 12% of schools have hygienic sanitation facilities (http://www.unicef.org/Viet Nam/wes.html. Accessed 20 April 2010).

Across the country there are a total of 974 hospitals, 781 regional polyclinics with 170,500 beds and approximately 6.7 doctors per 10,000 populations (General_Statistics_Office 2010). The health expenditure per capita was US$58 per year in the year 2007 by World Bank estimation (Source: World Bank http:// data.worldbank.org/indicator/SH.XPD.PCAP, accessed 10 July 2010) and according to the World Health Organisation the total expenditure on health at purchasing power parity per capita of Viet Nam in 2006 was US$151 (WHO 2009).

2.1.3 The Hospital for Tropical Diseases, Ho Chi Minh City:

Ho Chi Minh City is the largest city in Viet Nam (2095.5 sq km) with an estimated population of 7,165,200. The population density is 3419/ sq km (General_Statistics_Office 2010). The Hospital for Tropical Diseases (HTD) in Ho Chi Minh City is a tertiary referral centre for patients with infectious diseases and serves the whole of southern Viet Nam (population approximately 42 million). It also acts as a primary and secondary care health facility for patients living around the hospital. The hospital has 550 beds, with 200 beds for paediatric patients. The HTD has laboratories for haematology, biochemistry, microbiology and parasitology.

Paediatrics Ward B located at the 4th floor of the HTD has 44 beds dedicated to the treatment of children with enteric infections (diarrhoeal diseases and typhoid fever) from Ho Chi Minh City and adjacent areas including the provinces of the Mekong Delta, Long An, Tien Giang, Vinh Long, Ben Tre and Binh Duong.

Figure 2.2 The Hospital for Tropical Diseases in Ho Chi Minh City, Viet Nam.

2.1.4 Dong Thap Provincial Hospital:

Dong Thap is a province in the Mekong Delta of southern Viet Nam with a population of 1,667,700 living in an area of 3375 sq km. The population density is 494/sq km. Dong Thap province has many rivers and canals and is predominantly a rural rice farming region. Between June 2006 and March 2009 the randomised clinical trial (EG study – see Chapter 6) was conducted at the Infectious Diseases Ward, Dong Thap Provincial Hospital (DTH). DTH is the general hospital of Dong Thap Province with 740 patient beds 120 km south of Ho Chi Minh City. The Infectious Diseases Ward has 60 beds for adults and children.

Figure 2.3 Dong Thap General Hospital in Cao Lanh City at the Mekong Delta, Southern Viet Nam. (Photo by Mai Ngoc Lanh)

2.1.5 Oxford University Clinical Research Unit (OUCRU):

The Oxford University Clinical Research Unit opened in 1991, funded by the Wellcome Trust of Great Britain. The unit, located within the HTD, serves as a collaborative centre between the HTD in Ho Chi Minh City, the Department of Health, Ho Chi Minh City and Oxford University. The Unit started as an 8- bed ward for the treatment and

research on severe and complicated malaria. Over two decades the Unit has expanded with a modern 4-storey laboratory with all facilities including classical medical microbiology but also with sophisticated molecular techniques for the diagnosis and investigation of most severe infections including influenza, malaria, dengue, tuberculosis, typhoid fever and other enteric infections, central nervous system infection and tetanus. The OUCRU has developed strong links not only with HTD, but also other hospitals, institutes and universities in Ho Chi Minh City, Hanoi, Hue, the Mekong Delta and other centres in Asia (Nepal, Indonesia and China).

Figure 2.4 The Oxford University Clinical Research Unit, Ho Chi Minh City, Viet Nam.

In the following parts of this chapter (2.2 and 2.3) I describe the general clinical and microbiology methodology used subsequently in the whole thesis. Further relevant details are provided in subsequent chapters.

2.2 Microbiological methods:

From all studies, stool samples were collected from patients and cultured directly within 2 hours on the day of sampling. All specimens were processed in the microbiology laboratory of the HTD.

2.2.1 Stool culture:

2.2.1.1 Bacteria identification:

Samples were cultured directly, and after overnight enrichment in selenite F broth (Oxoid, Basingstoke, UK) onto MacConkey and XLD agar (Oxoid) at 37°C. Colonies suggestive of *Salmonella* or *Shigella* (non-lactose fermenting) were sub-cultured on to nutrient agar and were identified using a 'short set' of sugar fermentation reactions (Kliger iron agar, urea agar, citrate agar, SIM motility-indole media (Oxoid)). After incubation for 18 – 24 h at 37 °C, the test media were read for characteristic *Shigella* reactions. Serology and API 20E test strips of biochemical reactions (BioMerieux, Paris, France) were used to confirm the identity of *Shigella spp.* (Figure 2.5).

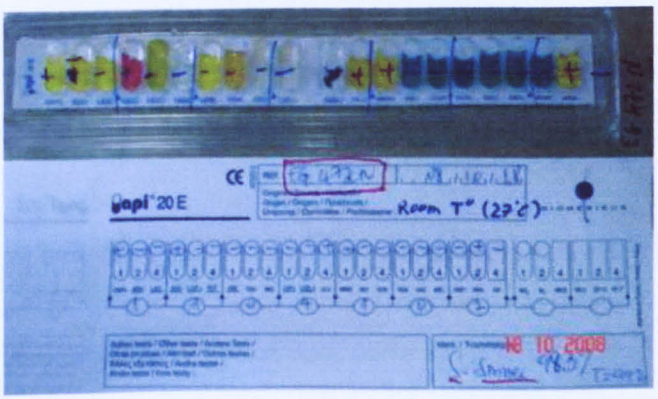

Figure 2.5 Example of API 20E test (bioMerieux, Paris, France) for identification of enteropathogens. Upper half: biochemical reaction, lower half: interpretation of reaction and result of online identification at *https://apiweb.biomerieux.com/.*

Serologic identification was performed by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed by testing with available monovalent antisera for specific serotype identification as per the manufacturer's recommendations (Denka Seiken, Japan). API 20E test was performed as directed by the manufacturer (BioMerieux, Paris, France).

2.2.1.2 Antibiotic susceptibility testing:

Antimicrobial susceptibility testing of all *Shigella* isolates against Ampicillin (AMP), Chloramphenicol (CHL), Trimethoprim – Sulfamethoxazole (SXT), Tetracycline (TET), Nalidixic Acid (NAL), Ofloxacin (OFX;), Ciprofloxacin (CIP), Gatifloxacin (GAT) and Ceftriaxone (CRO) was performed by Kirby-Bauer's disk diffusion test following standardized Clinical and Laboratory Standards Institute methods (Figure 2.6) (CLSI, 2007). The minimum inhibitory concentrations (MICs) were additionally calculated for all isolates by E-test, according to manufacturer's recommendations (AB Biodisk, Sweden) (Figure 2.7).

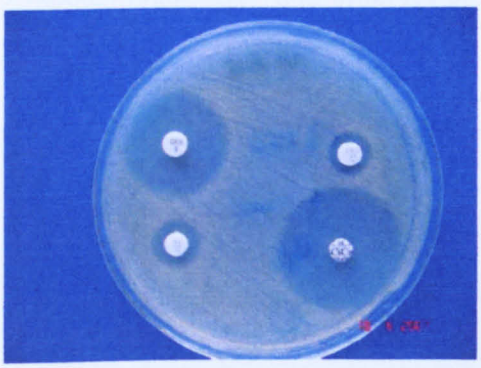

Figure 2.6 Example of Kirby-Bauer antibiotics susceptibility test. This strain is susceptible to ciprofloxacin (CIP, lower right) and ofloxacin (OFX, upper left) but resistant to nalidixic acid (NAL, lower left) and ceftriaxone (CRO, upper right).

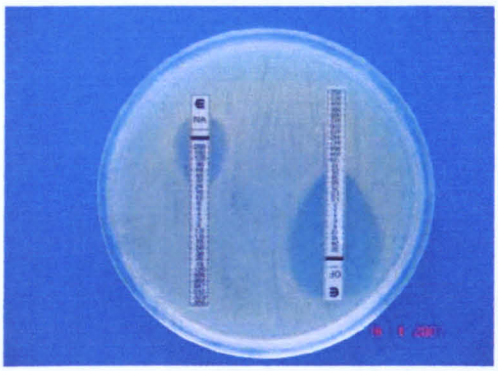

Figure 2.7 Example of E-test to measure the Minimum Inhibitory Concentration (MIC) of bacteria with antibiotics (NAL: nalidixic acid, OFX: ofloxacin).

2.2.1.3 Extended-spectrum-beta-lactamase (ESBL) investigation:

Those strains that were identified as resistant to ceftriaxone using the disk diffusion susceptibility test were further subjected to the "combination disc method" or modified double disc synergy test (DDST) to confirm ESBL production. The combination disc method utilizes discs containing only cefotaxime (CTX) (30 µg) and ceftazidime (CAZ) (30 µg) and both antimicrobials combined with clavulanic acid (CLA) (10 µg). ESBL producing strains were identified as those with a greater than 5 mm increase in zone of inhibition with the single antimicrobial compared to the combined antimicrobial, i.e. demonstrating ESBL inhibition (Figure 2.8).

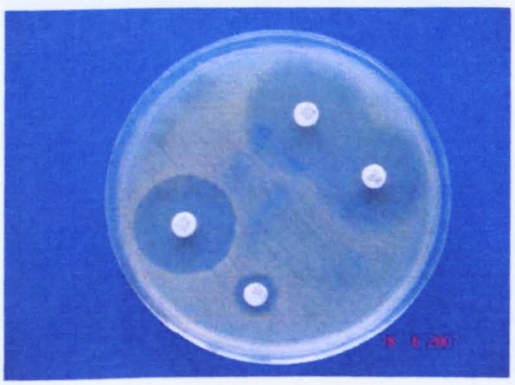

Figure 2.8 Combination disc test for confirming the presence of ESBL. The increase of >5mm of inhibition zone diameter of CTX-CLA (upper left) compare with CTX alone (lower) confirms the presence of ESBL produced by this strain.

In the modified double disc synergy test, a disc containing amoxycillin plus clavulanic acid was placed at the centre of the plate surrounded by discs of CTX, CTX-CLA, CAZ, CAZ-CLA, FEP at regular distances (Figure 2.9).

All antimicrobial testing was performed on Mueller-Hinton agar and interpreted according to the Clinical and Laboratory Standards Institute Guidelines (CLSI, 2007). Quality control of disk diffusion testing is performed weekly with ATCC 700603 *Klebsiella pneumoniae* and *E. coli* ATCC 25922 following CLSI guidelines (CLSI, 2007).

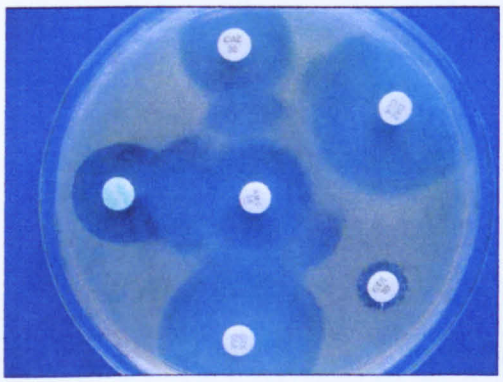

Figure 2.9 Modified double disc synergy test (DDST) to confirm the presence of ESBL. Note the keyhole formations between the pairs CAZ (upper most) and AMC (centre), FEP (left) and AMC, CTX (lower right) and AMC are typical of ESBL producing strains. (CAZ: Ceftazidime; CTX: Ceftriaxone; FEP: Cefepime; CLA: Clavulanic acid; AMC: Amoxycillin plus Clavulanic acid). In this modified DDST, the placement of CTX-CLA and CZA-CLA disc allows comparing their inhibition zone diameters with those of CTX and CZA alone as practiced in combination disc test.

2.2.2 Stool microscopy:

Stool was examined under microscope (HPF (x400)) for white blood cell, red blood cell and parasite. White blood cell and red blood cell count were scored on scale from zero to 4, scale 0 cells = 0/10 HPF, scale 1 = 1 to 10 cells/HPF, scale 2 = 11 to 20 cells/HPF, scale 3 = >20 cells/HPF.

2.3 Clinical methods:

All children admitted to the study were examined by research doctors, a detailed history of illness and physical examination was recorded at enrolment. Body temperature, number and characteristics of stools passed were recorded every 6 hours until discharge. The detailed methodology is described in chapter 6.

2.4 Molecular methods:

2.4.1 Bacterial Conjugation Experiment:

Bacterial conjugation experiments were performed at a 1:1 ratio in liquid cultures. The detailed procedure is described in chapter 5,

2.4.1.1 Transfer of ceftriaxone-resistant genes from wild-type Shigella strains to ceftriaxone-sensitive laboratory *E.coli* strain:

The donor strains were *Shigella* clinical isolates carrying ceftriaxone-resistant genes and the recipient was *E. coli* J53 (sodium azide resistant, ceftriaxone susceptible) (Figure 2.10).

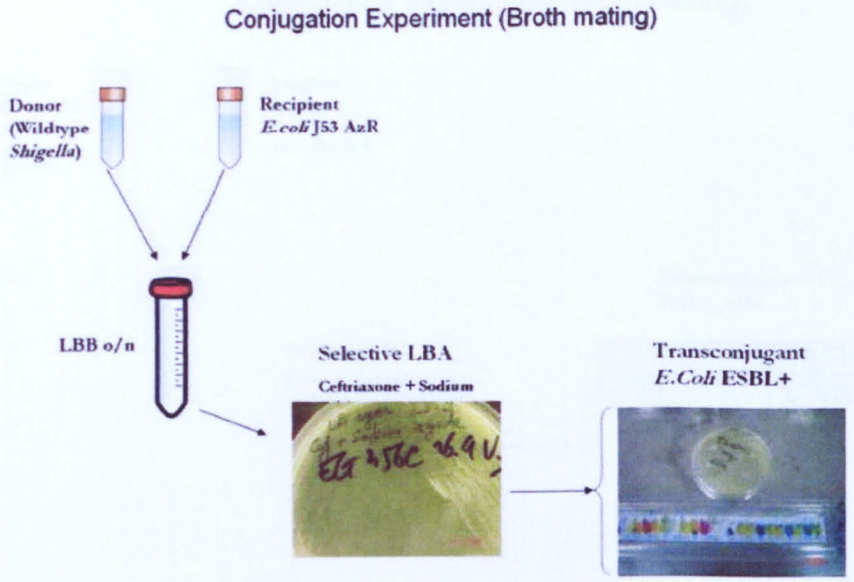

Figure 2.10 Schema of conjugation experiment to confirm the transfer of ESBL genes from the donor wild-type *Shigella* to laboratory *E.coli* J53 AzR.

2.4.1.2 Transfer of ceftriaxone-resistant genes from ceftriaxone-resistant transconjugant back to wild-type ceftriaxone-susceptible *S.sonnei*:

The donor strain in this experiment was a transconjugant resulting from the conjugation of ceftriaxone-resistant strain EG0187 with *E. coli* J53 (the transconjugant was named EG187c), and the recipient was wild-type *S.sonnei* EG 0211, which was a ceftriaxone-susceptible nalidixic acid-resistant *S.sonnei*. Both EG0187 and EG0211 strains were isolated at Dong Thap Provincial Hospital in 2008. The final transconjugant selection was carried out in selective LB media containing nalidixic acid 30 mg/L and ceftriaxone 6 mg/L.

Potential transconjugants were verified by biochemical tests using API 20E set, antibiotic susceptibility test and ESBL confirmation test (Figure 2.11).

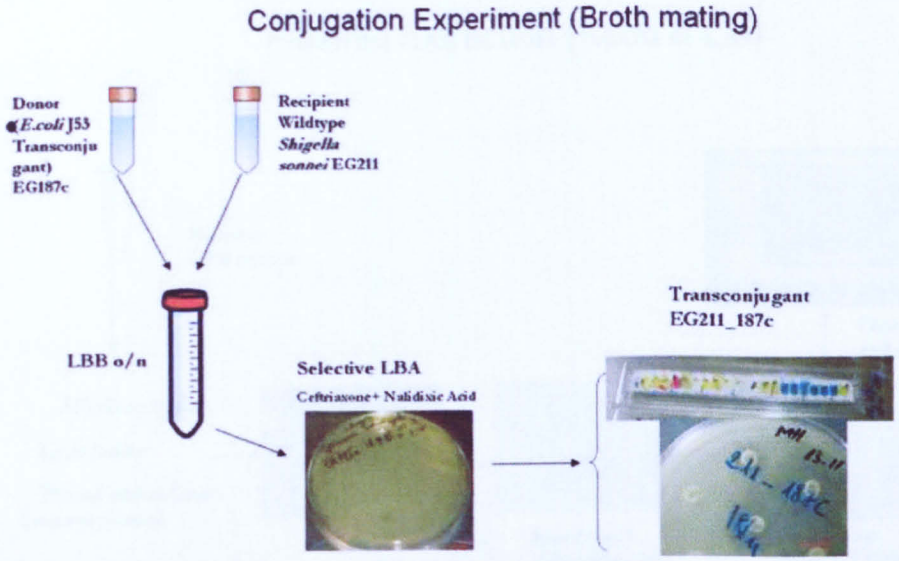

Figure 2.11 Schema of conjugation experiment to confirm the transfer of ESBL genes from the donor transconjugant EG187c (ceftriaxone-resistant) to the wildtype *S.sonnei* EG211 (ceftriaxone-susceptible, nalidixic acid resistant). The resultant transconjugant EG211_187c was resistant to both nalidixic acid and ceftriaxone.

2.4.2 Plasmid extraction and visualization:

Plasmid DNA was isolated from ESBL negative *Shigella* isolates, ESBL positive *Shigella* isolates, their transconjugants, and the laboratory recipient strain *E.coli* J53 AzR using a modified version of the methodology previously described by Kado and Liu (Kado and Liu 1981). *E. coli* 39R861 containing plasmids of 7, 36, 63 and 147kbp were used for sizing plasmid extractions on agarose gels (Figure 2.12).

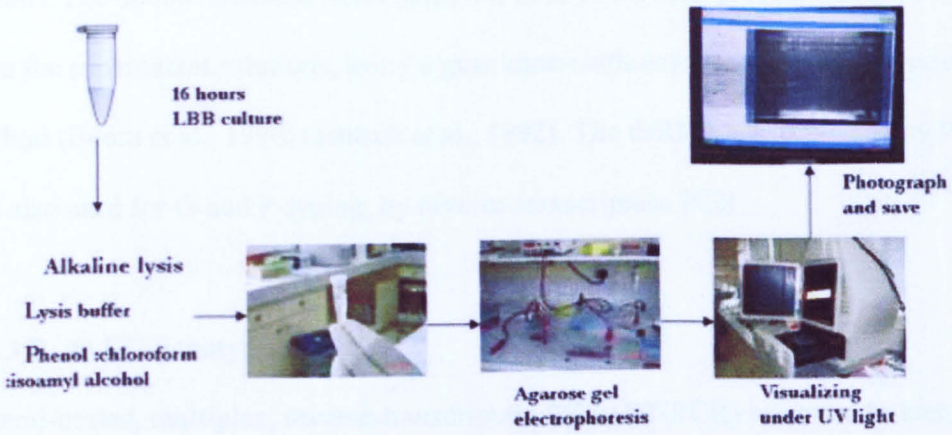

Figure 2.12 Steps in plasmid extractions (Modified Kado& Liu method)

2.4.3 ESBL gene PCR amplification and characterization is described in detail at chapter 5.

2.5. Virology methods:

2.5.1 Electron microscopy:

Faecal suspensions (10%–20%, v/v) were prepared in phosphate-buffered saline (pH 7.2). Copper, electron-microscopy grids (3mmin diameter; 400 mesh) that had been precoated in polyvinyl formal resin (Formvar®) were dipped into the suspension and air-dried. The grids were then negatively stained with 2% (v/v) phosphotungstic acid and then viewed on a Philip's 301 electron microscope at an initial screen magnification of ×45,000.

2.5.2 Extraction of rotavirus dsRNA:

The faecal suspensions were centrifuged in a bench-top microcentrifuge (13,000×g for 10min). The double-stranded RNA (dsRNA) of any rotavirus present was then extracted from the supernatant solutions, using a guanidine-isothiocyanate–silica glass-powder method (Boom et al., 1990; Gentsch et al., 1992). The dsRNA was analysed by PAGE and also used for G-and P-typing, by reverse-transcriptase PCR.

2.5.3 P- and G-genotyping:

A semi-nested, multiplex, reverse-transcriptase PCR (RT-PCR) was used to identify the P- and G-genotypes represented in each dsRNA sample. The primers used enable the detection not only of P[4], P[6], P[8], P[9], P[10], G1, G2, G3, G4, G8 and G9, but also of a novel variant of G1 (G1*) that has been detected in Malawi and a novel variant of P[8] — P[8*] — that has been detected in Malawi and the U.K. Amplicons were subjected to electrophoresis in 2% (w/v) agarose, stained with ethidium bromide and then visualized by ultra-violet trans-illumination. Genotypes were assigned by comparison of the banding patterns with those produced with reference samples of known genotype.

2.5.4 Sequencing:

The variant G1* and P[8*] amplicons were cut from the agarose gels, eluted and purified using a commercial gel purification kit (Qiagen, Crawley, U.K.), cloned into pGEM-T (Promega, Madison, WI) and then transformed into Escherichia coli TG2. Plasmid DNA containing the insert was isolated from the E. coli, using another commercial kit (Qiagen), and then sequenced, using MI2 primers, by Lark Technologies (SaVron Walden, U.K.). The sequences were compared using the clustal x software

package (Institut de Ge´ne´tique et de Biologie Mole´culaire et Cellulaire, Strasbourg; Thompson et al., 1994) and weighted residue tables.

2.6. Statistical methods:

2.6.1 Sample size calculation:

We calculated the sample size of the EG study as follows: based on the result of previous study, the cure rate of the current treatment (ciprofloxacin) was 65% (ZimBaSA_Dysentery_Study_Group 2002), and the expected cure rate of the gatifloxacin was 90%. For 80% power and 5% significance each group needed 50 culture-confirmed cases. Because the stool culture positivity rate was anticipated to be approximately 25%, and the dropped out rate 5%, we enrolled estimated that we needed to enrol 250 cases in each treatment arm.

2.6.2 Statistical analysis:

Data were double entered into Microsoft Excel 2003, Microsoft Access 2003 (Microsoft Corporation, Redmond, 2003) or Epidata version 3.1 (www.epidata.dk) for storage and manipulation. The Mann-Whitney U, Wilcoxon rank sum, and Kruskal-Wallis test were used for non-normally distributed data. Chi^2 test with Yates' correction was used for categorical variables or Fisher's exact test if the number in any cell was less than 5. The survival analysis with log rank test was used to compare the fever clearance time, diarrhoea clearance time and bacteriological clearance time.

2.6.3 Statistical software:

Softwares used for data analysis:

EpiInfo version 6.04d, CDC, Georgia, 2001,

Epidata version 3.1 (www.epidata.dk)

MapInfo Professional software (PitneyBowes, MapInfo Corporation, USA)

SPSS for Windows version 15, SPSS Inc., Chicago, 2006,

and R (http://www.r-project.org/).

2.7. Ethical approvals:

All studies were approved ethical assessment by the Scientific and Ethical Committees of:

- The Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam

-Dong Thap Provincial Hospital, Viet Nam

-Oxford Tropical Research Ethics Committee, UK (ref: OXTREC 010-06).

The clinical trial was registered at International Standard Randomized Controlled Trials Registry (ISRCTN55945881).

Chapter Three

Acute Childhood Diarrhoea: *Shigella* versus Rotavirus

3.1 Introduction:

Acute diarrhoea is a familiar clinical presentation to paediatricians around the world. In the United States alone, an estimated 211–375 million episodes of diarrhoeal illness occur each year, resulting in 73 million physician consultations, 1.8 million hospitalizations, and 3100 deaths (Guerrant 2001). For children under 5 years of age living in developing countries, there are a median of 3.2 episodes of diarrhoea per child-year. Estimates of mortality in developing countries based on studies published between 1992 and 2000 showed a median mortality rate of 4.9 children per 1000 per year in the first 5 years of their lives (Kosek et al. 2003). An update of the World Health Organisation Global Burden of Diseases estimates in 2007 showed that mortality from diarrhoea has declined over the past two decades from an estimated 5 million deaths among children under-five to 1.5 million deaths in 2004, which parallels downward trends in overall under-five mortality during this period. Despite these declines, diarrhoea remained the second most common cause of death among children under-five, following closely behind pneumonia, the leading killer of young children. Together, pneumonia and diarrhoea account for an estimated 40 per cent of all child deaths around the world each year. Nearly one in five child deaths is due to diarrhoea, a loss of about 1.5 million lives each year. The toll is greater than that caused by AIDS, malaria and measles combined (UNICEF/WHO 2009).

Acute diarrhoea may be fatal because of serious complications developing particularly in young children including severe dehydration and acidosis (Farthing 2007). In children in whom the illness lasts two weeks or more (i.e. persistent diarrhoea), nutrition status may deteriorate leading to malnutrition and hence further predispose them to pneumonia and other infections.

More than twenty viral, bacterial, and parasitic enteropathogens are currently associated with acute diarrhoea (O'Ryan et al. 2005). The aetiology of infectious diarrhoeal illness varies in time and places of studies, but generally viruses are the main agents, bacteria rank second and parasites contribute a relatively small part of the overall burden of disease.

In Viet Nam, there have been relatively few studies on the aetiology of acute diarrhoeal diseases and most have been conducted in Ha Noi. These have shown that rotavirus was the main agent, and *Shigella* was the most important bacteria associated with acute diarrhoeal diseases in children under-5 years old admitted to hospitals (Bodhidatta et al. 2007; Hien et al. 2008; Vu et al. 2006).

Knowledge of the agents causing acute diarrhoea in a region is important in informing the public health authorities and guiding the choice of appropriate prevention measures. Because a microbiological diagnosis of acute diarrhoea is not feasible in all cases, clinicians working at the bed-side also need information about aetiology of acute diarrhoea to guide clinical management. Until now comprehensive data on the aetiology of childhood diarrhoeal diseases in Ho Chi Minh City and surrounding provinces at the southern part of Viet Nam was lacking.

In this chapter I describe the aetiology of acute diarrhoea in children admitted to The Hospital for Tropical Diseases at Ho Chi Minh City and compare the clinical and epidemiological features of the two most important enteropathogens in the region: rotavirus and *Shigellae*. The aim of this study is to develop an algorithm to distinguish acute invasive diarrhoeal diseases (the prototype is shigellosis) from acute non-invasive diarrhoeal diseases (the prototype is rotavirus diarrhoea).

3.2 Materials and methods:

Children under 6 year-old of either sex admitted to Paediatric Ward B (See section 2.1.3), The Hospital for Tropical Diseases in Ho Chi Minh City, Viet Nam with a primary history of diarrhoea for less than 7 days were entered into the study provided their parents or care-givers gave informed consent. One admission stool sample from each patient was collected for investigation. Because of laboratory facility limited, only the first 5 patients admitted each day were considered eligible for the study. Microbiological, virological and clinical methods were described in section 2.2, 2.3 and 2.5 in chapter two. Briefly, stool samples examined by microscopy for white cells, red cells and parasites (*E.histolytica*, Ascaris); a smear was made using the modified Ziehl-Nielson stain for detecting *Cryptosporidium* and *Cyclospsora*. Standard culture methods were used for *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Vibrio* spp., *Aeromonas* spp., *Clostridium dificile* and *Yersinia* spp. *C.dificile* toxin was detected by antigen detection kits. Virus was detected by negative-stain electro-microscopy and genotyping (Landaeta et al. 2003). For *E.coli* identification, 5 *E.coli*-like colonies on McConkey agar from each sample was saved at -20°C for later molecular diagnosis of diarrhoeagenic *E.coli* [ETEC, EPEC, EIEC, EHEC, EAEC] by multiplex PCR.

Patients were treated according to the Hospital for Tropical Diseases guidelines which were adopted from the World Health Organisation and American Academy of Pediatrics Guidelines (Anonymous 1996). Fluid rehydration (oral or parenteral) and antibiotics were used under the physician's decision not waiting for the stool culture results. Details on demography, epidemiology and basic laboratory results, antibiotics used, clinical response and outcome were recorded in a standard clinical record form.

The study was reviewed and approved by The Scientific and Ethical Committee of The Hospital for Tropical Diseases in Ho Chi Minh City, Viet Nam.

3.3 Results

In the initial twelve month phase of the study there were 556 patients from which comprehensive investigation of enteropathogens (virus, bacteria and parasites) were done. In the second twelve month phase of the study 866 samples were cultured only for *Shigella* spp. (Figure 3.1).

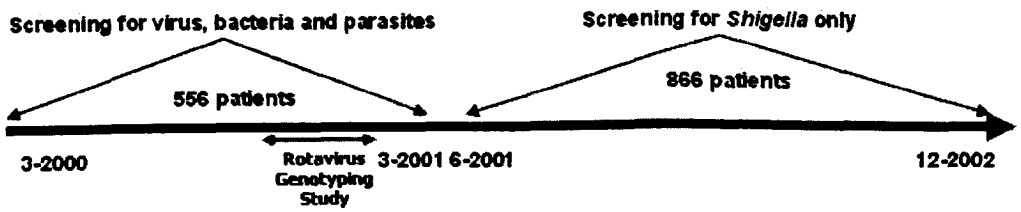

Figure 3.1 Diagram of participant recruitment for diarrhoeal aetiology investigation.

3.3.1 Aetiology of acute diarrhoea:

The microbiological result of Phase I study is presented in Table 3.2. At least one enteropathogen was found in faecal samples of 346 children (62% of total samples). Rotavirus was found in 259 patients (46%) and was the most common agent detected

including 25 mixed infection with other pathogens. *Shigella* was found in 52 cases (9% of total) in which *S.flexneri* 30, *S.sonnei* 20, *S.dysenteriae* 1 (not type 1) and *S.boydii* 1. There were 33 mixed infections in which 2 pathogens were found (6% of total) (Among which rotavirus + *Shigella* in 8 cases and rotavirus + *Campylobacter* in 6 cases) (Table 3.1). The molecular diagnosis of *E.coli* was not successful because the saved samples were damaged during storage.

Table 3.1 Enteropathogens found in 556 children with acute diarrhoea, including cases with mix infections

Pathogen	**No. of cases**	**(%)**
Rotavirus	259	46
Adenovirus	16	2.8
Calicivirus	6	1
Astrovirus	5	1
Shigella: S.flexneri	30	
S.sonnei	20	9
S.dysenteriae	1	
S.boydii	1	
Campylobacter spp.	20	3.6
Salmonella spp.	12	2.1
C.difficile	3	0.5
Ascaris lumbricoides	3	0.5
Cryptosporidium	3	0.5
E.histolytica	1	
None	210	38

* Rotavirus-*Shigella* 8, rotavirus-*Campylobacter* 6, rotavirus-*Salmonella* 2, Adenovirus-*Shigella* 2

In Phase II of the study there were 866 samples cultured and 61 strains of *Shigella* was isolated (33 *S.sonnei*, 22 *S.flexneri*, 6 *Shigella* spp.). Total *Shigella* strains isolated in two periods was 113.

3.3.2 Epidemiological features of rotavirus and *Shigella* infection:

In order to help clincians to distinguish *Shigella* infection from rotavirus infection in children I compared the clinical and epidemiological features of rotavirus and *Shigella* infection after excluding cases with mixed infection. 234 children with confirmed rotavirus infection and 99 Shigellosis cases were available for comparison.

Seasonal variation of rotavirus and Shigella infection:

There was no peak incidence during the year for either rotavirus or *Shigella* diarrhoea cases in the first period of study (Figure 3.2).

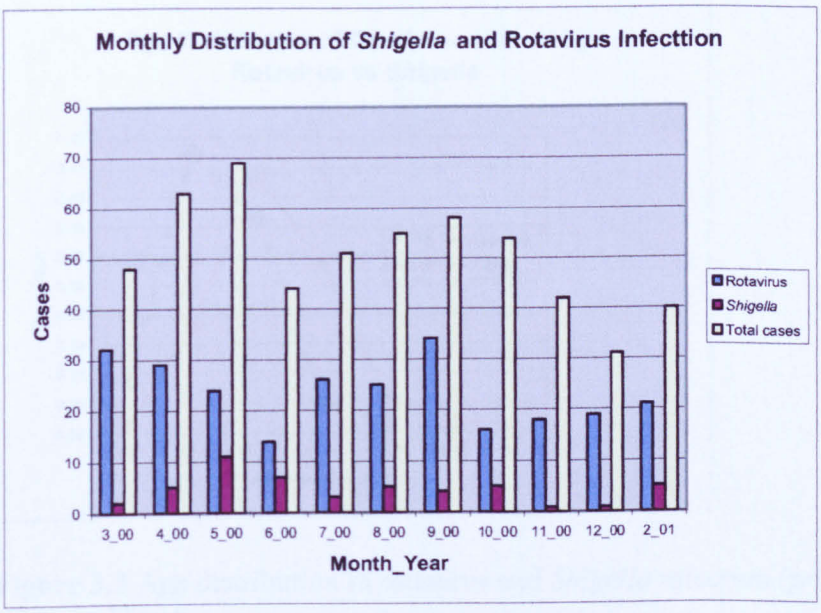

Figure 3.2 Monthly distribution of childhood diarrhoeal cases caused by rotavirus (234 cases) and *Shigella* (99 cases) from March 2000 (3_00) to February 2001 (2_01).

Age distribution of rotavirus and Shigella infection:

The distribution of age group in rotavirus and *Shigella* infection (Figure 3.3) showed that diarrhoea caused by rotavirus peaked in children between 7 to 12 months old. Ninety percent of rotavirus-associated diarrhoea cases were in children $\leq$ 24 months old,

including 8% in children 3 to 6 months old. *Shigella* spp. affected children in an older age group 13-24 months group, and was rarely seen in children less than 6 months old. The median age of children infected by rotavirus was 11 months (IQ range 8-17 months) while the median age of shigellosis patients was 23 months (IQ range 15-30 months). The difference was statistically significant (p=0.001, Mann-Whitney U test) and clinically relevant. Children younger 6 months may be infected by rotavirus but rarely by *Shigella*, suggesting that maternally derived immunity offered some protection against shigellosis but not against rotavirus in this period of the children life; in children older than 24 months the chance of getting shigellosis is higher than for rotavirus.

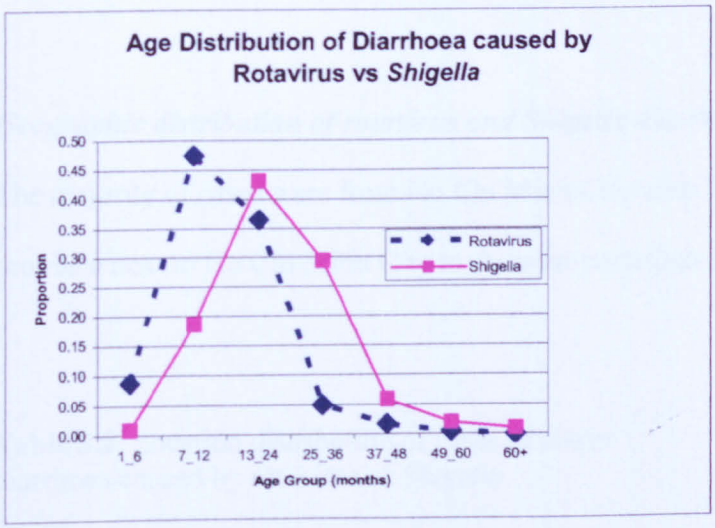

Figure 3.3 Age distribution in rotavirus and *Shigella* infection (p=0.001, Mann-Whitney U test).

Gender in acute diarrhoea caused by rotavirus and Shigella:

More boys suffered from rotavirus than girls with the ratio of boy : girl was 1.5. In contrary the boy : girl ratio of shigellosis cases was 1.

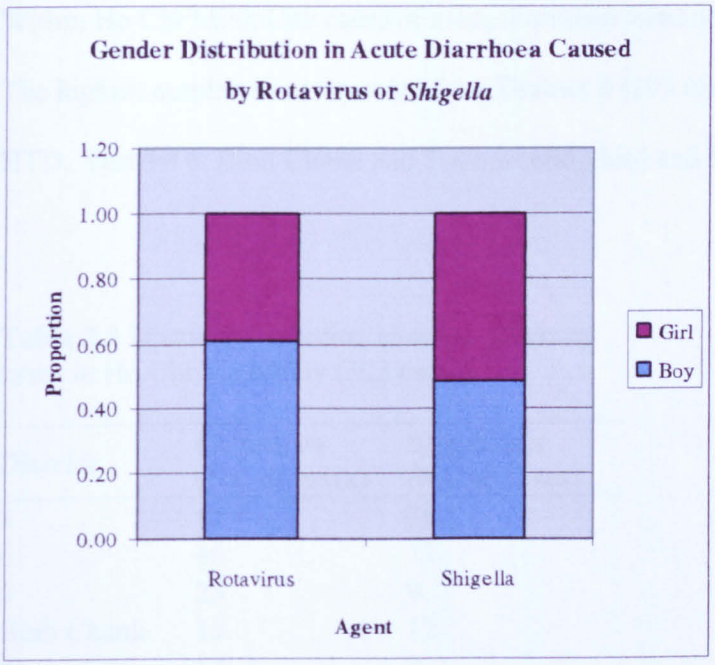

Figure 3.4 Gender in acute diarrhoea caused by rotavirus or *Shigella.*

Geographic distribution of rotavirus and Shigella diarrhoea:

The majority of cases were from Ho Chi Minh City with 303 cases. Long An, the province next to Ho Chi Minh City to the west contributed 25 cases (Table 3.2).

Table 3.2 Location distribution of cases of acute diarrhoea caused by rotavirus or *Shigella*

Province	**Rotavirus (No. cases)**	***Shigella* (No. cases)**
Ho Chi Minh City	215	88
Long An	16	9
Tien Giang	2	1
Lam Dong	1	0
Ben Tre	0	1

Within Ho Chi Minh City cases of acute diarrhoea were distributed in all 14 districts. The highest number of cases came from District 8 (101 cases) the closest district to HTD. District 6, Binh Chanh and 5 are second, third and forth place (Table 3.3).

Table 3.3 District distribution of acute diarrhoea cases in Ho Chi Minh City (303 cases)

District	Rotavirus (No. of cases)	Shigellosis (No. of cases)
8	65	36
6	46	11
5	23	9
Binh Chanh	15	12
11	14	3
10	12	2
Tan Binh	10	5
7	8	4
1	6	2
Nha Be	4	3
4	4	1
Go Vap	3	0
3	3	0
9	1	0
Thu Duc	1	0

3.3.3 Genotypes of rotavirus in Ho Chi Minh City.[1]

Stool samples which were positive for rotavirus by negative-stain electron microscopy were subjected to reverse transcriptase polymerase chain reaction (RT-PCR) to identify the P- and G-genotypes. The primers used enable the detection of not only the P[4], P[6], P[8], P[9], P[10], G1, G2, G3, G4, G8 and G9, but also the variant of G1 (G1*) and of P8 (P8*). The results of the analysis of 49 isolates were presented on Table 3.4 (Landaeta et al. 2003). G1 [P8] and it's variants G1* P[8], G1* P[8*], G1/G1* P[8]/P[4]

[1] This part of the thesis had been published in details else where: M. E. Landaeta et al., 'Characterization of Rotaviruses Causing Diarrhoea in Vietnamese Children', *Ann Trop Med Parasitol*, 97/1 (Jan 2003), 53-9.

was the predominant genotype (20 isolates).Of note was the second place G2 [P4] which has been isolated in 16 patients.

Table 3.4 Genotypes of rotavirus isolated in Ho Chi Minh City, 9-12/2000.

G1 P[8]	2
G1* P[8]	5
G1* P[8*]	12
G1/G1* P[8]/P[4]	1
G1* P[4]	4
G1* P[4]/P[6]	1
G2 P[4]	16
G2/G1 P[4]/P[8]	1
G2 [P6]	1
G2 P[8*]	1
G2 P[NT]	1
G4 P[6]/P[4]	1
G4 P[8*]	2

* mutation variation of G or P genes;
NT: not typed

3.3.4 Clinical features of acute diarrhoea caused by rotavirus and *Shigella*.

The symptoms and laboratory characteristics of 333 children with acute diarrhoea is shown in Table 3.5. Patients with diarrhoea caused by rotavirus were more likely to present with the triad "fever-vomiting-watery diarrhoea"; whereas children with shigellosis more typically presented with "fever-mucoid diarrhoea-abdominal pain" while about half of them also suffered from vomiting and passed watery diarrhoea. Shigellosis cases also had higher blood white cell count than patients with rotavirus diarrhoea (p=0.001), and higher chance of having WBC (94% versus 32%, p= 0.0001) and RBC (70% versus 10%, p=0.0001) in their stools (Table 3.5).

Table 3.5 Clinical symptoms and laboratory findings in children with acute diarrhoea caused by rotavirus or *Shigella* (n=333)

Symptoms	Rotavirus (n=234)	*Shigella* (n=99)	P value
Fever	87%	94%	0.1*
Vomiting	**89%**	56%	0.001*
Watery diarrhoea	**93%**	46%	0.001*
Mucoid diarrhoea	7%	**54%**	0.001*
Abdominal pain	11%	**38%**	0.001*
Convulsion	1%	**7%**	0.01*
Dehydration	1%	6%	.5*
Temperature (°C)	38.0 (37-38.5)	38.0 (37.5-39.5)	0.05**
Blood WBC /μL	7400 (5800-9300)	10150 (7700-13100)	0.001**
Presence of Faecal WBC	32%	94%	<0.001*
Presence of Faecal RBC	10%	70%	<0.001*

RBC: red blood cell; WBC: white blood cell
* Fisher exact test
** Mann-Whitney U test, values are medians (IQ range)

Of note 14/99 (14%) shigellosis cases recovered well despite not having been treated with antibiotics, and 85/234 (36%) cases of rotavirus diarrhoea has been treated with antibiotics (Table 3.6). Five antibiotics were used in these cases: Ofloxacin (41), Norfloxacin (34), Spiramycin (6), Cefaclor (3) and Ceftriaxone (1).

Table 3.6 Clinical outcomes and antibiotic use in acute childhood diarrhoea cases caused by rotavirus or *Shigella* (n=333)

	Rotavirus	***Shigella***	**P value**
Illness before admission (days)[#]	3 ±1.8	2.2 ±1.2	0.001*
Fever Clearance Time (days)[#]	2.1 ±1.6	1.7 ±1.0	0.3*
Diarrhoea Clearance Time (days)[#]	3.1 ±1.8	2.5 ±1.5	0.1*
Hospital stay (days)[#]	4.7 ±2.1	4.0 ±2	0.001*
Treated with Antibiotics	36%	86%	p=0.001 **

* (Mann-Whitney U test)
** Chi^2 test
[#]Values are means ±SD

3.4 Discussion:

3.4.1 Aetiology of acute diarrhoea:

Confirming the causative agent in acute diarrhoea is important not only to inform public health authorities but also in guiding appropriate intervention measures for prevention and treatment. The aetiology of acute diarrhoea varies by location, timing of investigation and the setting in which the study was carried out (hospital-based or community-based). In this hospital-based study in Ho Chi Minh City in the southern part of Viet Nam, at least one enteropathogen was detected in 346 out of 556 (62%) children hospitalised for acute diarrhoea. Rotavirus is the most common agent causing acute diarrhoea in children with 259/556 (46%); *Shigella* spp. ranks second with 52/556 (9%) of all cases. These two were the most important enteropathogens associated with acute diarrhoea in children in Ho Chi Minh City and surrounding provinces. This finding is concordant with data from northern Viet Nam where an study in Ha Noi revealed rotavirus 33/104 (31%) and *Shigella* 21/249 (8.4%) to be the most important

enteropathogens associated with acute diarrhoea in children under 5 years of age admitted to hospitals (Bodhidatta et al. 2007; Hien et al. 2008).

Other viruses were also present in childhood diarrhoea cases in our study: adenovirus 16/556 (2.8%) and astrovirus 5/556 (1%). Calicivirus (including norovirus and sapovirus) were detected in our study in Ho Chi Minh City at low percentage 1% (6/556 samples). However the prevalence of norovirus has been noted to have increased in to 5.5% (56/1010 samples) in a study from October 2002 to September 2003 at Children Hospital Number 1 Ho Chi Minh City (Nguyen et al. 2007), and recently (year 2009) to 27.8% of 187 stool samples from paediatric acute diarrhoea patients in 3 hospitals at Ho Chi Minh city (Steve Baker, personal communication). *Campylobacter* spp. was detected in 20/556 (3.6%) and *Salmonella* spp. in 12/556 (2.1%) of all cases in our study, and were the second and third most important bacteria associated with acute diarrhoea in children. This ranking is different from that reported in Ha Noi where *Salmonella* was the second more important bacteria with 19/291 cases (7%) and *Campylobacter* third with 11/291 (4%). A community-based study carried out in a rural area along the Red River approximately 50 kilometres northwest of Ha Noi isolated *Campylobacter* from 150/2160 (6.8%) of children presenting with acute diarrhoea but also from 8/203 (3.9%) of healthy control children (Isenbarger et al. 2001). The presence of *Campylobacter* as an important bacteria associated with acute childhood diarrhoea may reflect a change of life style in Viet Nam where poultry, increasingly raised by intensive farming methods, processed and sold through supermarkets is being consumed. *Cryptosporidium* was also found in a small number 3/559 (0.5%) of our children with diarrhoea. Enterotoxigenic *Bacterioides fragilis*, an emerging agent causing acute diarrhoea which has been described recently from Ha Noi was not detected in our study (Vu et al. 2006).

Unfortunately for unexpected prolong power cut to the freezer, the *E.coli* identification was unable to perform. Hien and colleagues in their study on aetiology of diarrhoeal diseases in children at Saint Paul Hospital, Ha Noi, detected diarrhoeagenic *E.coli* in 64/249 (25.7%) of diarrhoea children and in 13/126 (10.5%) of healthy children control group (Hien et al. 2008). The pathogenicity index[2] of diarrhoeagenic *E.coli* as a whole in Ha Noi is 2.5 (25.7% / 10.5%) suggesting their role in acute diarrhoea; but separately EPEC, E/EEC and EAggEC were isolated at a high frequency from both cases and controls (Hien et al. 2008). Using multiplex PCR technique to detect diarrhoeagenic *E.coli* in stool samples, Vu *et al* in Ha Noi also reported the prevalence of isolation of diarrhoeagenic *E.coli* strains from under-5 years old children with diarrhoea was slightly higher than that from the age-match healthy controls: 132/587 (22.5 %) in cases versus 30/249 (12 %) in controls (T. V. Nguyen et al. 2005). Further in-depth studies of *E.coli* are needed before firm conclusions can be reached about the role of these potential enteropathogens in Vietnamese children (Hien et al. 2008; Vu et al. 2006).

In northern Ghana, the most common pathogens in paediatric patients were rotavirus (55%), adenovirus (28%) and norovirus (10%); intestinal parasites (5%) and bacteria (5%) were rare. Rotavirus was the only pathogen found significantly more frequently in patients than in controls (odds ratio 7.7; 95% CI, 4.2–14.2) (Reither et al. 2007). In The United States, enteric bacteria contributed only ~5 % while virus contributed 21 % as the cause of acute diarrhoea in children evaluated in outpatient settings (Denno et al. 2005). In a study involved patients who presented with diarrhoea to a children's hospital emergency department at Washington, United States, bacteria were isolated in 119/1626 (7.3%) and virus in 138/417 (33%) of cases (Klein et al. 2006). Rotavirus is the most

[2] Pathogenicity Index (PI): the percentage of diarrhea patients shedding a particular pathogen or shedding *E. coli* carrying a target gene, divided by the respective percentage of control patients with positive test results for that same pathogen or factor.

important virus detected in diarrhoeic children in the United States, just the same with that in Viet Nam in this study and other studies (Bodhidatta et al. 2007; Vu et al. 2006). In contrast, while *Shigella* is the most important bacteria associated with childhood acute diarrhoea in Viet Nam (Hien et al. 2008), in the United States it is *Campylobacter* (Denno et al. 2005).

In our study there were 22 cases (4%) in which two enteropathogens were detected in faecal samples and one case with 3 pathogens (rotavirus plus astrovirus and *Campylobacter* spp.). The presence of poly-microbial infection has been reported in 56/1010 stool samples (5.5%) in another study at Ho Chi Minh City (Nguyen et al. 2007). Mixed infection was also found 2.4% in Pakistan (Khan et al. 1988), 5% in a study in Spain (Roman et al. 2003) and 6% in Greece (Kafetzis et al. 2001). Mixed infection did not cause a more severe clinical picture, particularly the severity of dehydration (Nguyen et al. 2007).

3.4.2 Genotypes of rotavirus in Ho Chi Minh City:

The predominant genotype was G1 [P8] and variants of this genotype (in 20 cases). Interestingly the G2 genotype [P4] was found in 16 patients (Table 3.4). In a study undertaken at the same time at Children Hospital No.1 at Ho Chi Minh City G1 [P8] was the most prevalent with 517/889 (58.2%) of all isolates (Doan et al. 2003). Another study in 2002-2003 also in Children Hospital No.1 at Ho Chi Minh City revealed for the first time the emergence of genotype G9 as the third most common rotavirus G-type in 67/681 samples (13.7%) (Nguyen et al. 2007).

The diversity of rotavirus G and P-genotypes should be taken account when considering vaccine implementation for the prevention of rotavirus diarrhoeal diseases in Viet Nam. A good rotavirus vaccine should provide protection against the majority of viral

genotypes circulating in the region. In the case of Viet Nam, where rotavirus presented with many important genotypes, a monovalent vaccine may not offer sufficient protection against the circulating strains of rotavirus. In March 2006, Brasil innitiated universal immunisation of infants with 2 doses of a monovalent G1[P8] rotavirus vaccine. Two years after that, the hospitalisation of acute rotavirus diarrhoea cases decreased 59%, the number of all-cause acute gastroenteritis hospitalisation reduced 29% among under-five children (Safadi et al. 2010). Among 133 faecal samples collected from February 2005 to December 2007 the genotype G2[P4] was found in 1.4% in 2005, in 44% in 2006, and in 96% in 2007 (Carvalho-Costa et al. 2009). In a study allocating 926 children with acute diarrhoea in Recife City, Brasil, 119 cases were found rotavirus positive in faecal samples. Among 80 severe rotavirus positive cases (defined as cases requiring intravenous fluid rehydration at the Emergency Department or hospitalisation), 70 cases were of G2 [P4] genotype (Correia et al. 2010). The shift of dominant rotavirus genotype from G1 [P8] to G2 [P4] may reflect the cyclic genotype fluctuation in Brasil or may be a consequence of low protection of the monovalent vaccine against G2 [P4] genotype infection.

3.4.3 The difference in clinical and epidemiological features of acute diarrhoea caused by rotavirus and *Shigella:*

An important question for the clinician confronted with a sick child with acute diarrhoea is whether an antibiotic is indicated. To answer this question, the clinician has to make a presumptive diagnosis: if it is thought to be viral then no antibiotic is required, if thought to be caused by cholera or other invasive bacteria, such as shigellosis, antibiotic should be administered (WHO 2005a). This study revealed a number of important differences in the clinical presentation of diarrhoea caused by rotavirus and by *Shigella.* The typical case of diarrhoea caused by rotavirus is a boy 7-12 months old, admitted to

hospital with the triad of fever-vomiting-watery diarrhoea, blood white cell count within normal limit and without white cells in the stool. A typical shigellosis cases presented with fever-mucoid diarrhoea-abdominal pain with leukocytosis and the presence of white blood cell in stool. However the reality is that in practice, there is significant overlap in the clinical and laboratory symptoms and signs of these two diseases. Data from this study showed that 46% of 99 children with shigellosis passed only watery stool, not mucoid stools. This is similar to Hien et al's finding in Ha Noi where 48% (10/22 cases) of shigellosis in their study passed only watery diarrhoea (Hien et al. 2008). von Seidlein found that less than one-third of culture-proven shigellosis episodes presented with dysentery (von Seidlein et al. 2006). The proportion of *Shigella* infection episodes presented as non-dysenteric diarrhoea is as high as 80% (50/63 cases in Peru) to 89% (106/119 cases in Egypt) in community-based studies (Abu-Elyazeed et al. 2004; Kosek et al. 2008). In our study, 32% of 234 rotavirus infection cases had white blood cell in their stool and 10% of all rotavirus diarrhoea cases even had red blood cell in stools. These findings underscore the difficulty in making a differential diagnosis of cases presented with acute diarrhoea in children. *Shigella*, in addition to their invasiveness, also produced enterotoxins ShET1 and ShET2 through the control of the *set* and *sen* genes. These toxins may explain the watery diarrhoea phase seen early in the course of the illness (Niyogi 2005). In many patients the dysenteric phase of bloody diarrhoea may never be a feature of the illness. Because of this, the clinical case definition that includes only patients with a history of dysentery, frequently used in government data collections may miss more than two-thirds of shigellosis cases (von Seidlein et al. 2006). The diagnosis of shigellosis should not be excluded in those patients with watery diarrhoea as the sole presentation (Wu et al. 2009). A call has been launched for revision of clinical guidelines for the diagnosis and treatment of diarrhoeal illness in limitted source health settings such as rural areas where primary care

physicians are unlikely capable of performing bacterial culture (Abu-Elyazeed et al. 2004). Rapid tests, such as immuno-chromatographic dipstick which have been available for rotavirus (Weitzel et al. 2007) and on-developing for *Shigella* diagnosis (Nato et al. 2007b), may be of great value to help clinician having the correct diagnosis sooner. Early diagnosis of rotavirus prevents unnecessary use of antibiotics (which were used in 38% of our cohort) thus minimizes the chance of emerging resistance to antibiotics in gut flora. Early diagnosis of *Shigella* infection may helps clinicians giving antimicrobials earlier to prevent the spread of the bacteria to house-hold members or to the community.

From the data of my study, I suggest a simple algorithm to help clinicians making a differential diagnosis between acute invasive diarrhoea (the prototype is shigellosis in which antibiotics are indicated) with non-invasive acute diarrhoea (the prototype is rotavirus infection in which antibiotics are usually not indicated) (Figure 3.5). The algorithm was developed using simple clinical data (bloody/non-bloody diarrhoea, with/without abdominal pain ± tenesmus, Blood WBC high/not high, presence/absence WBC in stool). The value of the algorithm needs to be validated in a larger diarrhoea population in the future.

3.4.4 Limitations of the study:

The present study had several limitations which need to be taken into account when interpreting the data. Firstly, the lack of a control group may lead to overestimate the role of a potential pathogen such as Enterovirus or *Salmonella* spp.. Secondly, the *E.coli* identification by PCR techniques has not been carried out because of reasons beyond our control. Without data on the *E.coli* isolation rate, the importance of this genus as a cause of acute diarrhoea in children cannot be evaluated. Studies in Viet Nam as well as

Tunisia reported a high rate of isolation of diarrhoeagenic *E.coli* in both cases and control population (Al-Gallas et al. 2007; Hien et al. 2008; T. V. Nguyen et al. 2005). Because of these limitations more studies are needed to clarify the magnitude of *E.coli* in causing childhood diarrhoea in the setting of tropical region such as Viet Nam. The technique used for virus detection (negative-stain electro-microscopy) in this study is time consuming and labour-intensive and has been replaced recently either by rapid immunochromographic tests or enzyme-link immunosorbant assay (Atchison et al. 2009) or real-time reverse transcription (RT)-PCR assays with specific primers (Svraka et al. 2009).

3.5 Conclusion:

Despite the limitations mentioned above, this study was the first comprehensive investigation of the aetiology of acute childhood diarrhoea in in-patients at Ho Chi Minh City, which showed that rotavirus and *Shigella* spp. to be the most importance virus and bacteria associated with acute diarrhoea.

The study has also revealed the diversity of the rotavirus genotypes circulating in the region, in which variants of G1 [P8] and G2 [P4] contributed more than 69% (34/49 isolates). This should be considered by the health policy decision-makers in designing the appropriate rotavirus vaccine for children in the region. Norovirus and *Campylobacter* spp are emerging causes of acute diarrhoea in this region. Regular surveillance studies are needed to document the aetiology of acute diarrhoea from which appropriate public health and clinical interventions can be applied.

The overlap in the clinical presentations and results of basic laboratory test of acute diarrhoea caused by rotavirus and *Shigella* spp. also presents a challenge for the

clinician. A practical algorithm based on simple clinical data and easy-to-use rapid tests to diagnose these two important causative agents would be useful in helping the clinician making a diagnosis and plan treatment.

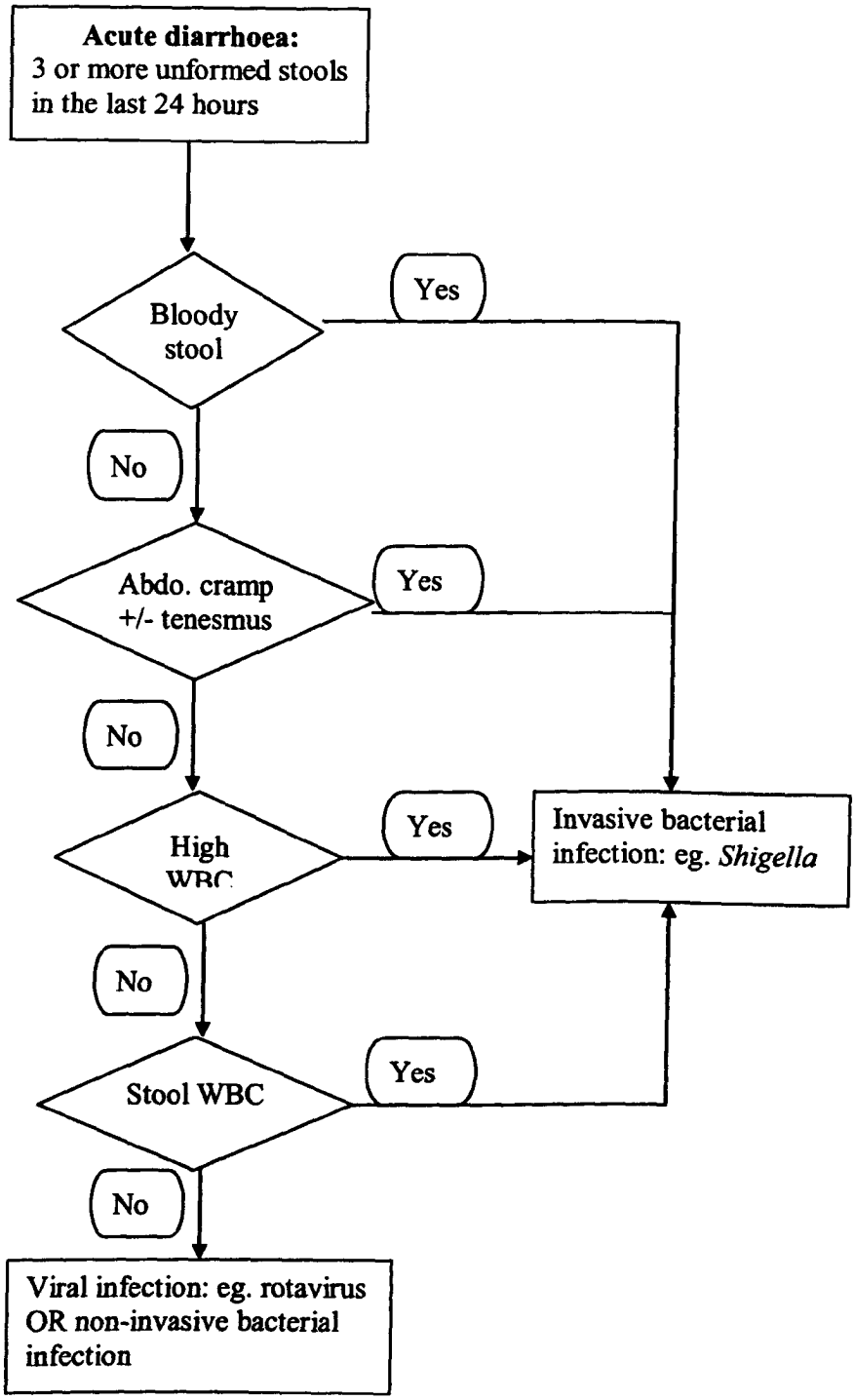

Figure 3.5 Algorithm for differential diagnosis of acute invasive diarrhoea (e.g. shigellosis) with acute non-invasive diarrhoea caused by viral infection (e.g. rotavirus) or enterotoxin-producing bacteria (e.g. cholera).

Chapter Four

Changing Epidemiology, Antimicrobial Susceptibility and Clinical Features of *Shigella* Infection in Southern Viet Nam

4.1 Introduction:

Shigellosis is a major global public health problem. Each year approximately 1.1 million people are estimated to die following infection and there are an estimated 165 million episodes of *Shigella* infection (Kotloff et al. 1999). *Shigella* infection occurs mainly in developing countries where people live in overcrowded conditions often with personal hygiene, sanitation and safe water supply often not optimal. The incidence of *Shigella* infection varied from 0.6 episodes/1000 person-years in Thailand to 107/1000 person-years in Egypt (Ram et al. 2008). Although on a different scale shigellosis remains an important public health issue in developed countries. A report from the National Center for Infectious Diseases in the United States of America found the incidence of shigellosis to be 5.6 cases per 100,000 persons in the 1989-2002 (Gupta et al. 2004).

Four species (i.e. sero-groups) of the genus *Shigella* can cause diarrhoeal diseases in human: *Shigella dysenteriae* (sero-group A), *S.flexneri* (sero-group B), *S.boydii* (sero-group C) and *S.sonnei* (sero-group D). All the members of the genus *Shigella* are human

restricted pathogens (and higher primates) and exert their effects on the gastrointestinal mucosa via the production of a number of virulence factors and enterotoxins (Niebuhr and Sansonetti 2000; Philpott et al. 2000). *S.dysenteriae* (15 serotypes) is the most dangerous *Shigella* species in which serotype 1 has been associated with epidemics of severe dysentery that have killed thousands of people in Central America, India sub-continent and Africa (Levine et al. 2007). *S.flexneri* (6 serotypes) is the predominant endemic sero-group in developing countries while *S. sonnei* is the most commonly isolated species in developed countries, over 70% of the total worldwide cases, are reported from the Unites States of America and Israel suggesting a reporting bias and the probability of massive under-reporting (Gupta et al. 2004; Mates et al. 2000). *S.boydii* contributes a very small part of Shigellosis, mainly in India where it first isolated.

In a recent community-based surveillance, von Seidlein *et al.* found variation in the dominant *Shigella* species across Asia (*S. sonnei* predominated in Thailand, *S. flexneri* was dominant in other Asian countries including China, Viet Nam, Bangladesh, Pakistan and Indonesia), with fluctuations in *S. flexneri* serotypes in the same location over the duration of the study (von Seidlein et al. 2006). The authors concluded that "*Shigella* appears to be more ubiquitous in Asian impoverished populations than previously thought and antibiotic-resistant strains of different species and serotypes have emerged" (von Seidlein et al. 2006). Such findings have important implications for treatment and prevention strategies of shigellosis. Increasing antimicrobial resistance is a constant threat and suggests that current treatment regimes may be compromised.

It is known that the circulating species and serotypes may be considered as a marker for the socio-economic conditions in an individual setting (Chompook et al. 2005). It is

clear that Viet Nam, in particular Ho Chi Minh City has undergone rapid economic development since the early 1990's. The improvement of living condition of people in the region may influence to the epidemiology and clinical feature of *Shigella* infection.

In this chapter I have retrospectively compared the microbiological, epidemiological and clinical data from a series of studies on childhood shigellosis conducted in southern Viet Nam between 1995 and 2009 to gain a broader understanding of the changing epidemiology and antibiotic resistance pattern of *Shigella* infection in this region.

4.2 Materials and methods:

All the studies were carried out at the Paediatric Ward B of the Hospital for Tropical Diseases in Ho Chi Minh City (HTD). In addition, from June 2006 to December 2008 part of the clinical trial (EG study) was also carried out at The Infectious Diseases Ward, Dong Thap Provincial Hospital (DTP). Both locations are in urban settings, however due to the referral patterns, both hospitals have a wide catchment area and accept patients from their locality and also from other rural provinces in the vicinity (Figure 2.1, chapter two).

Data from paediatric in-patients enrolled in this thesis come from 3 studies covering 3 periods.

(i) The first period (referred to as Period A) was data taken from a study performed at the Paediatric Ward B between January 1995 and August 1996. This was a randomized controlled trial comparing ofloxacin (15mg/kg divided in 2 doses in a single day) with nalidixic acid (55mg/kg/day divided in 4 doses for 5 days) in the treatment of childhood bacillary dysentery and has been published elsewhere (Vinh et al. 2000). Additional strains for microbiological assessment only (nine in total) were collected for comparison

within the same period of the study duration from Dong Thap Provincial Hospital. Overall 80 strains were isolated from enrolled children over this period; clinical data was available for analysis on 63 patients with culture confirmed shigellosis.

(ii) The second period (referred to as Period B) used data available from a study conducted at the HTD, between March 2000 and December 2002. This period was a clinical and microbiological investigation of the aetiology of diarrhoea in the paediatric population admitted to the HTD in Ho Chi Minh City. Whilst the treatment criteria for this descriptive study were not controlled (> 90% of patients received treatment with fluoroquinolones (either norfloxacin 20mg/kg/day or ofloxacin 10mg/kg/day for 3-5 days), the remainder of the criteria for admission to the study were comparable, children were eligible for enrolment to the study if consent was given and they were aged less than 14 years. The obvious variation in the enrolment for this study was that children were enrolled on the basis of having any diarrhoeal syndrome, rather than specifically targeting those with dysentery and suspected shigellosis. One hundred and fourteen *Shigella* isolates were recovered during this period; clinical data was available for analysis on 113 patients.

(iii) The final period (referred to as Period C) in which data was combined in this retrospective assessment was a trial conducted at the HTD and at DTP between June 2006 and March 2009. This was a randomized controlled trial comparing ciprofloxacin (30 mg/kg/day for 3 days) with gatifloxacin (10mg/kg/day for 3 days) for the treatment of dysentery in Vietnamese children. The inclusion criteria were as Period A. One hundred and three isolates were collected during this period and clinical data on all admitted children were available for analysis. All three studies were approved ethical assessment by the Scientific and Ethical Committee of the Hospital for Tropical

Diseases, Oxford University Tropical Ethics Committee (OxTREC) and Dong Thap Provincial Hospital.

Microbiological methods used were described in section 2.3 of chapter 2.

Clinical data was recorded on specialized clinical report forms for all three studies by clinical staff involved in the studies. The data collected included basic demographic details of the patient, age (months), sex, location of residence and weight (kg). A history from all patients was also recorded, including; duration of illness prior to admission to hospital (days), fever (defined as a prolonged temperature > 37.5°C), abdominal discomfort, vomiting, watery diarrhoea (defined as three or more loose bowel movements during a 24-h period), bloody or mucoid diarrhoea (defined as ≥3 loose stools with obvious blood or mucus), estimated number of episodes of diarrhoea before attending hospital, convulsions believed to be related to fever and/or infection and if there was any known pre-treatment with antimicrobials.

4.3 Results:

Over the duration of the three studies spanning 14 years (1995-2009), 297 strains of *Shigellae* were isolated.

4.3.1 Epidemiological characteristics:

Spatial distribution of *Shigella* strains:

Among 297 strains of *Shigellae*, 228 were from children living within the districts that constitute Ho Chi Minh City. These children, who were treated as in-patients for shigellosis, came from 13 different districts in Ho Chi Minh City (Figure 4.1).

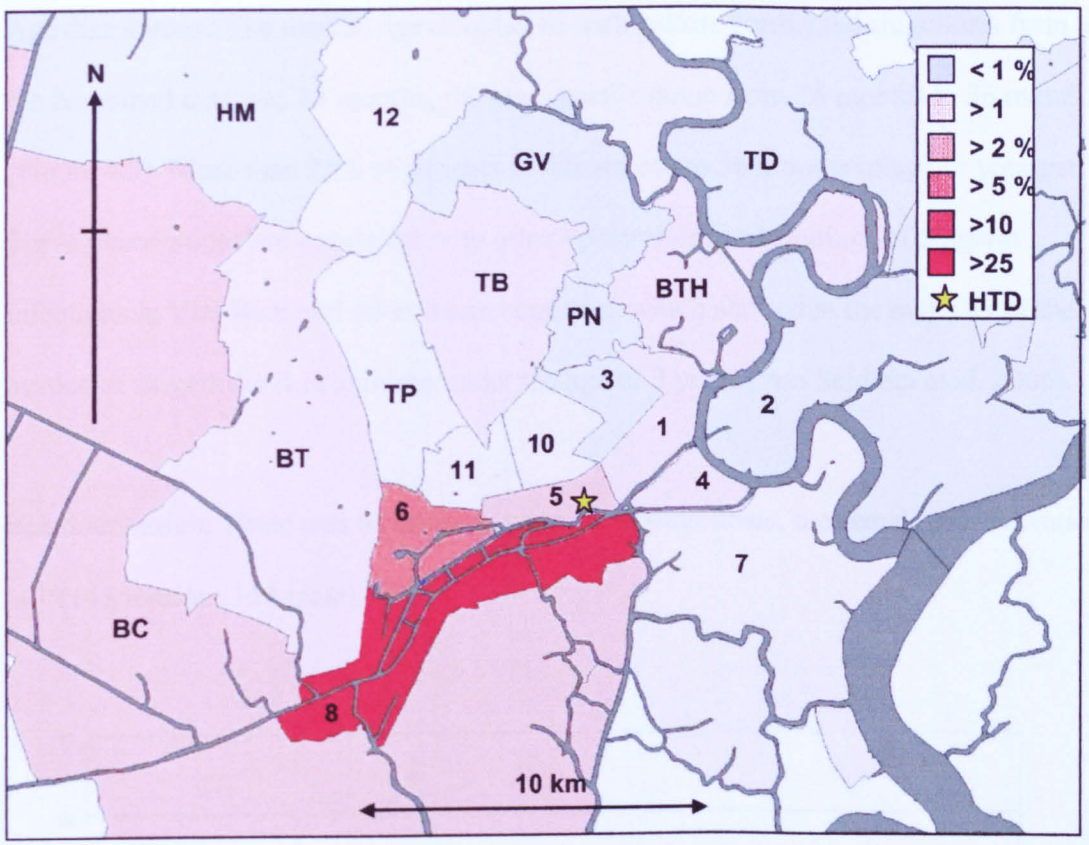

Figure 4.1 The distribution of household residences of cases of childhood shigellosis admitted to the Hospital for Tropical Diseases in Ho Chi Minh City.

Whilst the distribution of the location of the residences of these patients is biased by referral patterns and people attending the local hospital (HTD is one of several hospitals in the City where children may be treated for gastrointestinal infections), the majority of children attending HTD with culture confirmed shigellosis came from the three districts within the locality of the Hospital (districts 5, 6 and 8), which constitutes a total population of approximately 800,000 people. In total, the majority of patients resided in district eight (n = 88) within approximately 6 Km of the hospital. There was no significant change in the locality of patients over the three studies, or any relationship between serotype and location of the residence of the patients (including in those residing outside Ho Chi Minh City).

Age distribution: The median age of children with culture confirmed shigellosis from all the combined data was 24 months, the interquartile range from 16 months to 36 months (Figure 4.2). More than 75% of patients in this series are 36 months of age or younger. These observations are consistent with other epidemiological findings of *Shigella* infections in Viet Nam and other Asian countries, which show that the majority of the burden of shigellosis is in children under the age of 3 years (von Seidlein et al. 2006).

Sex distribution: There was no dominant gender in shigellosis, the female to male ratio is 1 (145 female : 152 male).

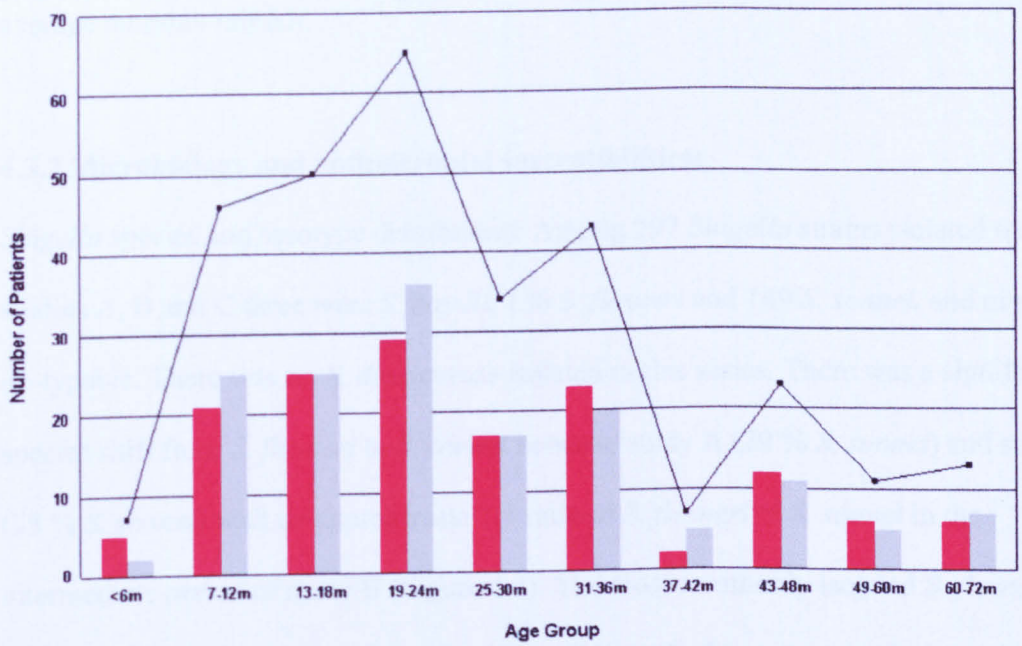

Figure 4.2 The combined distribution of sex and age of 297 children with shigellosis in southern Viet Nam. Graph depicts the combined age and sex distribution (female-red, male-grey). The black line represents the total number of cases per age group specified.

Seasonal variation of *Shigella* infection: The combined data from studies A, B and C demonstrated some seasonality related to the times of peak infection, with the majority of cases (> 60 %) occurring in the wet season (May and September) (Figure 4.3).

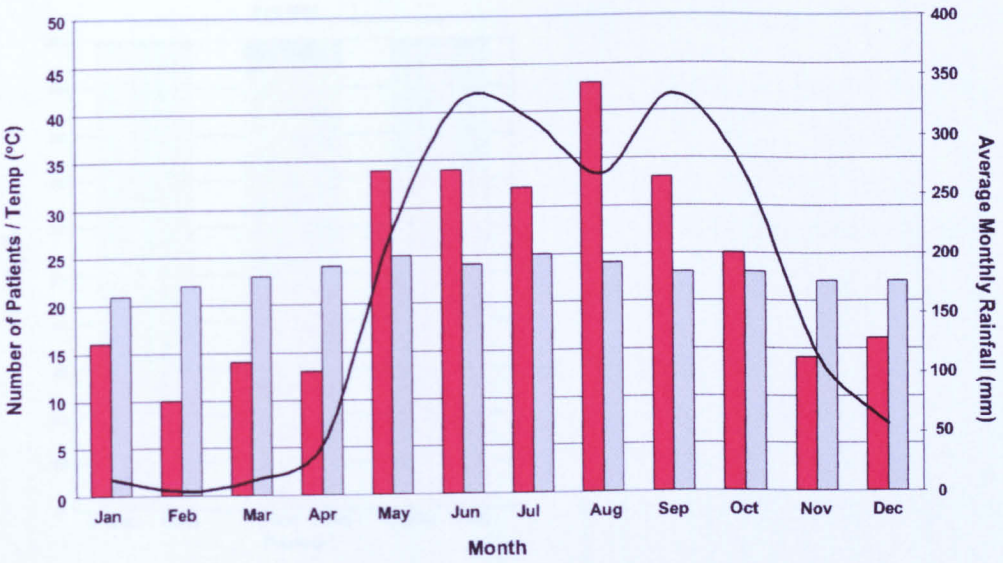

Figure 4.3 The seasonal distribution of shigellosis in southern Viet Nam. Data represent the average number of cases, rainfall and temperature per month for Ho Chi Minh City. Red bar: total number of cases, grey bar: average monthly temperature and black line: average monthly rainfall.

4.3.2 Microbiology and antimicrobial susceptibilities:

Shigella species and serotype distribution: Among 297 *Shigella* strains isolated from studies A, B and C three were *S. boydii*, 136 *S. flexneri* and 149 *S. sonnei*, and nine were un-typable. There was no *S. dysenteriae* isolated in this series. There was a significant species shift from *S. flexneri* to *S. sonnei* between study A (29 % *S. sonnei*) and study C (78 % *S. sonnei*) with an approximate 1:1 ratio of *S. flexneri* to *S. sonnei* in the intermediate period of study B (Figure 4.4). The most commonly isolated *S. flexneri* serotype was serotype 2a representing 43% of all the *S. flexneri* strains isolated (Table 4.1).

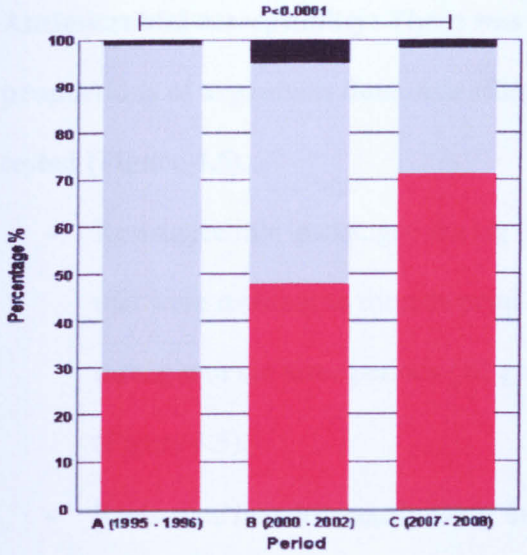

Figure 4.4 Distribution of *Shigella* species and serotypes from three childhood shigellosis studies in Southern Viet Nam over fourteen years. The distribution of *Shigella* species from study A (1995 - 1996) n = 80, study B (2000 - 2001) n = 114 and study C (2006 - 2008) n = 103. The percentages of organisms that are *S. sonnei*, *S. flexneri* are coloured red and grey respectively. Other *Shigella* species are coloured black. The p value was calculated using a chi – squared test.

Table 4.1 *S. flexneri* serotypes isolated in southern Viet Nam 1995-2008

S.flexneri serotpye	No. of	(%)
1a	0	0
1b	0	0
1c	4	2.9
2a	59	43.4
2b	8	5.9
3a	13	9.6
3b	2	1.5
3c	16	11.8
4	7	5.1
4a	5	3.7
4x	0	0
5a	0	0
6	13	9.6
x	0	0
y	0	0
Not typed	8	5.9
Total	136	100

Antimicrobial susceptibility: There was a significant change in the profile of the proportions of organisms demonstrating resistance to the seven antimicrobials tested (Figure 4.5).

- Resistance rate unchanged during the 3 periods: The proportion of organisms that were resistant to trimethoprim–sulfamethoxazole and tetracycline was high throughout the three periods (all greater than 95% resistance in all groups) (Figure 4.5).
- Resistance rate decreased during the 3 periods: There were significant decreases in the proportions of organisms resistant to ampicillin from 75 % to 48 % ($p = 0.0003$) and of chloramphenicol from 66 % to 30 % ($p = <0.0001$) between period A and period C.
- Resistance rate increased during the 3 periods: The number of Shigellae isolated that were resistant to nalidixic acid increased from 8% in study A to 68% in study C. There was only one *S.flexneri* strain isolated in September 2008 in Dong Thap Province which was fully resistant to fluoroquinolones (ciprofloxacin MIC = 6 mg/L, ofloxacin MIC = 16 mg/L, gatifloxacin MIC = 8 mg/L).
- Occurrence of ceftriaxone resistance: *Shigella* strain resistant to ceftriaxone was not recognized until the first case in February 2001. After a silent period, ceftriaxone resistant strain was also identified in May 2007 and then increased rapidly reaching 23 % of all *Shigella* strains in Period C (Figure 4.5).

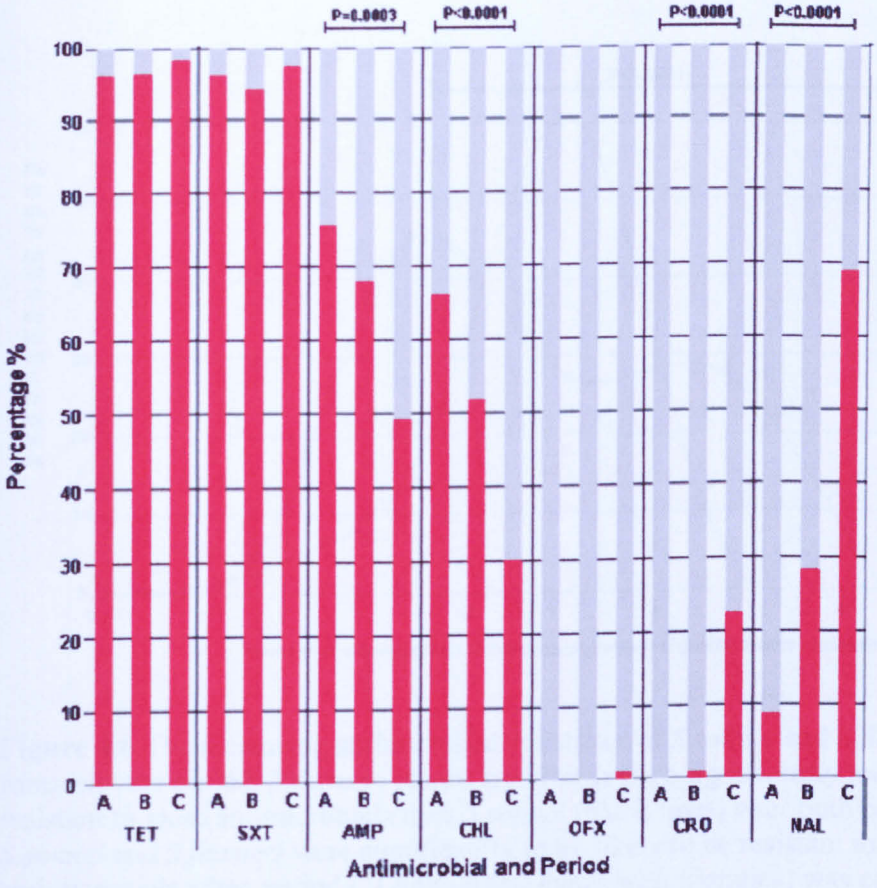

Figure 4.5 The changing antimicrobial resistance patterns of *Shigella* spp. Graph shows the percentage of resistant (red) and sensitive (grey) organisms isolated from period A, B and C. Statistical significant changes were calculated using a chi squared test.

There was an overall increase in the number of antimicrobials that the organisms were resistant to (Figure 4.6). In study A, 62 % of all Shigellae were resistant to three or more of the seven antimicrobials tested, this percentage increased to 87 % in study B and decreased slightly to 83 % in study C; the increase was statistically significant when we compared period C with period A ($p = <0.0001$, Chi squared test).

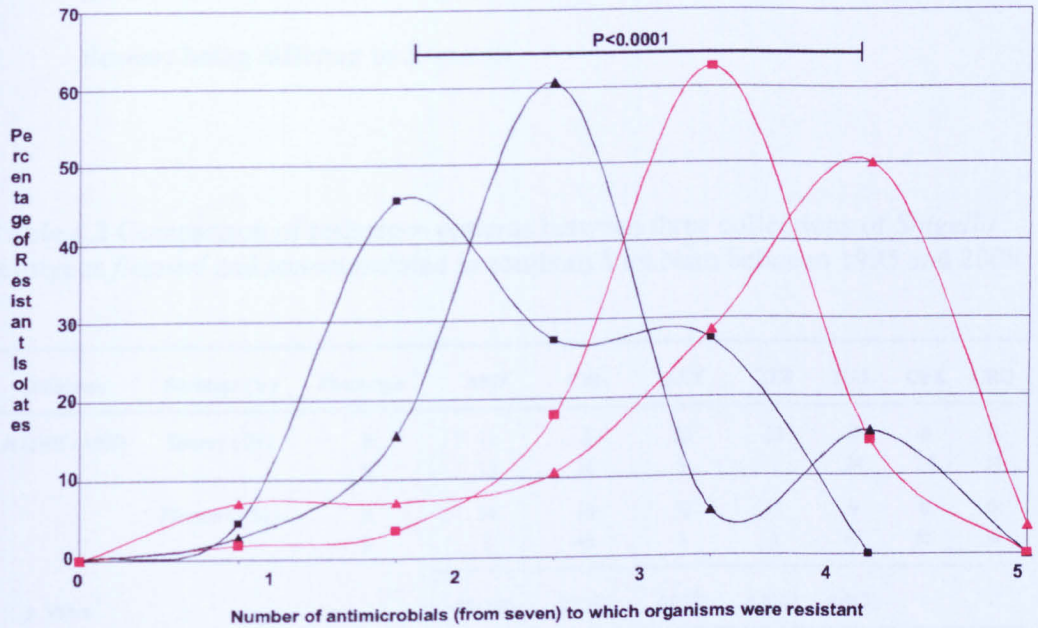

Figure 4.6 The increasing antimicrobial resistance of *S.sonnei* and *S.flexneri* during a fourteen year period. *S.flexneri* strains (red lines) were significantly more likely to be resistant to more antimicrobials than *S.sonnei* (black lines) over both collection periods. *S.sonnei* and *S.flexneri* were significantly more likely to be resistant to more antimicrobials when period C (2006-2008) (lines with triangles) was compared to period A (1995-1996) (lines with squares).

- Difference in antimicrobial resistance pattern between *S.flexneri* and *S.sonnei*:

  There was a significant change in sensitivity patterns over time related to the type of *Shigella* sero-group isolated (Table 4.2). *S. flexneri* was significantly more likely to be resistant to ampicillin in study A, study C and when combined over all three studies. *S. flexneri* was also significant more likely to be resistant to chloramphenicol in study B, study C and overall (Table 4.2). The combined data demonstrated that *S. sonnei* was significantly more likely to be resistant to both trimethoprim–sulfamethoxazole and ceftriaxone. The overall pattern of reversion of sensitivity to ampicillin and chloramphenicol was mainly observed with respect to *S. sonnei* isolates. The reversion of *S. sonnei* sensitivity to older

generation antimicrobials may account for the overall sensitivity patterns of *S. flexneri* being different to *S. sonnei*.

Table 4.2 Comparison of resistance patterns between three collections of *Shigella* serotypes *flexneri* and *sonnei* isolated in southern Viet Nam between 1995 and 2008

Collection	Serotype (n)	Phenotype [b]	AMP	CHL	SXT	TET	NAL	OFX	CRO
A (1995 - 1996)	*Sonnei* (24)	R	11	8	23	23	0	0	0
		S	13	16	1	1	24	24	24
	Flexneri (56)	R	54	10	53	53	9	0	0
		S	2	46	3	3	47	56	56
p Value [a]			<0.0001	0.1287	0.8102	0.8102	0.0371	-	-
B (2000 - 2002)	*Sonnei* (54)	R	50	10	53	52	9	0	1
		S	4	44	1	2	45	54	53
	Flexneri (50)	R	46	38	44	49	21	0	0
		S	4	12	6	1	29	50	50
p Value [a]			0.9316	<0.0001	0.0415	0.5577	0.0052	-	0.329
C (2007 - 2008)	*Sonnei* (71)	R	17	5	71	69	51	0	12
		S	54	66	0	2	20	71	59
	Flexneri (30)	R	25	28	28	30	19	1	1
		S	5	2	2	0	11	29	29
p Value [a]			<0.0001	<0.0001	<0.0001	0.3696	0.4619	0.297	0.076
Combined	*Sonnei* (148)	R	78	23	147	144	60	0	13
		S	70	125	1	4	88	148	135
	Flexneri (136)	R	125	76	125	132	49	1	1
		S	11	60	11	4	87	135	135
p Value [a]			<0.0001	<0.0001	0.001	0.613	0.365	0.478	0.002

- An increase in the number of organisms resistant to multiple antimicrobials over time was seen in both *Shigella* species. However, between study A and study C, *S. flexneri* was more likely to be resistant to more antimicrobials than *S. sonnei* (p = <0.0001). Resistance to multiple antimicrobials increased from two to three out of the seven tested from study A to C for *S. sonnei* and from four to five

from the seven antimicrobials tested from study A to C for *S. flexneri* (Figure 4.5).

- MIC of Ciprofloxacin in Nalidixic acid resistant versus Nalidixic acid susceptible *Shigella* spp.: The median of MIC of ciprofloxacin is 10 times higher in Nalidixic acid resistant strains than in Nalidixic acid susceptible strains (0.125ug/mL versus 0.012ug/mL). The hospital stay was longer in patients infected with Nalidixic acid resistant *Shigella* than patients with Nalidixic acid susceptible strains (Table 4.3).

Table 4.3 The ciprofloxacin MIC and duration of hospital stay in 2 groups of children infected by Nalidixic acid-resistant or -susceptible *Shigella* spp.

	Nalidixic acid - resistant n=83	**Nalidixic acid - susceptible n=161**	**P values[a]**
Ciprofloxacin MIC (μg/mL)[b]			
All cases	0.125 (0.094-0.190)	0.012 (0.008-0.012)	0.0001
S.flexneri	0.190 (0.125-0.190)	0.012 (0.012-0.016)	0.0001
S.sonnei	0.094 (0.094-0.125)	0.012 (0.008-0.012)	0.0001
Hospital stay (days)[b]			
All cases	5 (4-6)	3 (2-5)	0.005
S.flexneri	5 (4-6)	4(2.5-5)	0.01
S.sonnei	4.5 (4-5)	3(2.5-5)	0.0003
FCT[c] (hrs)	23.5 (12-36)	24 (12-41)	0.07
DCT[d] (hrs)	48 (30-72)	48 (29-74)	0.07

[a] P values calculated using the Mann-Whitney U test test
[b] Value were median (interquartile range) [c]FCT: Fever clearance time
[d]DCT: Diarrhoea clearance time

4.3.3 Clinical features associated with *Shigella* infection:

The clinical observation data was combined and analyzed from all three studies; this permitted a comparison of clinical characteristics of the patients with confirmed shigellosis over the three periods. Data was available for analysis from 279 patients: 63 patients from study A, 113 patients from study B and 103 patients from study C (Table

4.4). These data demonstrated several changes in disease profile over the three studies, particularly between study A and study C, which is more than 12 years apart.

- Change in age of patients:

There was a statistically significant increase in age, which corresponded with an increase in weight of the children from study A to study C (Table 4.4).

- Patients were admitted earlier in study C than in study A:

There was decrease in the number of days of history of the disease symptoms prior to admission to hospital (decrease in median days from 2 to 1).

- More indicators of severity in patients of study C:

There was a statistically significant increase in the number of children with watery diarrhoea (44 % to 71 % from study A to C), abdominal pain (52 % to 76 % from study A to C) and febrile convulsions (4 % to 20 % from study A to C).

These clinical features combined all suggest progressively more severe illness in patients being admitted to hospital between 1995 and 2008. Additionally, patients in study C had higher white blood cell counts, higher density of white cells in their stool and had longer stays in hospital (an increase from 3 to 5 median days from study A to study C). The combined duration of disease (days of history + duration of stay in hospital) was significantly increased from study A to study C (Table 4.5).

The increase in the severity of the disease coincided with a change in the dominant *Shigella* species isolated (*S. sonnei* becoming more common) and a change in antimicrobial resistance profile of the organisms.

Table 4.4 Clinical results and observations of *Shigella* infections in southern Viet Nam

	A (1995 - 1996)	B (2000 - 2002)	C (2006 - 2008)	Combined	*p* value [a]
Pateints	*n* = 63	*n* = 113	*n* = 103	*n* = 279	
Age (months) [d]	23 (17 - 48)	21 (14 - 29)	30 (19 - 42)	24 (16 - 36)	< 0.001
Weight (Kg)	10 (9 - 13)	10 (9 - 12)	11.5 (10 - 15)	10.5 (9 - 13)	0.004
Male Sex (%)	31 (49)	50 (44)	61 (59)	184 (59)	0.085
Patient History					
Days	2 (1 - 7)	2 (1 - 9)	1 (1 - 4)	2 (1 - 9)	< 0.001
Fever (%)	62 (98)	104 (92)	100 (97)	266 (95)	0.09
Abdominal Pain (%)	33 (52)	41 (36)	79 (76)	153 (54)	< 0.001
Vomitting (%)	24 (38)	64 (56)	51 (49)	139 (50)	0.062
Watery Diarrhoea (%)	28 (44)	67 (59)	74 (71)	169 (60)	0.002
Bloody / Mucoid Diarrhoea (%)	63 (100)	60 (53)	98 (95)	221 (79)	< 0.001
Diarrhoeal episodes per day	NA	8 (5 -10)	8 (5 -10)	8 (5 -10)	0.595
Convulsions (%)	4 (6)	7 (6)	20 (19.4)	31 (11)	< 0.001
Known pretreatment (%)	3 (5)	8 (7)	4 (4)	14 (5)	0.543
Clinical Details					
Serotype *Sonnei* (%)	21 / 63 (33)	55 / 113 (49)	71 / 103 (69)	153 / 279 (55)	< 0.001
White Cell Count (x 10^3 / mm^3)	10 (8.3 - 15)	10.1 (7.7 - 12.8)	13.1 (10.1 - 17.3)	11.3 (8.7 - 15.4)	< 0.001
Red Cells in Stool [b]	NA	1	1	1	0.715
White Cells in Stool [b]	NA	3	3	3	0.02
Mucoid Duration (hrs)	31.5 (24 - 53.5)	36 (24 - 54)	28 (18 - 48)	30 (19.5 - 48)	0.113
Diahorrea duration (hrs)	48.5 (29.25 - 87)	48 (24 - 72)	48 (30 - 72)	48 (26.75 - 72)	0.402
Duration of Illness					
Hospital stay (days)	3 (1 - 12)	4 (1 -15)	5 (2 - 14)	4 (1 -15)	< 0.001
Disease duration (days) [c]	4 (2 - 15)	6 (3 - 18)	6 (3 - 15)	6 (2 - 18)	< 0.001

[a] p Values calculated using either Chi-square test or Kruskal-Wallis test
[b] Cells in Stool assessed over a range from not seen to highly prevalent (0 – 4)
[c] Disease duration calculated by addition of history of disease and stay in hospital
[d] Interquartile range values in brackets unless stated

The data presented in Table 4.5 demonstrates only subtle differences between the syndromes synonymous with the two differing species. *S. flexneri* is associated with an increase in the number of days of illness prior to admission in hospital, the number of

episodes of diarrhoea, an increase in the duration of mucoid/bloody diarrhoea and the duration of stay in hospital.

Table 4.5 Clinical presentation of *S. flexneri* and *S.sonnei* infections

	S. flexneri	*S. sonnei*	*p* value [a]
Pateints	*n* = 123	*n* = 147	
Age (months) [d]	25 (12 - 42)	23 (14 - 36)	0.105
Weight (Kg)	11 (8.5 - 14)	10 (9.9 -13)	0.558
Male Sex (%)	55 (44.7)	83 (56.5)	0.055
Patient History			
Days	2 (2 - 3)	1 (1 - 2)	< 0.001
Fever (%)	117 (95)	141 (96)	0.761
Abdominal Pain (%)	64 (52)	84 (57.1)	0.48
Vomitting (%)	60 (48.8)	74 (50.3)	0.78
Watery Dirrhoea (%)	78 (63.4)	86 (58.5)	0.41
Bloody / Mucoid Diarrhoea (%)	97 (78.9)	117 (80)	0.88
Diarrhoeal episodes per day	10 (5 - 10)	8 (5 - 10)	0.051
Convulsions (%)	9 (7.3)	21 (14.3)	0.07
Known pretreatment (%)	7 (5.7)	7 (4.8)	0.585
Clinical Details			
White Cell Count (x 10^3 / mm^3)	10 (8 - 13.6)	12 (10.5 - 15.5)	0.029
Red Cells in Stool [b]	1	1	0.056
White Cells in Stool [b]	3	3	0.173
Mucoid Duration (hrs)	36 (24 - 53.5)	25 (18 - 48)	0.054
Diahorrea duration (hrs)	48 (39 - 72)	48 (27 - 72)	0.088
Duration of Illness			
Hospital stay (days)	5 (4 - 5)	4 (3 - 5)	0.276
Disease duration (days) [c]	7 (6 - 8)	5 (4 - 7)	0.009

[a] p Values calculated using either Chi-square test or Kruskal-Wallis test
[b] Cells in Stool assessed over a range from not seen to highly prevalent (0 – 4)
[c] Disease duration calculated by addition of history of disease and stay in hospital
[d] Interquartile range values in brackets unless stated

4.4 Discussion:

Our findings demonstrate that the epidemiology of shigellosis infection is similar in southern Viet Nam to other locations in Asia. The main burden of infection is in those under three years of age (Agtini et al. 2005; Chompook et al. 2005; von Seidlein et al. 2006; Wang et al. 2005). The median age of patients in this investigation was 24 months; this is slightly less than a previous study in Nha Trang, Central Viet Nam (von Seidlein et al. 2006). A discrepancy in age in the two settings may be related to the epidemiological study in Nha Trang being performed with ongoing community surveillance, rather than those admitted to a paediatric ward of the hospital for treatment as in this study.

We also found a pattern of infection which correlated with the rainy season. The observation that *Shigella* infections generally coincide with the wet season in a tropical setting has been noted before in an urban setting in North Jakarta, Indonesia (Agtini et al. 2005). Transmission of *Shigella* has been linked to waste water and river water in Viet Nam in two independent locations in Viet Nam, Hanoi and Nha Trang respectively (Hien et al. 2007; Kim et al. 2008). An increase in faecal contamination of the water supply due to increased ground water may account for this pattern as distance to a water source/river was found to be associated with higher risk of shigellosis in Nha Trang (Kim et al. 2008). The majority of patients enrolled in the studies combined here resided in District 8 of Ho Chi Minh City. Although we are unable to draw strong conclusions from the residences of these patients owning to referral and catchment areas of the HTD, District 8 represents the area of the city with the greatest density of canal networks and waterways.

Our observations from 1995 to 2008 recognized for the first time a shift of dominant *Shigella* species from *S.flexneri* to *S.sonnei* at the turn of the 21st century in Ho Chi Minh City and adjacent provinces in southern Viet Nam (Figure 4.3). The shift in dominant *Shigella* species has been recognized previously. In Japan, where the prototype *Shigella* was first described, *S.dysenteriae* type 1 was the most common species in the latter half of 19th century and early 20th century. But after that most of the *Shigella* strains isolated in hospitalized patients in Tokyo were *S.flexneri*. The percentage of *S.sonnei* increased gradually from 10% in 1953 to 50% in 1963 (Hiroshi 1964) and in recent years *S.sonnei* is the most prevalent *Shigella* species in Japan. In Britain and Europe during the 1920s *S.flexneri* and *S.sonnei* were of approximately equal prevalence, but the former gradually faded and after the Second World War *S.sonnei* has accounted for the majority of the endemic shigellosis in these areas. Similar trends have also been observed in the United States. (Skirrow M.B. 1996). Statistics in Korea showed that *S.sonnei* constituted 4.8% of all *Shigella* isolated in the 1961-1968 period, increased to 23.6% in 1980-1987, and then became the most prevalent species in 1991-1997 period with 77.1% of all isolates (Lee 2006). This change has also been seen recently in many newly industrialized countries including Thailand, Turkey, Israel (Ashkenazi et al. 1993; Ceyhan et al. 1996; von Seidlein et al. 2006). The cause of the shift of *Shigella* species is not fully understood but probably reflects the expanding economies in these countries because there is a significant correlation between the Gross Domestic Product (GDP) and the frequency of *S.sonnei* isolation (Ram et al. 2008).

In addition to the shift in the dominant *Shigella* species over time, there has also been a shift in antimicrobial resistance with a marked increase in resistance to nalidixic acid and ceftriaxone. We have previously reported the alarming increase in ceftriaxone

resistant *Shigellae* in southern Viet Nam (Vinh et al. 2009a). This is important as ceftriaxone is an alternative drug for the treatment of shigellosis and often used in patients with severe illness and in those in whom the first line drugs have not been successful (WHO 2005). However in these studies patients with shigellosis caused by a ceftriaxone resistant *Shigellae* strain still responded well to ciprofloxacin (Vinh et al. 2009a).

Whilst nalidixic acid is no longer recommended for use in the treatment of Shigellosis, resistance to nalidixic acid increases the MIC to fluoroquinolones (ciprofloxacin or other drugs in the class), which do remain recommended drugs for the treatment of *Shigella* infections (WHO 2005a). Therefore, resistance to nalidixic acid may delay recovery in those treated with fluoroquinolones, as showed in Table 4.5.

Shigellae circulating in Ho Chi Minh City would seem to be under drug selective pressure in this population, an idea supported by the sequential decrease in resistance to older antimicrobials, such as ampicillin and chloramphenicol which are rarely used in the community to treat gastrointestinal infections. The uncontrolled use of antimicrobials in this setting may fuel the spread of multiple drug resistant organisms. However, due to the promiscuous nature of the *Shigellae* it is likely that resistance genes are transferred regularly to and from other enteric bacteria and maintained by continued selective drug pressure in the community.

Currently there are several potential *Shigella* vaccines undergoing development, of which some have already been tested in early phase clinical trials (Coster et al. 1999; Katz et al. 2004; Kotloff et al. 1995b; Kotloff et al. 2000; Launay et al. 2009; Levine et al. 2007). The development of *Shigella* vaccines may be hindered by the number of

different species and serotypes circulating in one setting and in differing locations. For example, *S. flexneri* serotypes are known to fluctuate over time as shown in India, Indonesia, Bangladesh, and Pakistan (Dutta et al. 2002; von Seidlein et al. 2006). Here, we have demonstrated a significant longitudinal transition of species from *S. flexneri* to *S. sonnei*. Vaccine development for shigellosis is challenging as primary infection offers only serotype specific immunity (Kotloff et al. 1995a). A study concerning a cohort of Chilean children found infection conferred 76% protective efficacy against re-infection with the same serotype (Ferreccio et al. 1991). An option for controlling shigellosis would be the development of a series of single serotype vaccines which could be implemented in individual locations with a known serotype profile. Alternatively, the most cost effective method of control would be the development of a polyvalent vaccine offering protection to a number of known dominant serotypes, this approach may aid in tackling the global burden of shigellosis. The transition of dominant *Shigella* species in southern Viet Nam has occurred on a background of economic development and may predict a continuing cycle in other areas under going similar rapid economic changes.

In summary, on the basis of data collected from three studies covering 14 years from 1995 to 2008, a transition in dominant *Shigella* species from *S.flexneri* to *S.sonnei* has been recognized for the first time in Viet Nam, and an increase of antimicrobial resistance overtime, particularly resistance to ceftriaxone and nalidixic acid, which appears to predict a more severe clinical disease presentation and prolonging the illness course.

Chapter Five

Extended-Spectrum β-Lactamase Producing *Shigella spp.* in Southern Viet Nam

5.1 Introduction:

Enterobacteriaceae that have the capability to express CTX-M (so named because of their hydrolytic activity against CefoTaXime) a family of extended spectrum beta-lactamases (ESBLs) have emerged as a major public health threat worldwide (Livermore et al. 2007; Rossolini et al. 2008). Most of the research in this area is conducted in industrialized countries, where organisms such as *Escherichia coli* and *Klebsiella* spp., mostly from urinary tract infections, are the commonest source (Pitout and Laupland 2008; Valverde et al. 2008; Heffernan et al. 2009). Relatively little is known about the distribution of such genes in organisms found in developing or countries undergoing economic transition, where the circulating pathogens may differ.

Enterobacteriaceae capable of producing ESBLs have been described previously in South East Asia (Cao et al. 2002); Kiratisin et al. 2008; Ruppe et al. 2009). Ho Chi Minh City in southern Viet Nam is typical of many cities where patterns of infectious diseases are changing due to rapid economic growth, better access to health care and improving infrastructure. Le recently showed that 42% of healthy people carried ESBL producing bacteria as part of their regular intestinal flora (Le et al. 2009). This work suggested that commensal organisms may play an important role in the dissemination

and maintenance of such antimicrobial resistance genes in the population. Furthermore, the uncontrolled use of antimicrobials in the human population and in livestock rearing may lead to further problems with drug resistance and more limited treatment options

Shigellosis is a gastrointestinal infection caused by members of *Shigella* spp and transmitted by the faecal-oral route. Children less than five years old and living in developing countries bear the brunt of the disease (Kotloff et al. 1999; Kim et al. 2008). In our hospital in Ho Chi Minh City, shigellosis is the commonest cause of paediatric admission in those with a confirmed bacterial aetiology (See Chapter Three). Although diarrhoeal diseases caused by *S.sonnei* is generally mild and self-limited in healthy adults in industrialized countries, antibiotic therapy is necessary for the treatment for severe forms of dysentery caused by *Shigella* spp. (Christopher et al. 2009). Infection with any *Shigella* species can be lethal to children in developing countries, particularly the very young, malnourished or immuno-compromised. Without effective antibiotic treatment, mortality due to *Shigella* infection, especially from infection with *S.dysenteriae* type 1 may exceed 10% (Bennish and Wojtyniak 1991). Antibiotics shorten the duration of symptoms, prevent severe complications and eradicate *Shigellae* from the stool more quickly and hence prevent the spread of the disease in the community (Salam and Bennish 1991; Vinh et al. 2000).

Fluoroquinolones are the drugs of choice to treat *Shigella* infections in both adults and children (WHO 2005). However, as with many other members of the Enterobacteriaceae, mutations in the genes encoding the target proteins for fluoroquinolones are common in *Shigella* (Chau et al. 2007; Hu et al. 2007). Our recent findings have shown that patients with shigellosis remain in hospital for longer periods compared with ten years ago and the disease severity has concurrently increased (Vinh

et al. 2009b). Interestingly, at the same time there has been a significant species shift from *S.flexneri* to *S.sonnei* isolated from patients (Vinh et al. 2009b). Patients are treated with fluoroquinolones; however, those patients that do not respond to the standard therapy are treated with third generation cephalosporin (mainly intravenous ceftriaxone). The intravenous third generation cephalosporins are amongst the most commonly used antimicrobials in hospitals in Ho Chi Minh City and the oral second and third generation cephalosporin are also widely available without prescription in the community. Antimicrobial resistance in the Shigellae is common; these organisms are closely related to *E.coli* and are readily transformed by exogenous DNA (Bratoeva and John 1994; Dutta et al. 2002; Iversen et al. 2003). The distribution of antimicrobial resistance is, however, often different depending on the species. A multi-centre study across Asia demonstrated that *S.flexneri* were more likely to be resistant to ampicillin, whilst *S.sonnei* were more likely to be resistant to cotrimoxazole (von Seidlein et al. 2006). Resistance patterns and species dominance are variable depending on the specific location and can vary with time (Anh et al. 2001; Lartigue et al. 2005; Kuo et al. 2008).

Shigellosis caused by third generation cephalosporin resistant *Shigella* in Viet Nam had not been previously reported. In this chapter, I report the clinical and epidemiological aspects of shigellosis caused by ESBLs producing *Shigella spp.* In addition, the transferability and molecular characteristics of ESBLs genes in clinical *Shigella* strains is also described.

5.2 Materials and methods:

The work described in this chapter was conducted at 2 sites: (i) The Paediatric Ward B, Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Viet Nam, and (ii) Infectious Diseases Ward, Dong Thap Provincial Hospital (DTP).

All the *Shigella* spp. strains described in this chapter were isolated from patients enrolled into a randomized controlled trial comparing treatment with ciprofloxacin and gatifloxacin as described in Chapter Six.

Microbiological culture, antimicrobial susceptibility testing and molecular techniques were described briefly in chapter two. The detailed procedure is described here.

Procedure of bacterial conjugation experiment:

Step 1: Revival of parental strains: Sub-culture donor and recipient strain onto Nutrient Agar (NA) plates. Incubate at 37°C overnight.

Step 2: Pick single colony each from the donor and recipient plate and inoculate into separate 15-ml Falcon tube containing 4 ml of Luria-Bertani (LB) broth. Incubate at 37°C (tilt 30°) overnight with gentle shaking.

Step 3: Mating experiment: Inoculate 0.5 ml of donor and 0.5 ml of recipient broth culture into a 50-ml Falcon tube containing 4 ml of LB broth. Also inoculate 0.5 ml of donor and 0.5 ml of recipient into 2 separate 50-ml Falcon tube containing 4 ml of LB broth as control. Incubate all tubes at 37°C (tilt 30°) 12-16 hours without shaking.

Step 4: Culture of mating broth and control donor and recipient onto selective LB agar plate containing sodium azide 100mg/L and ceftriaxone 6 mg/L:

- Centrifuge Falcon tubes 4000 RPM at 4 °C, in 5 minutes.
- Discard supernatant.
- Re-suspend sediment of each Falcon tube with 1 ml NaCl 0.9%.
- Spread 100 µL of mating culture onto separate selective LB agar plate containing sodium azide 100mg/L plus ceftriaxone 6 mg/L firstly without dilution, then at 10^{-1} dilution, 10^{-2} dilution, 10^{-3} dilution with NaCl 0.9%.
- Spread 100 µL of donor as well as recipient control onto separate selective LB agar plate containing sodium azide 100mg/L and ceftriaxone 6 mg/L.

- Incubate the plates at 37°C overnight.

Step 5: Checking plates for transconjugants:

Nothing grew on donor and recipient control plate

Spread one colony from mating plate onto LB agar plate containing sodium azide 100mg/L and ceftriaxone 6 mg/L.

Step 6: Re-identify transconjugant by API 20E.

Performe antibiogram and ESBL confirmation test for transconjugants.

Procedure of plasmid extraction:

1. Inoculate pure culture of ESBL negative *Shigella* isolates, ESBL positive *Shigella* isolates, their transconjugants, *E. coli* 39R861 and *E.coli* J53 AzR strain each into 3ml of LB broth. Leave overnight at 37°C (gentle shaking).
2. Centrifuge to harvest cells. To the cell pellet, add 150µl of E buffer and re-suspend.
3. Add 300µl of lysis buffer and mix gently. The solution should begin to clear quickly.
4. Heat sample to 55°C for 1 hour precisely.
5. Add 600µl of Phenol/Chloroform and mix gently until the solutions have emulsified.
6. Centrifuge for 30 minutes to separate layers.
7. Remove a 35 to 45µl aliquot from the middle of the top layer and add final sample buffer (treat as a 5x concentrate)
8. Load samples onto 0.7% agarose gel. Run gel at 90 volts for 3 hours.
9. When Blue dye is near bottom of gel, remove to a box containing Ethidium Bromide solution (5µl of a 10mg/ml stock per 100mls of water) to stain
10. Leave 45 minutes to stain gel.

11. Rinse in distilled water and visualize under UV light.

12. Photograph and save as digital files.

ESBL gene PCR amplification and characterisation.

Genomic DNA was isolated from strains that were subjected to PCR from 1 ml of a 5 ml overnight bacterial culture using the wizard genomic DNA extraction kit (Promega, USA), as per the manufacturer's recommendations.

Genomic DNA was subjected to PCR amplification targeting known classes of *bla* genes using, initially, primers that would recognize sequences encoding ESBLs:

SHV (F; 5′ TCTCCCTGTTAGCCACCCTG,

R; 5′; CCACTGCAGCAGCTGC),

TEM (F; 5′ TGCGGTATTATCCCGTGTTG,

R; 5′ TCGTCGTTTGGTATGGCTTC),

CTX-M (F; 5′ CGATGTGCAGTACCAGTAA,

R; 5′ TTAGTGACCAGAATCAGCGG).

Further characterization of the various sub-classes of bla_{CTX} ESBL genes was performed using primers

CTX-M-1; (F 5′ ATGGTTAAAAAATCA CTGCG,

R 5′ TTACAAACCGTCGGTGAC),

CTX-M-2; (F 5′ TGGAAGCCCTGGAGAAA AGT

R 5′ CTTATCGCTCTCGCTCT GT);

CTX-M-9 (F 5′ATGGTGACA AAGAGAGTGCAAC,

R 5′ TTACAGCCCTTCGGCGATG)

in previously outlined PCR amplification conditions.

All sequencing reactions were performed twice to ensure correct sequencing and sequences were verified aligned and manipulated using Bioedit software

(http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All ESBL gene sequences were compared to other ESBL sequences by BLASTn at NCBI. The DNA sequence of various classes of bla_{CTX} were downloaded and aligned with the produced sequences.

5.3 Results:

5.3.1 The rapid occurrence of Ceftriaxone-resistant *Shigella spp.* in Ho Chi Minh City and adjacent provinces:

During a 24 month period between April 2007 and March 2009 we isolated 94 *Shigella* strains from the stools of children admitted with dysentery. Of these 94 strains, 24 were *S. flexneri* and 70 were *S. sonnei*. There were 35 ceftriaxone-resistant strains: 33 *S.sonnei* and 2 *S.flexneri*.

Timing of occurrence: The first isolation of a ceftriaxone resistant organism during the transitional period occurred in May 2007. The numbers of *Shigellae* isolated that were resistant to ceftriaxone fluctuated over the following months. However, there was increase in the proportion of resistant to sensitive isolates from 19% to 41% (5 / 11 strains) between the periods from April 2007–September 2007 and April 2008–September 2008, respectively. This trend peaked in March 2009, with six out of seven *Shigella* strains isolated resistant to ceftriaxone (MIC >256). The rate of resistance to ceftriaxone between September 2008 and March 2009 was 75%. The increasing proportion of ceftriaxone-resistant to sensitive isolates in the late period from April 2008 to March 2009 (23 / 22 strains) was significantly increased in comparison to the earlier period from April 2007 to Mar 2008 (12 / 33 strains) was statistically significant ($p=0.02$, Fisher exact test, Figure 5.1).

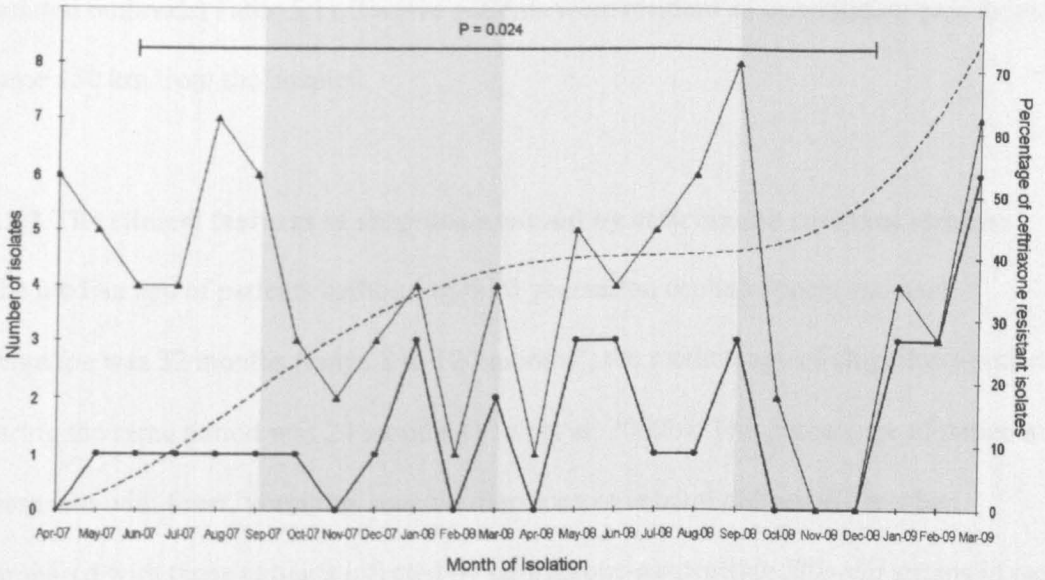

Figure 5.1 Graph depicting an increase in number and proportion of ceftriaxone-resistant *Shigella* spp. isolated between April 2007 and March 2009 at The Hospital for Tropical Diseases in Ho Chi Minh City.

The overall number of strains in this period is 35. While tracking back our *Shigella* collection in period 2000-2002, one strain of *S.sonnei* (strain DE0611) isolated in February 2001, which had high MIC for ceftriaxone (MIC>256mg/L), was confirmed as ESBL-positive, and we now believe this to be the first ESBL-producing *Shigella* isolate in our 1995-2009 series, and the first ESBL producing *S.sonnei* to be confirmed in Viet Nam.

Location of cases with ceftriaxone-resistant Shigellae: 24 strains were isolated from patients living in Ho Chi Minh City, 6 in Long An Province (which has a border with Ho Chi Minh City), 5 in Dong Thap Province, and 1 from Vinh Long Province. Owing to the rapid increase in the isolation of such organisms we hypothesised that an individual dominant strain had began circulating in one area of Ho Chi Minh City from May 2007. However, residence data procured on the time of admission showed that such strains were circulating over a wide area of the city and not purely limited to an

isolated outbreak (Table 5.1). Twelve patients were resident in surrounding provinces, some 150 km from the hospital.

5.3.2 The clinical features of shigellosis caused by ceftriaxone resistant strains:

The median age of patients harbouring third generation cephalosporin resistant *Shigellae* was 32 months (range 8 to 120 months), the median age of shigellosis patients during the same period was 24 months (Vinh et al. 2009b). The percentage of patients presented with fever, vomiting, mucoid diarrhoea or seizure did not differ when compared with those patients infected by ceftriaxone-susceptible *Shigella* strains in the same period. The white cell count in blood was not different between the two groups. The presence of faecal leukocyte was higher in patients with ceftriaxone-resistant *Shigella*. All patients were treated with fluoroquinolones (ciprofloxacin or gatifloxacin) and responded well irrespective to the susceptibility status to ceftriaxone. As a result of treatment, the fever clearance time was the same in two groups, but the diarrhoea clearance time is shorter in patients infected by ceftriaxone-resistant strains. There were no deaths in our series (Table 5.1).

5.3.3 The combined resistance patterns of ESBL producing *Shigella spp.*:

Between 2007 and 2009, 35 (34 %) *Shigella* isolates cultured were resistant to ceftriaxone. Of these strains, 33 were *S. sonnei* and the other two isolates were *S. flexneri*. The remaining strain was a solitary sporadic *S. sonnei* isolate previously cultured in 2001 (strain DE 0611).

Resistance to 3rd generation cephalosporin: All the 36 ceftriaxone resistant strains were examined by the combination disc method to identify ESBL producing organisms (CLSI, 2007). Thirty four *S. sonnei* strains and one *S. flexneri* strain (35 from 36 ceftriaxone resistant *Shigella*) produced the characteristic ESBL pattern on

investigation, whereas the hydrolysing activity of the other *S. flexneri* (strain EG0419) was not inhibited by clavulanic acid (Table 5.2).

Table 5.1 Clinical features of childhood shigellosis caused by ceftriaxone-resistant versus ceftriaxone-susceptible *Shigella* strains

	Ceftriaxone-resistant N=34	**Ceftriaxone-susceptible N=60**	**P value**
Febrile (cases / total)	32 / 34	59 / 60	0.6*
Vomiting (cases / total)	24 / 34	45 / 60	0.9*
Bloody diarrhoea (cases / total)	29 / 34	56 / 60	0.36*
Seizure (cases / total)	8 / 34	10 / 60	0.4*
Number of stool daily #	6 (5-10)	8.5 (5-10)	0.1**
Blood white cell count ($10^3/mm^3$) #	14.75 (11.4-17.5)	13.35 (9.95-17.25)	0.44**
Stool white cell (score 0-3) #	3 (1-4)	1 (0-3)	0.007**
Fever clearance time (hours) #	18 (12-30)	20 (10-30)	0.89**
Diarrhoea clearance time (hours) #	36 (12-48)	48 (30-70)	0.03**
Death	0	0	

* Fisher exact test,
** Mann-Whitney U test
# Number are median (interquartile ranger)

Combined resistance to other antibiotics: Along with ceftriaxone, all strains were examined for resistance to an additional five antimicrobials by disc diffusion method and MIC measurement (Table 5.1). As predicted, all strains demonstrated co-resistance to ampicillin. Thirty five of the 36 strains (97 %) were resistant to trimethoprim–sulfamethoxazole and tetracycline, whilst 33/36 strains were resistant to nalidixic acid. Only three isolates: DE0611, EG0419 and EG0471 were co-resistant to chloramphenicol, of which two *S.flexneri* strains EG0419 and EG0471, were resistant to five of the six antimicrobials tested (Table 5.1).

Table 5.2 Temporal and spatial distribution of ceftriaxone resistance *Shigella* strains in southern Viet Nam.

Strain ID	Serotype	Age (months)	Sex	Month	Year	Province	ESBL (+/-)	Antimicrobial Tested *													
								AMP		CHL		SXT		TET		NAL		OFX		CRO	
								Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC
DB0611	*S. sonnei*	10	M	February	2001	HCMC	+	R	>256	R	8	R	>32	R	128	S	2	S	0.06	R	>255
EG0356	*S. sonnei*	48	M	May	2007	HCMC	+	R	>256	S	6.0	R	>32	R	64	R	64	S	0.38	R	>256
EG0373	*S. sonnei*	30	M	June	2007	HCMC	+	R	>256	S	6.0	R	>32	R	128	S	1.5	S	0.064	R	>256
EG0384	*S. sonnei*	36	M	July	2007	HCMC	+	R	>256	S	6	R	>32	R	256	R	32	S	0.38	R	>256
EG0390	*S. sonnei*	17	M	August	2007	VINH LONG	+	R	>256	S	6	R	>32	R	128	R	>256	S	0.38	R	>256
EG0395	*S. sonnei*	36	F	September	2007	HCMC	+	R	>256	S	12	R	>32	R	96	R	>256	S	0.5	R	>256
EG0162	*S. sonnei*	28	M	October	2007	DONG THAP	+	R	>256	S	8	R	>32	R	48	R	64	S	0.38	R	>256
EG0419	*S. flexneri*	23	F	December	2007	HCMC	-	R	>256	R	>256	R	>32	R	48	R	>256	S	0.5	R	128
EG0187	*S. sonnei*	16	M	January	2008	DONG THAP	+	R	>256	S	3	R	>32	R	192	S	1.5	S	0.047	R	24
EG0421	*S. sonnei*	36	F	January	2008	HCMC	+	R	>256	S	4	R	>32	R	>256	R	128	S	0.38	R	>32
EG0424	*S. sonnei*	48	F	January	2008	HCMC	+	R	>256	S	6	R	>32	R	64	R	>256	S	0.38	R	>256
EG0204	*S. sonnei*	26	F	March	2008	DONG THAP	+	R	>256	S	6	R	>32	R	32	R	64	S	0.38	R	>256
EG0430	*S. sonnei*	36	F	March	2008	HCMC	+	R	>256	S	6	R	>32	R	>256	R	48	S	0.25	R	128
EG1008	*S. sonnei*	18	M	May	2008	LONG AN	+	R	>256	S	8	R	>32	R	96	R	128	S	0.38	R	>256
EG1009	*S. sonnei*	8	M	May	2008	HCMC	+	R	>256	S	8	R	>32	R	96	R	192	S	0.38	R	>256
EG1010	*S. sonnei*	60	F	May	2008	HCMC	+	R	>256	S	6	R	>32	R	96	R	>256	S	0.5	R	>256
EG1013	*S. sonnei*	25	M	June	2008	HCMC	+	R	>256	S	6	R	>32	R	96	R	>256	S	0.25	R	>256
EG1012	*S. sonnei*	15	F	June	2008	HCMC	+	R	>256	S	8	R	>32	R	96	R	192	S	0.38	R	>256
EG1011	*S. sonnei*	108	F	June	2008	HCMC	+	R	>256	S	8	R	>32	R	96	R	128	S	0.38	R	>256
EG1007	*S. sonnei*	48	M	July	2008	LONG AN	+	R	>256	S	6	R	>32	R	64	R	48	S	0.38	R	192
EG0250	*S. sonnei*	35	M	August	2008	DONG THAP	+	R	>256	S	6	R	>32	R	48	R	48	S	0.25	R	>256
EG0250a	*S. sonnei*	36	M	September	2008	DONG THAP	+	R	>256	S	6	R	>32	R	48	R	48	S	0.25	R	>256
EG0471	*S. flexneri*	49	M	September	2008	HCMC	+	R	>256	R	>256	R	>32	R	128	R	>256	S	0.5	R	>256
EG0472	*S. sonnei*	66	M	September	2008	HCMC	+	R	>256	S	4	R	>32	R	96	R	48	S	0.38	R	>256
EG1014	*S. sonnei*	29	M	January	2009	LONG AN	+	R	>256	S	6	R	>32	R	>256	R	>256	S	0.25	R	>256
EG1015	*S. sonnei*	72	F	January	2009	HCMC	+	R	>256	S	4	R	>32	R	32	R	48	S	0.25	R	>256
EG1016	*S. sonnei*	39	M	January	2009	HCMC	+	R	>256	S	6	S	0.38	S	1.5	R	48	S	0.25	R	>256
EG1017	*S. sonnei*	11	F	February	2009	HCMC	+	R	>256	S	5	R	>33	R	97	R	49	S	1.38	R	>256
EG1018	*S. sonnei*	29	M	February	2009	HCMC	+	R	>256	S	6	R	>32	R	48	R	>256	S	0.38	R	>256
EG1019	*S. sonnei*	120	F	February	2009	HCMC	+	R	>256	S	6	R	>32	R	>256	R	48	S	0.25	R	>256
EG1020	*S. sonnei*	48	M	March	2009	HCMC	+	R	>256	S	8	R	>32	R	64	R	192	S	0.38	R	>256
EG1021	*S.sonnei*	20	M	March	2009	HCMC	+	R	>256	S	8	R	>32	R	64	R	>256	S	0.25	R	>256
EG1022	*S.sonnei*	29	M	March	2009	HCMC	+	R	>256	S	8	R	>32	R	48	R	>256	S	0.25	R	>256
EG1023	*S.sonnei*	9	F	March	2009	LONG AN	+	R	>256	S	6	R	>32	R	48	R	96	S	0.38	R	>256
EG1024	*S.sonnei*	84	M	March	2009	LONG AN	+	R	>256	S	6	R	>32	R	64	R	96	S	0.25	R	>256
EG1025	*S.sonnei*	30	M	March	2009	LONG AN	+	R	>256	S	6	R	>32	R	48	R	96	S	0.25	R	>256

5.3.4 The transferability of ceftriaxone resistance in *Shigella spp.*

The rapid rise in the prevalence of *Shigella* spp. which are resistant to third generation cephalosporin lead us to hypothesise that this resistance pattern arose by plasmid-transfer (Vinh et al. 2009a). Conjugation experiments were conducted to try transfer the ESBL genes from wild-type ESBL-positive *Shigella* strains to laboratory *E.coli* strain, and then from transconjugant back to ESBL-negative wild-type *Shigella* strains.

Transfer of ESBL genes from wildtype ESBL-positive *Shigella* strains to laboratory E.coli J53 (Sodium Azide resistant) strain: All 36 ceftriaxone resistant strains were subjected to conjugation experiments with the laboratory *E.coli* J53 (AziR). Thirty five *Shigella* strains gave positive results (Table 5.3). The exception is strain EG1020 where conjugation experiment was not successful.

Table 5.3 Characterisation of *bla*$_{CTX-M}$ genes and the corresponding plasmids of ESBL expressing *Shigella* spp. Strain EG0471 was *S.flexneri*, the rest were *S.sonnei.*

Strain ID	Ceftazidime zone size (mm)	*bla* $_{CTX-M}$	Plasmid size (kbp)	Conjugative (+/-)
DE0611	28	CTX-M-24	70	+
EG0162	18	CTX-M15	100	+
EG0187	27	CTX-M-24	70	+
EG0204	19	CTX-M15	100	+
EG0250	19	CTX-M15	100	+
EG0250a	19	CTX-M15	100	+
EG0356	28	CTX-M-24	70	+
EG0373	18	CTX-M15	100	+
EG0384	20	CTX-M15	100	+
EG0390	22	CTX-M15	100	+
EG0395	20	CTX-M15	100	+
EG0421	20	CTX-M15	100	+
EG0424	21	CTX-M15	100	+
EG0430	21	CTX-M15	100	+
EG0471	20	CTX-M15	100	+
EG0472	20	CTX-M15	100	+
EG1007	22	CTX-M15	100	+
EG1008	20	CTX-M15	100	+
EG1009	21	CTX-M15	100	+
EG1010	21	CTX-M15	100	+
EG1011	21	CTX-M15	100	+
EG1012	20	CTX-M15	100	+
EG1013	19	CTX-M15	100	+
EG1014	19	CTX-M15	100	+
EG1015	22	CTX-M15	100	+
EG1016	20	CTX-M15	100	+
EG1017	20	CTX-M15	100	+
EG1018	20	CTX-M15	100	+
EG1019	20	CTX-M15	100	+
EG1020	20	CTX-M15	100	-
EG1021	21	CTX-M15	100	+
EG1022	21	CTX-M15	100	+
EG1023	21	CTX-M15	100	+
EG1024	20	CTX-M15	100	+
EG1025	20	CTX-M15	100	+

There was 1 plasmid transferred from each wildtype *Shigella* donor to the recipient (Figure 5.2).

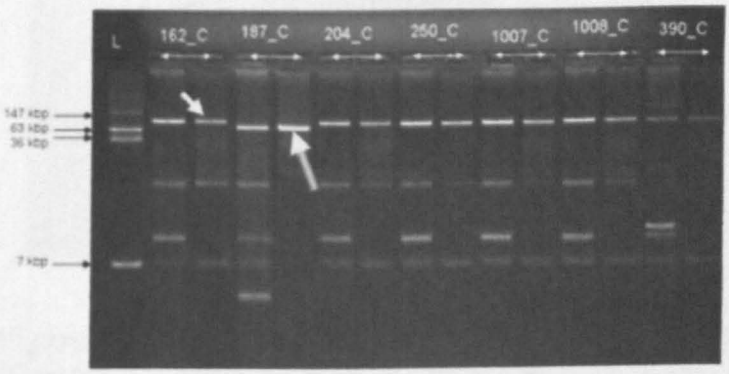

Figure 5.2 Transferability of ESBL containing plasmids from wildtype *Shigella* strains to *E.coli* J53 AziR. L: plasmids of *E.coli* 39R861 with known size of 7, 36, 63 and 147 kbp; numbers in upper row denote ID number of wildtype *Shigella* strains, the letter C denotes the corresponding transconjugants of those strains. Solid short arrow indicates the ~100 kbp plasmid; the long arrow indicates the ~70 kbp plasmid.

The *S. flexneri* (strain EG0419) which was not confirmed as ESBL-producing in combination disc test did transfer the ceftriaxone resistant gene to the laboratory strain *E.coli* J53 (AziR) also (Figure 5.3).

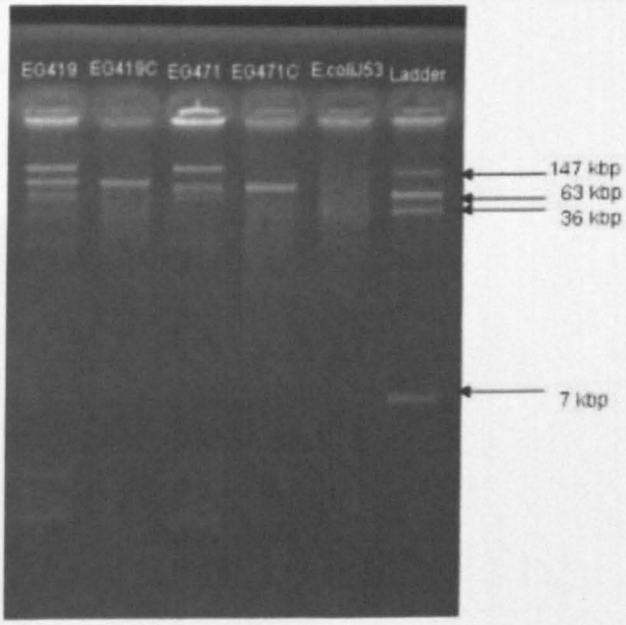

Figure 5.3 Plasmid transfer in 2 *S.flexneri* strains EG419 and EG471. Upper row: ID number of *Shigella* strains, the letter C after ID number denotes the corresponding transconjugants of those strains. *E.coli* J53 was the plasmid-free laboratory strain used as a receiver in the experiment. Ladder: plasmids of *E.coli* 39R861 with known size of 7, 36, 63 and 147 kbp.

Transfer of ESBL genes from transconjugant back to ESBL-negative wildtype *Shigella* strain: The transconjugant EG187c (which is a result of the mating between wild-type ceftriaxone-resistant *S.sonnei* strain EG187 and the laboratory ceftriaxone-sensitive sodium azide-resistant *E.coli* J53, hence ceftriaxone-resistant but nalidixic acid-sensitive) was used as donor, and the wild-type ceftriaxone-sensitive nalidixic acid-resistant *S.sonnei* strain EG211 as a receiver. The conjugation was carried out in LB media containing ceftriaxone 6mg/L plus nalidixic acid 30mg/L. The final transconjugant (named EG187c-211tc) was a *S.sonnei* strain which was resistant to both ceftriaxone and nalidixic acid. The plasmids from these strains were extracted using a modification of Kado and Liu method- and the DNA separated on a 0.7% agarose gel and photographed (Kado and Liu 1981). The plasmid sizes calculated in agarose gel electrophoresis of the transferable plasmid in the clinical isolate *S.sonnei* EG0187, in

the transconjugant EG187c and in the final transconjugant (EG187c-211tc) were the same (Figure 5.4).

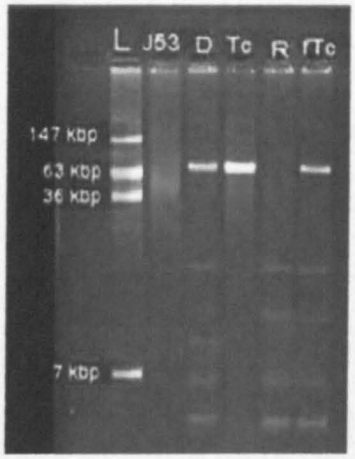

Figure 5.4 Transfer of ESBL-carrying plasmid from the wildtype *S.sonnei* EG187 (D) to *E.coli* J53, then from the transconjugant (Tc) to a wildtype *S.sonnei*. The wildtype *S.sonnei* EG187 (D) as the donor to the laboratory *E.coli* J53, then from the transconjugant (Tc) to a wildtype nalidixic acid resistant ceftriaxone susceptible *S.sonnei* receiver (R); the resulting final transconjugant (fTc) was a *S.sonnei* strain which was resistant to both nalidixic acid and ceftriaxone. The size of ESBL-carrying plasmid in this case was ~70 kbp; the ESBL in this strain was CTX-M-24. L: plasmids of *E.coli* 39R861 with known size of 7, 36, 63 and 147 kbp.

5.3.5 Characterisation of *bla* genes:

Genomic DNA of all ESBL producing *Shigella* strains were extracted, this DNA was subjected to PCR amplification to characterise the ESBL genes. Primers were selected specific for bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ classes of ESBL genes. All ESBL-positive strains (34 *S. sonnei* and 1 *S.flexneri*) strains produced CTX-M primer-specific amplicons. No amplicons were observed from PCR with the bla_{TEM} and bla_{SHV} primers. Further PCR amplifications were performed on DNA from all strains that produced amplicons with the $bla_{CTX\text{-}M}$. Primers specific for the three major CTX clusters, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}2}$ (Bonnet 2004) were selected. Three strains (DE0611, EG0356 and EG0187) produced amplicons with the $bla_{CTX\text{-}M\text{-}9}$ primers and the

remaining 32 isolates produced amplicons with the $bla_{CTX\text{-}M\text{-}1}$ primers (Table 5.3). The amplicons were cloned into cloning vector pCR 2.1 (Invitrogen, USA) and sequencing reactions were carried out on an ABI 3700 sequencing machine (ABI, USA) at the Sanger Institute, UK. All ESBL gene sequences were compared to other ESBL sequences by BLASTn at NCBI. The DNA sequence of various *bla*CTX-M ESBLs were downloaded and aligned with the produced sequences.

Sequence analysis demonstrated that there were two different types of $bla_{CTX\text{-}M}$ present in the *S. sonnei* population, $bla_{CTX\text{-}M\text{-}24}$ (n=3, 8 %) and $bla_{CTX\text{-}M\text{-}15}$ (n=32, 92 %). The only *S.flexneri* strain (EG0471) possessed $bla_{CTX\text{-}M\text{-}15}$ genes (Table 5.3). Both genes ($bla_{CTX\text{-}M\text{-}24}$ and $bla_{CTX\text{-}M\text{-}15}$) share 74 % DNA homology with each other; $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}24}$ differ by 12 and 6 nucleotides from their parent group ($bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$) respectively. The first ESBL producing *S. sonnei* strains in our series (strain DE0611) harbouring $bla_{CTX\text{-}M\text{-}24}$ was isolated in Ho Chi Minh City in February 2001 and 6 years later the second strain (EG0356, isolated in May 2007) was also found to harbouring the $bla_{CTX\text{-}M\text{-}24}$ and also from a resident of Ho Chi Minh City. Although the possibility of an outbreak of ceftriaxone resistant *Shigella* was a concern, in fact the next strain of ceftriaxone resistant *Shigella* was isolated in June 2007 and showed a different phenotype. These strains had less activity against ceftazidime when compared with the first two strains. The third *S. sonnei* possessing $bla_{CTX\text{-}M\text{-}24}$ was isolated from Dong Thap Province in January 2008. Thereafter, all the ESBL-producing *Shigella* strains harboured the $bla_{CTX\text{-}M\text{-}15}$ genes (Table 5.3).

Plasmid size estimation (calculated from comparing with plasmids of known sizes of the strain *E.coli* 39R861) demonstrated that the $bla_{CTX\text{-}M\text{-}15}$ was consistently carried on a large plasmid (~100 Kbp), whilst the $bla_{CTX\text{-}M\text{-}24}$ gene was carried on a smaller (~70 Kbp) plasmid. The differing plasmid sizes and ESBL genes correlated precisely with

two distinct zone clearance areas when strains were susceptibility tested with ceftazidime. The strains expressing CTX-M-24 demonstrated less activity against ceftazidime when compared to CTX-M-15 (median zone size, CTX-M-24: 28mm, CTX-M-15: 20mm respectively) (Table 5.2).

5.4 Discussion:

Multi-drug resistance is a well-known phenomenon in *Shigellae.* Almost all oral antibiotics used for the treatment of shigellosis have become useless after widespread use (David A. Sack et al. 2001). Resistance to currently recommended antibiotics such as ciprofloxacin, azithromycin has also been recognised (Boumghar-Bourtchai et al. 2008; Kansakar et al. 2007; von Seidlein et al. 2006). Members of the *Enterobacteriaceae* that carry CTX-M group ESBLs have been isolated from many parts of the world since the mid-1990s (Bonnet 2004). CTX-M genes were also detected in Thailand, Cambodia and Singapore (Cao et al. 2002; Kiratisin et al. 2008; Koh et al. 2004; Ruppe et al. 2009).

In Viet Nam CTX-M-14 and CTX-M-17 genes have been previously identified from pathogenic *K.pneumoniae* causing hospital-acquired infection (Cao et al. 2002). Work in our institution has shown that ESBLs are commonly expressed in organisms which constitute the "normal" gastrointestinal flora in the general population living in Ho Chi Minh City (Le et al. 2009). Such data predicts that intestinal flora may be a considerable reservoir of ESBL encoding genes carried on transposable genetic elements (plasmids and transposons) permitting transfer to pathogenic counterparts intermittently carried in the gastro-intestinal flora.

5.4.1 Increasing trend of ESBL-producing *Shigella* spp. but without increasing severity of the disease:

The occurrence of ESBL-producing *Shigella* strain (majority are *S.sonnei*) increased sharply from 2007 to 2009. The spatial distribution of 36 ESBL-producing *Shigella* strains in our series suggested that there was not a common-source outbreak of disease. The clinical features of children infected with ESBL-producing *Shigella* strains were not more severe when compared with those infected by ESBL-negative *Shigella* strain. All patients responded favourably to fluoroquinolone treatment and there were no severe complications or deaths in this case series.

5.4.2 Two types of CTX-M genes in *Shigella* spp. in southern Viet Nam:

Different CTX-M genes in the *Shigellae* have been reported from Argentina (CTX-M-2) (Radice et al. 2001), Korea (CTX-M-14) (S. Kim et al. 2004), China (CTX-M-14) (Xiong et al. 2007), Lebanon (CTX-M-15) (Matar et al. 2007) and from a Czech traveller returning from India (CTX-M-15) (Hrabak et al. 2008). More recently, Nagano *et al.* described a novel CTX-M-64 hybrid from a shigellosis patient infected with *S. sonnei* after returning to Japan from China (Nagano et al. 2009). Our data showed that there are 2 ESBL genes circulating in *Shigella* strains in southern Viet Nam, the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}24}$ genes. The $bla_{CTX\text{-}M\text{-}24}$ gene was first detected in *S.sonnei* strain DE0611 in February 2001 and re-appeared in May 2007 in Ho Chi Minh City. The last time this strain was isolated was in January 2008 in a patient resident in Dong Thap Province. The presence of the $bla_{CTX\text{-}M\text{-}15}$ was detected in June 2007 and has gradually become the dominant gene (comprised of 92% of all ESBLs) in *S.sonnei* strains isolated through 2009. The single *S.flexneri* strain that harboured the $bla_{CTX\text{-}M\text{-}15}$ gene was isolated in September 2008 from a 4 years old boy resident in Ho Chi Minh City.

5.4.3 Epidemics of CTX-M carrying plasmids in *Shigellae*.

Our findings demonstrate a switch from 0% to 75% ceftriaxone resistance in *Shigella* spp. over a two year period in the key age group (1 to 3 years) for this disease. By sampling across the Ho Chi Minh City area, covering approximately 150 sq kilometres of southern Viet Nam and a population of approximately 15 million people, we have shown that the genetic explanation for this resistance pattern is the dissemination of two novel plasmids that carry two distinct ESBL genes:

(i) The ~70Kbp plasmid first appeared in 2001, harbouring the $bla_{CTX\text{-}M\text{-}24}$ gene, and re-appeared in May 2007 in Ho Chi Minh City and was last identified in January 2008 in Dong Thap Province, and has not been detected since then. There are only 3 *Shigella* strains carrying the ~70 kbp plasmid in our series. The presence of the $bla_{CTX\text{-}M\text{-}24}$ gene has been previously reported in only one *S.flexneri* strain from Hong Kong (Cheung et al. 2005).

(ii) The second plasmid, which is ~100kbp and harbours the $bla_{CTX\text{-}M\text{-}15}$ gene, first detected in June 2007 in Ho Chi Minh City and later- in October 2007 Dong Thap Province, has replaced the ~70Kbp plasmid and has become the dominant plasmid carrying $bla_{CTX\text{-}M\text{-}15}$ gene. The total number of *Shigella* strains we have identified which carry this plasmid is 32, including 1 *S.flexneri* and 31 *S.sonnei*.

The *Shigellae* are closely related to *E. coli* (Wirth et al. 2006), and similar to *E. coli*, are capable of rapid population changes by the acquisition of extraneous DNA. *Shigella* spp. are capable of carrying multiple plasmids with an array of phenotypes including virulence and antimicrobial resistance (Dutta et al. 2002; Iversen et al. 2003). The presence of *Shigella* in the gastrointestinal tract of humans is an ideal environment to acquire horizontally transferred genetic material via conjugation. Highly transmissible

plasmids that impinge on the fitness of the host may be rapidly disseminated under appropriate conditions.

The detection of the plasmid-transferable *bla*CTX-M-15 in *Shigella* strains in southern Viet Nam is important in terms of the shifting pattern of the epidemiology of the disease. Our data suggests an increase in the prevalence of the CTX-M-15 carrying plasmid circulating in *Shigella* community in Ho Chi Minh City and surrounding provinces in the southern part of Viet Nam. CTX-M-15 was first detected in *E.coli* in India in 2001, then was rapidly distributed globally mainly through the ST131 clone of *E.coli*. Over the intervening nine years this has become the dominant transposable ESBL. CTX-M-15 has now been reported from all continents except Antarctica (Pitout 2010). CTX-M-15 belongs to the CTX-M-1 cluster and is derived from CTX-M-3 by one amino acid substitution at position 240 (Asp- Gly). The beta-lactamase CTX-M-15 has been detected in *S.sonnei* in France and Lebanon (Lartigue et al. 2005; Matar et al. 2007).

Shigellosis is primarily a community-acquired infection, the presence of CTX-M ESBLs in *Shigella* spp. may indicate that CTX-M-carrying plasmids are circulating not only in the hospital environment but also in the community. Characterizing the molecular nature of ESBL-carrying plasmids in other bacteria in the Enterobacteriaceae family such as *E.coli* and *K.pneumoniae* may help to address this question. Moreover, there is a dominant presence of CTX-M in *Shigella* isolates causing symptomatic disease and their rapid spread suggests that these organisms are under strong selection pressure either through drug pressure or other biological advantages. The use of third generation cephalosporins, such as oral cefpodoxime and cefixime in the community is

common in Viet Nam, and places the even the short term usage of ceftriaxone and other broad-spectrum cephalosporins in jeopardy.

Viet Nam is a country that is in many respects a microcosm of the rapidly changing developing world. The Vietnamese economy is developing rapidly and the country is undergoing transition with an increasing population, urbanisation and shifting patterns of infectious diseases. In the past decade there has been a transition in species from *S. flexneri* to *S. sonnei* in the southern provinces of Viet Nam (Vinh et al. 2009b). With this shift has come the emergence of ESBL-producing *Shigellae*, mainly *S. sonnei*, between 2007 and 2009. These findings from the Vietnamese population should serve as a warning for other countries encountering the same economic transition. The progressive evolution of pan-resistant *Shigella* makes vaccine development an increasingly important objective.

Chapter Six

A Randomised Controlled Trial of Gatifloxacin versus Ciprofloxacin for the Treatment of Bacillary Dysentery in Children

6.1 Introduction:

Shigella infection is a commonly seen enteric infection world-wide. In industrialized countries, small outbreaks of shigellosis may occur in infant day-care centres or nursery where optimal personal hygiene is difficult to maintain. Travellers from industrialised countries may get shigellosis during or short after their travel to developing countries. The Center for Disease Control and Prevention recently estimated that there are more than 440,000 cases of shigellosis per year in the United States (Mead et al. 1999). In developing countries, *Shigella* infection remains the most important bacterial cause of diarrhoeal diseases in children (Hien et al. 2008). The annual number of *Shigella* infection episode was estimated to be 163.2 million in developing countries, with 1.1 million deaths; a total of 69% of all episodes and 61% of all deaths attributable to shigellosis involved children less than 5 years of age (Kotloff et al. 1999). Despite the recently decrease in the case fatality rate *Shigella* infections remain an important public health issue (Sansonetti 2006).

The treatment of shigellosis includes providing water and electrolytes rehydration and nutrition in addition to the administration of appropriate antibiotics. Antimicrobial therapy is important for the treatment of shigellosis. Without antimicrobial treatment,

the case fatality rate associated with *S.dysenteriae* type 1 infection may exceed 10%, particularly in the young and the older patients (Bennish and Wojtyniak 1991). Antimicrobials shorten the duration of symptoms, eradicate *Shigellae* from the stool more quickly, and may prevent severe complications (Salam and Bennish 1991). The choice of antibiotics to use as first line against *Shigella* infection should be based on knowledge of the antibiotic susceptibility patterns of locally circulating strains. Currently, the World Health Organisation guidelines have recommended ciprofloxacin as the drug of choice for all patients with shigellosis, irrespective of their age. Pivmecillinam (amdinocillin pivoxil), ceftriaxone and azithromycin are considered as alternatives for the treatment of multi-resistant strains of *Shigella* in all age groups (WHO 2005a). Unfortunately, strains of *Shigella* developed resistance to ciprofloxacin and other antibiotics have been reported (Taneja et al. 2005).

Although drugs in the quinolone class have been reported to cause arthropathy in immature laboratory animals, the risk of joint damage in children treated with short course of fluoroquinolones appears to be minimal (Bethell et al. 1996; Sansone et al. 2009) and is clearly outweighed by the value of these drugs for treatment of potentially life-threatening disease (Leibovitz 2006).

Gatifloxacin is a new 8-methoxy-fluoroquinolone antibiotic. The presence of a methoxy group at position 8 in gatifloxacin increases the antibacterial activity of the drug against *Streptococcus pneumoniae* in addition to *Enterobacteriaceae* spp., and also a structural advantage that decreases the likelihood of emergence of resistance (Leibovitz 2006). The drug is distributed extensively into tissues. The MIC90 for *Shigella* spp. is 0.01-0.03mg/L (Douglas 2001). Gatifloxacin actively penetrates into phagocytic cells *in vitro*. Its significant accumulation in these cells (intracellular: extracellular

concentration ratios are between 5-7 times in macrophages and neutrophils) may result in enhanced activity against susceptible intracellular pathogens such as *Salmonellae* and *Shigellae* (Douglas 2001). In Viet Nam gatifloxacin has showed an excellent efficacy and safety for the treatment of typhoid fever caused by multidrug and nalidixic acid resistant organisms in both children and adults (Dolecek et al. 2008).

As a result of increasing multidrug resistance (including nalidixic acid and ciprofloxacin) in *Shigella* strains circulating in Viet Nam (von Seidlein et al. 2006), we conducted a controlled trial comparing the efficacy of gatifloxacin, a third generation quinolone, versus the currently recommended drug ciprofloxacin, of the second generation quinolone (Leibovitz 2006), in the treatment of childhood shigellosis in southern Viet Nam.

6.2 Materials and methods:

The study was conducted from June 2006 to March 2009 at two places in southern Viet Nam: The Paediatric Ward B at the Hospital for Tropical Diseases (HTD) of Ho Chi Minh City and The Infectious Diseases Ward, Dong Thap Provincial Hospital (DTP) (see section 2.1.3 and 2.1.4 chapter two).

The study protocol was approved by the Scientific and Ethical Committee of the Hospital for Tropical Diseases, Ho Chi Minh City, of Dong Thap Hospital and by The Oxford Tropical Research Ethics Committee (ref: OxTREC 010-06) and registered at International Standard Randomized Controlled Trials Registry (ISRCTN55945881).

Inclusion criteria to the randomised clinical trial (Code name EG):

Patients 3 months to 14 years old of either sex, who were admitted to the study wards with acute dysenteric syndrome (i.e. bloody stools, or mucoid stools accompanied by

abdominal pain and/or tenesmus) of less than 72 hours were eligible to be included in the study provided their parents or caregivers gave fully informed written consent.

Exclusion criteria:

Patients were excluded from the study if:

-They had documented been treated with any quinolones for this illness,

-Their clinical states were severe enough to require parenteral antibiotics (based on treating physician's decision)

-They had coexisting illnesses requiring antimicrobial therapy,

-There were trophozoites of *Entamoeba histolytica* observed in stool microscopic examination.

-Informed consent was not obtained

Random allocation of patients to the clinical trial (code name EG study):

A computer-generated randomization list for two study drugs in blocks of 10 was prepared by a person not involved in the care or evaluation of the patients or in data analysis. Whenever a patient fulfilled the entry criteria, a consecutively numbered sealed opaque envelop containing a small piece of paper (folded 4 times) printed the name of one of the study drugs was open to allocate her/him into one of two study arms:

- (1) Ciprofloxacin (OPV manufacturer, Viet Nam) 15 mg/kg by mouth every 12 hours for a total of 6 doses in 3 days,

- (2) Gatifloxacin (Stada Pharmaceuticals, Viet Nam) 10 mg/kg per day (orally in a single dose) in 3 days.

In case the clinical condition did not improve or worsened requiring parenteral antibiotics, ceftriaxone 50mg/kg of body weight per day in a single dose was used for 5 days. Other treatment included fluid and electrolytes (WHO Oral Rehydration Solution, or parenteral Lactated Ringer solution) and antipyretics were given to the two groups at the discretion of the treating physician. Seizures were treated with diazepam 0.25 mg/kg of body weight intravenously.

Clinical examination:

Study day 1 (D1) began with the first dose of the study drug and continued for the next 24 hours. The clinical history and physical examination of the patients were documented on standardized case record forms (CRF). Vital signs and stools characteristics (bloody, mucoid or watery) were recorded every 6 hours, and the number of bloody or watery stools recorded every 24 hours.

Haematology and biochemistry tests:

Full blood count and microscopic examination of the stools for red blood cells (RBC), white blood cells (WBC) and parasites including *Entamoeba histolytica* were done for all patients. Serum electrolytes, ultrasound examination of the abdomen, and other tests were done at the discretion of the treating physician.

Assessment parameters:

For the purpose of the EG study, outcome parameters were as follows:

Primary outcome:

Failure rate: percentage of patients who suffered from either clinical failure or microbiological failure:

(a) Clinical failure: the patient still had symptoms (fever and/or diarrhoea) at day 5 following treatment.

Diarrhoea was defined as:

Watery diarrhoea: passing 3 or more of liquid (un-formed) stools in the past 24 hours;

Bloody diarrhoea: passing at least one stool with visible blood;

Mucoid stool: visible mucus in stool;

Fever was defined as axillary temperature of >37.5°C.

Patients re-admitted to the hospital because of diarrhoea within 1 week of discharge were classified as clinical failure.

(b) Microbiological failure: *Shigella* was recovered from patient's stool after day 5 following treatment.

Secondary outcomes:

(a) Fever Clearance Time (FCT): time from the start of treatment to when the axillary temperature fell below 37.6°C and remained there for more than 24 hours.

(b) Diarrhoea Clearance Time (DCT): the time until the first formed stool.

(c) Bloody diarrhoea Clearance Time (BDCT) the time until the last stool containing visible blood was passed.

(d) Bacteria Clearance Time: the time from the start of treatment until the first culture negative stool (in cases where *Shigella* had been isolated on enrolment).

(e) Hospital stay: from admission to discharge (days);

6.3. Results:

6.3.1 Patients:

A total of 500 cases of childhood acute dysentery were included into the study, 200 cases at The Hospital for Tropical Diseases at Ho Chi Minh City and 300 cases at Dong Thap Provincial Hospital at the Mekong Delta. Half of these patients (250 cases) were allocated to the ciprofloxacin arm, and 250 cases in the gatifloxacin arm. Five patients in the ciprofloxacin arm and 1 child in the gatifloxacin arm were withdrawn from the study before taking study drug because their parents wanted to transfer them to other hospitals for their convenience. *Shigellae* were isolated from 108 admission stool culture (46 in Ciprofloxacin arm and 62 in gatifloxacin arm). Four hundred and thirty three patients returned to the hospital for follow-up 7-10 days after discharge, including 35 *Shigella* culture positive cases in ciprofloxacin group and 47 *Shigella* culture positive in gatifloxacin group (Figure 6.1).

The baseline characteristics of the patients enrolled in the two study arms were similar, except the number of cases with *Shigella* in admission stool in ciprofloxacin was lower than in gatifloxacin arm (46 versus 62 patients). The median age of patients was 19 months old, and the median weight 10 kg. The patients were ill for a mean of 24 hours before admission to the hospital. The percentage of nalidixic acid resistant *Shigella* strains was the same in two groups (72% in ciprofloxacin arm versus 68% in gatifloxacin arm); so was the percentage of patients had follow-up visit (Table 6.1). *S.sonnei* was the dominant species with 73 out of 108 *Shigella* strains isolated, equal to 68%. *S.flexneri* constituted 30% with 33 strains (12 in ciprofloxacin arm and 21 in gatifloxacin treatment arm). There were only 2 *S.boydii* strains, both in the gatifloxacin arm.

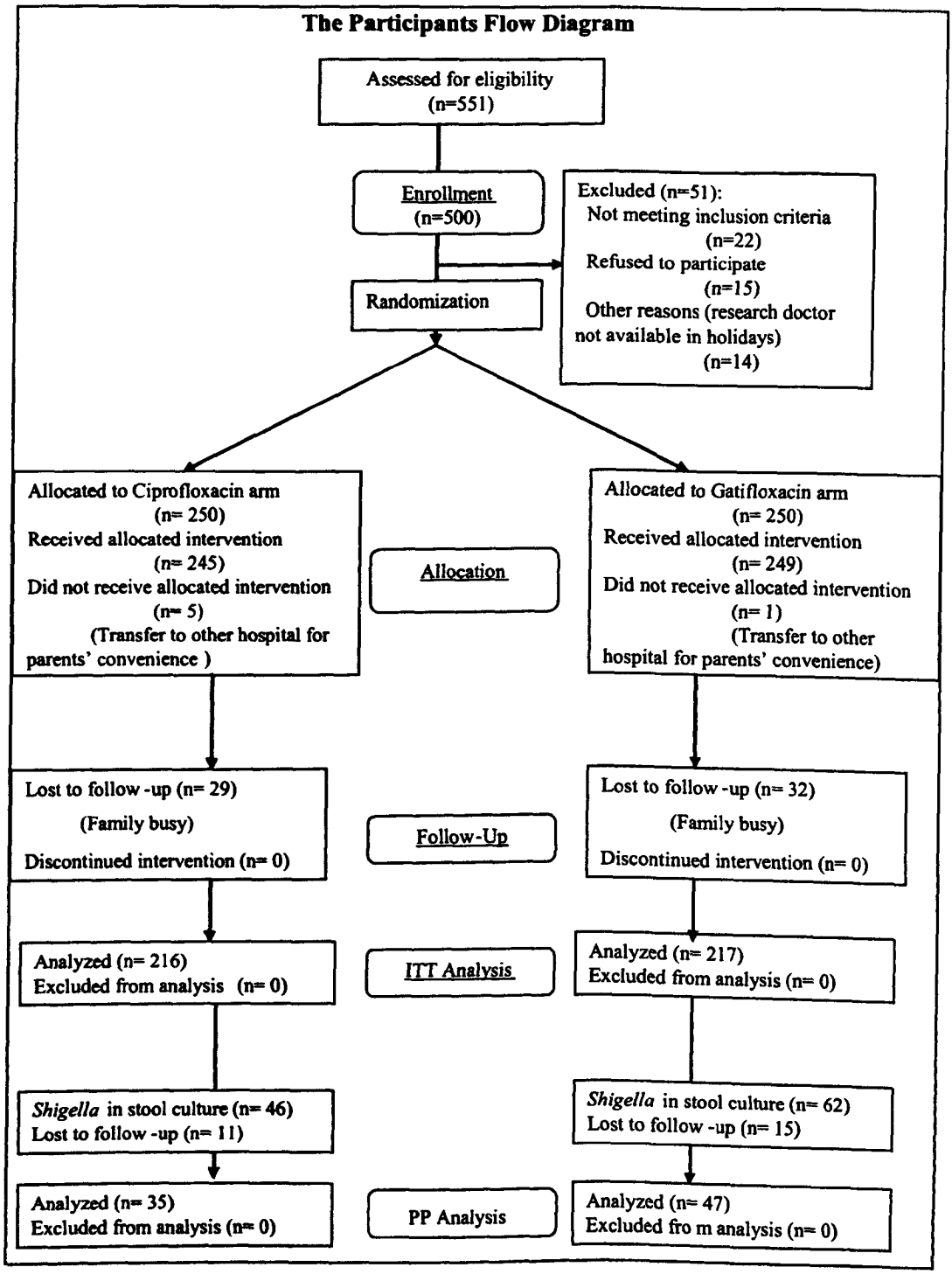

Figure 6.1 Participants flow diagram in the study.

Of particularly interest, there was one *S.flexneri* strain from a patient in gatifloxacin arm which was not only resistant to nalidixic acid but also to both ciprofloxacin and gatifloxacin. This patient responded uneventful to gatifloxacin treatment and was discharged healthy.

Table 6.1 Baseline clinical and laboratory characteristics of 494 children with acute dysentery.

Parameters	Ciprofloxacin N=245	Gatifloxacin N=249
Age (month)*	19.0 (11.0-33.0)	19.5 (10.0-32.0)
Weight (kg)*	10 (8.4-12.0)	10 (8.2-12.5)
Boy/Girl (No.)	148/97	146/103
Duration of Illness before admission (hrs)*	24 (16-48)	24 (16-48)
Body Temperature (°C)*	38.5 (37.5-39.0)	38.5 (37.5-39.0)
White cell count (/mm^3)*	11,450 (8,595-15,700)	11,150 (8,645-14,200)
Total *Shigella* strains (No.)	46	62
S.sonnei	34	39
S.flexneri	12	21
S.boydii	0	2
Nalidixic acid resistant *Shigella* (No.of strains (%))	33/46 (72%)	42/62 (68%)
Follow-up (No.of cases (%))	216 (88%)	217 (87%)
Shigella culture positive	35	47

* Median (interquartile range)

6.3.2 Efficacy:

All children receiving antimicrobial treatment responded well to the treatment; there were no death or severe complications during the course of treatment. There was 1 child in each treatment arm who received rescue treatment with intravenous ceftriaxone because their clinical status did not improve after 3 days of treatment. They all recovered and were discharged healthy.

Among 494 patients allocated to 2 treatment arms, there were 433 children who attended for follow-up 7 to 10 days after discharge from the hospital; the data of these patients was analysed on an intention-to-treat basis. The failure rate (the primary outcome) of the 2 treatment arms was 7.4% in ciprofloxacin arm versus 6.9% of gatifloxacin arm. This different was not statistically significant (OR=0.93, 95%CI= 0.42–2.05, p=0.9, Fisher exact test). The median time to first formed stool (diarrhoea

clearance time) was 67 hours in ciprofloxacin treated patients in comparison with 64 hours in gatifloxacin treated group (p=0.7) (Table 6.2 and Figure 6.2).

Other secondary outcome measures (bloody diarrhoea clearance time and fever clearance time) were also not different between the two treatment groups (Table 6.2).

Table 6.2 Responses to treatment with ciprofloxacin and gatifloxacin in 433 children with acute dysenteric syndrome attending follow-up.

Parameters	Ciprofloxacin (n=216)	Gatifloxacin (n=217)	P values
Failure, cases (%)	16 (7.4%)	15 (6.9%)	p=0.9 ‡
Clinical failure	11 (5.1%)	11 (5.0%)	
Relapse	5 (2.3%)	4 (1.8%)	
Microbiological failure	0	0	
Fever clearance time (hrs)*	24 (12-36)	24 (12-37)	0.5 #
Bloody diarrhoea clearance time (hrs) *	30 (21-54)	29 (21- 48)	0.5 #
Diarrhoea clearance time (hrs) *	67 (42-93)	64 (42-93)	0.7 #

* Median (Interquartile range)
‡ Chi^2 test
# Mann-Whitney U test

There were 82 children among 108 *Shigella* positive patients attending follow-up, 35 in the ciprofloxacin arm and 47 in the gatifloxacin arm. The analysis of these patients (per protocol analysis) showed that the failure rate was 8.5% in both treatment arms (Table 6.3).

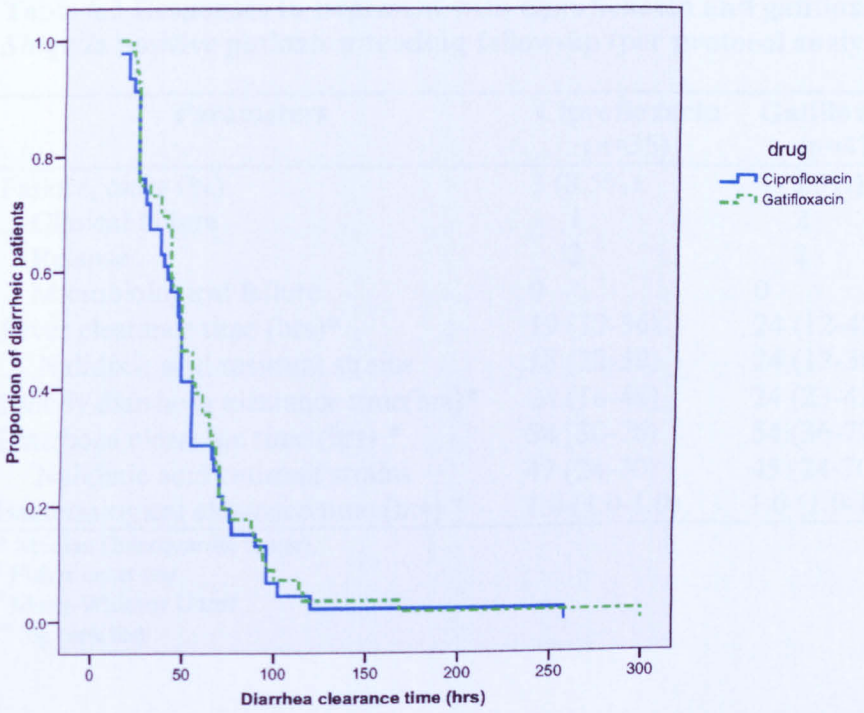

Figure 6.2 Comparison of diarrhoea clearance time in 433 dysentery patients treated with ciprofloxacin (continuous line) versus gatifloxacin (broken line) (p=0.7 Mann-Whitney U test).

There were 75 cases caused by nalidixic acid resistant *Shigella* strains, 33 in the ciprofloxacin arm and 42 in the gatifloxacin treatment arm. The fever clearance time as well as the diarrhoea clearance time did not differ significantly in the ciprofloxacin arm versus the gatifloxacin arm (Table 6.3 and Figure 6.3).

Table 6.3 Responses to treatment with ciprofloxacin and gatifloxacin of 82 *Shigella* positive patients attending follow-up (per protocol analysis).

Parameters	Ciprofloxacin (n=35)	Gatifloxacin (n=47)	P values
Failure, cases (%)	3 (8.5%)	4 (8.5%)	p=1 ‡
Clinical failure	1	2	
Relapse	2	2	
Microbiological failure	0	0	
Fever clearance time (hrs)*	19 (12-36)	24 (12-48)	0.5 #
Nalidixic acid resistant strains	18 (12-30)	24 (12-36)	0.4 #
Bloody diarrhoea clearance time(hrs)*	24 (18-48)	24 (23-42)	0.6 #
Diarrhoea clearance time (hrs) *	54 (30-76)	54 (36-77)	0.9 #
Nalidixic acid resistant strains	47 (24-70)	45 (24-70)	0.8 #
Bacteriological clearance time (hrs) *	1.0 (1.0-1.0)	1.0 (1.0-1.0)	0.11 ##

* Median (Interquartile range)
‡ Fisher exact test
# Mann-Whitney U test
log rank test

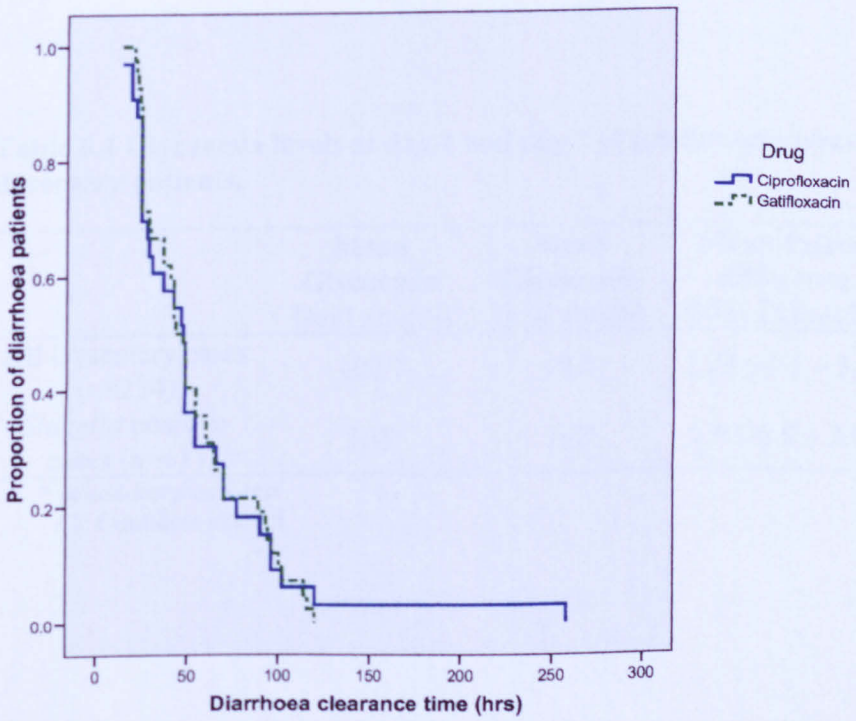

Figure 6.3 Comparison of diarrhoea clearance time in shigellosis caused by nalidixic acid resistant strains, ciprofloxacin versus gatifloxacin.

6.3.3 Adverse effect:

This study was not designed to compare adverse effects of the 2 study drugs. All patients revealed no joint pain during the course of treatment and on follow-up visit one week after discharge. The concern was that dysglycaemia (hypoglycaemia and hyperglycaemia) has been recognized in elderly patients treated with gatifloxacin. We compared glycaemia levels on day 1 (just before taking gatifloxacin) and day 3 (at the same time of day) in patients in the gatifloxacin arm. In 234 patients whose pair glycaemia data were available, the means of glycaemia in D1 and D3 was 100.7 mg% and 98.4 mg% respectively. Of 61 patients with *Shigella* in stool culture the glycaemia was 108 mg% versus 102 mg% in D1 and D3. Using paired-samples T-test the difference between glycaemia of D1 and D3 was not significant in both intended-to-treat (p=0.18) and per-protocol analyses (p=0.09) (Table 6.4). There was no mortality in this study population.

Table 6.4 Glycaemia levels at day 1 and day 3 of gatifloxacin treatment in dysentery patients.

	Mean Glycaemia Day1 (mg%)	**Mean Glycaemia** Day3 (mg%)	**Mean Paired difference** (95% CI)(mg%)	**P value** (2-tailed)*
All Dysentery cases (n=234)	100.7	98.4	2.28 (-1.1 – 5.6)	0.18
Shigella positive cases (n=61)	108	102	6.0 (-0.8 – 2.9)	0.09

* paired-samples T-test
CI: Confident interval

6.4. Discussions:

An effective antimicrobial is important for the management of shigellosis. It shortens the course of the illness, prevents the occurrence of severe complications, and reduces the transmission of the bacteria to close contacts, such as household members, classmates or neighbour (Traa et al. 2010). A 3-day course of ciprofloxacin is the current recommendation treatment for shigellosis including those caused by *S.dysenteriae* type 1 (WHO 2005a). The results of our study showed that gatifloxacin was as effective as ciprofloxacin in the treatment of childhood dysentery including culture-confirmed childhood shigellosis.

In this study, 494 children hospitalized for acute dysentery (in which 108 cases with positive faecal culture for *Shigella*) were treated by either the WHO recommended treatment of ciprofloxacin for 3 days or gatifloxacin once a day for three days. There were 433 patients who attended for follow-up at 7–10 days after discharge and were eligible for primary outcome comparison. The failure rate was not different between the two treatment groups (7.4% in ciprofloxacin group versus 6.9% in gatifloxacin group). Time to become afebrile and time to first normal stool were also similar in the two groups (Table 6.2). In the subset of children with *Shigella* cultured on admission stool the failure rate, fever clearance time and diarrhoea clearance time were not significant different in both treatment groups (Table 6.3). The duration of faecal excretion of *Shigella* following antimicrobial treatment was slightly longer in the ciprofloxacin group (means 1.2 days in ciprofloxacin group versus 1.05 days in gatifloxacin group), but this was neither a clinically important difference nor was it significant statistically ($p=0.11$, log rank test, Table 6.3).

The treatment failure rate of 8.5% in the culture confirmed shigellosis in this study was much lower than the 31% to 35% reported in the treatment of dysentery caused by *S.dysenteriae* type 1 (ZimBaSA_Dysentery_Study_Group 2002). The difference may reflect the fact that the main serogroup of Shigellae in our study was *S.sonnei,* which is considered to cause a more benign illness than *S.dysenteriae* type 1. In a study in Bangladesh the failure rate in adults treated with ciprofloxacin was 18% (Khan et al. 1997). In our previous study at the same institution in Ho Chi Minh City, in which *S.flexneri* was the dominant sero-group, the failure rate was 10% in the ofloxacin group (Vinh et al. 2000). One possibility is that shigellosis has become a more benign illness over time (Sansonetti 2006), or the patients admitted latterly were seen and admitted earlier and the case management at health facilities was better than in previous years.

Although gatifloxacin is a newer fluoroquinolone, it's efficacy in the treatment of shigellosis did not appear to be superior to the older fluoroquinolone ciprofloxacin. However, because of its long half-life, the once-a-day administration is simpler and more convenient than the twice-a-day needed for ciprofloxacin. In this series 70% of *Shigella* strains were nalidixic acid resistant, but both study drugs cleared *Shigella* from stools effectively (Table 6.3). Of interest in this study is that one child was infected with a gatifloxacin-resistant *S.flexneri* strain (MIC of gatifloxacin was 8 μg/ml) but responded very well clinically to treatment with gatifloxacin. The bacteria were cleared from the patient's stool after one day of gatifloxacin treatment. Possibly the clinical response to treatment depends on a variety of factors of which antibiotics are only a part.

The use of fluoroquinolones in children has been controversial for many years because of the fear of causing arthropathy, an adverse effect observed in immature laboratory

animals during the pre-clinical testing of this class of drugs. However, the overwhelming clinical evidence suggests that these fears are unfounded. A two year follow-up children treated with short course ciprofloxacin or ofloxacin for typhoid fever in Viet Nam did not showed any adverse effects on the children's growth nor did it reveal any evidence of arthropathy (Bethell et al. 1996). Recent studies in young lambs have shown that ciprofloxacin and gatifloxacin did not affect growth velocity in ovine model when administered in a dose regimen similar to that used in paediatric populations (Sansone et al. 2009). The World Health Organisation, after considering the potential risks and benefits, has recommended ciprofloxacin as the drug of first choice for the treatment of shigellosis in both adults and children (WHO 2005a) as well as the use of fluoroquinolones in the treatment of enteric fever.

One other issue with gatifloxacin has been the association with dysglycaemic episodes both in pre-clinical studies and retrospective studies in the elderly. The propriety drug "Tequine" (Gatifloxacin manufactured by Bristol Myers Squibb) was voluntarily withdrawn from the market in May 2006 because of concerns about dysglycaemic. Although hypoglycaemia and hyperglycaemia were reported rarely in clinical trials with gatifloxacin, dysglycaemic events (mainly involved elderly patients with diabetes) were reported during the post-marketing period and published in medical literature (Park-Wyllie et al. 2006; Yadav and Deopujari 2006). In 1504 patients $\geq$ 18 years old treated with either gatifloxacin or ceftriaxone for up to 5 days for acute community-acquired pneumonia or acute exacerbation of chronic bronchitis in the United States, the risk of dysglycaemia with gatifloxacin was not as high as previously reported in ambulatory patients (Onyenwenyi et al. 2008). Dysglycaemia was not reported in the pre-clinical testing in younger animals and it has not been reported in younger patients treated with gatifloxacin. Gatifloxacin maybe a very important drug both for enteric fevers in the

setting of high rates of nalidixic acid resistance and fluoroquinolone reduced susceptibility or resistance and for tuberculosis. Further work on its efficacy and its adverse event profile is needed before this drug is potentially lost from our potential therapeutic options. This is particularly true when most of the time we are using this drug to treat young adults and children in whom problems with dysglycaemia have not been reported.

The design of this study may confound our interpretation. The study was not double-blind, the treating doctors and nurses knew the drugs their patients received, although neither the patients nor their parents did. However, because the randomization was prepared by individuals unrelated to the allocation or treating patients, none of the treating clinicians or nurses knew which drug the patients received before the envelop was opened, so treatment allocation was concealed.

Another potential weakness of the study is that because gatifloxacin was withdrawn from the market by the primary manufacturer after the start of this trial, the future of gatifloxacin remains unclear. However, there are plans to prepare a dossier to submit to the World Health Organisation bringing together all the clinical experience of the use of this drug in enteric fever and tuberculosis and assess whether its continued production is required (C. Dolecek personal communication).

In conclusion, gatifloxacin was as effective as ciprofloxacin in the treatment of acute dysentery, specifically shigellosis, in Vietnamese children. A simple once-a-day oral administration may lead to better adherence. Acute dysglycaemia episodes were not seen in children treated with the 3-day course of gatifloxacin in this study. With the

increasing trend of quinolone-resistance in Shigellae, it is worth to consider other antimicrobials for the treatment of shigellosis.

Chapter Seven

Summary and Directions for Future Research

Acute diarrhoea is a clinical syndrome every paediatrician sees in their daily practice. The case fatality rate of acute diarrhoeal disease has been decreased substantially in recent years presumably due to better case management at health care facilities. Shigellosis remains a significant global public health problem particularly affecting disadvantaged children.

In Viet Nam, studies on the prevalence and antibiotic susceptibility of *Shigella* infection have been conducted in Ha Noi in northern Viet Nam and in Nha Trang in the central part of the country. There has been much less data on childhood *Shigella* infection in southern Viet Nam. This has been the focus of my thesis.

This thesis focused upon the epidemiology, clinical features, antibiotic resistance and treatment of *Shigella* infection in Vietnamese children and aimed to address four questions:

(i) What is the magnitude of *Shigella* infections as a causative agent of childhood acute diarrhoea in southern Viet Nam? Is clinical picture specific enough to differentiate acute diarrhoea caused by *Shigella* from that caused by other agents including rotavirus?

(ii) What have been the changes in the epidemiology, antibiotic susceptibility and clinical features of childhood shigellosis in southern Viet Nam in the last 15 years?

(iii) What are the molecular characteristics of the ESBL genes in the *Shigella* population circulating in southern Viet Nam? What transferable genetic elements have mediated the transmission of ESBL genes between bacteria?

(iv) Is gatifloxacin an effective drug for the treatment of *Shigella* infection in children?

In this chapter the key findings from my thesis are summarised, the limitations of the studies identified and some personal thoughts on the areas of research that demand further investigations are discussed.

7.1 Research questions revisited:

Question 1: What is the magnitude of *Shigella* infections as a causative agent of childhood acute diarrhoea in southern Viet Nam? Is the clinical picture specific enough to differentiate acute diarrhoea caused by *Shigella* from that by rotavirus?

The study to answer the above question was described in Chapter Three. A total of 556 children admitted to the hospital because of acute diarrhoeal illness were included in a 12-month study during the period 2000-2001. Standard microbiology methods were used to detect enteropathogenic bacteria and parasites. The negative-stain electro-

microscopy was performed for detecting potential enteropathogenic virus in stools. There was no enteropathogen detected in 210 samples (38% of all samples) and at least one enteropathogen was detected in 62% of samples. Among the enteropathogens, rotavirus was the most important agent (46%) and *Shigella* ranked second with 9% of all cases. This result was similar to that reported from other developing countries. The most frequent genotype of rotavirus strains was G1 [P8] and its variants (20/49) and of interest genotype G2 [P4] ranked second with 16 strains. These data should be considered when choosing the type of rotavirus vaccine (monovalent or polyvalent vaccine) for this community in Viet Nam. Next to these pathogens norovirus and *Campylobacter* spp. were frequently identified.

To answer the second part of the question I compared the clinical features of cases of acute childhood diarrhoea in which a single enteropathogen were isolated in admission stools, either rotavirus or *Shigella* spp.. There were 234 cases with rotavirus and 99 cases with *Shigella*. Rotavirus caused diarrhoea in a younger age group (peaked in 7-12 months old group) than that of *Shigella* (peaked in 13-24 months old group). Shigellosis in children less than 6-months was very rare but 8% of rotavirus diarrhoea cases were in this very young group. A typical case of rotavirus diarrhoea is a child 7-12 months old, admitted to hospital with the triad of "fever-vomiting-watery diarrhoea", the blood white cell count is within normal limits and with no white cells in the stool. A typical shigellosis cases presented with "fever-mucoid diarrhoea-abdominal pain" with leukocytosis and the presence of white blood cells in stool. In practice of course there is significant overlap in the clinical and laboratory signs of these two diseases. Data from this study showed that 46% of children with shigellosis passed only watery stool and not mucoid stools. In addition 32% of rotavirus infection cases had white blood cells in their stool and 10% of all rotavirus diarrhoea cases had red blood cells in their stools.

These findings underscore the difficulty in making the clinical diagnosis of cases presented with acute diarrhoea in children. More studies using simple clinical and laboratory tests would be useful to help clinicians answer this issue. It has major implications for which microbiological tests to order in a financially constrained environment and what treatment to offer.

Question 2: Are there changes in epidemiology, antibiotic susceptibility and clinical aspects of childhood shigellosis in southern Viet Nam in the last 15 years?

This question was answered in Chapter Four. I had a chance to look at a collection of *Shigella* spp. strains isolated from hospitalised children over 3 time periods: 1995-1996, 2000-2002 and 2006-2009 with clinical data recorded. A prominent change in dominant *Shigella* species was seen: *S.flexneri* was the dominant species in 1995-1996 (~70% of all strains) while *S.sonnei* was the most frequent in 2006-2009 (~70%), in the transitional period 2000-2002 the proportion of *S.flexneri* and *S.sonnei* was mixed. The second important change in the 15 year time period was the sharp increase of *Shigella* strains which were resistant to nalidixic acid from ~7% in 1995-1996 to ~70% in 2006-2009. The third change was the clinical features of in-hospital shigellosis cases suggested that the clinical presentation had become more severe; the body temperature and the blood white cell counts were higher and the hospital stay was longer than seen previously. However there was no fatality in the 3 periods. Lastly of note was the identification and rapid development of ESBL-producing *S. sonnei* strains as the dominant species. The details of these strains were described in Chapter Five.

Question 3: What is the molecular characteristic of ESBL genes in *Shigella* population circulating in southern Viet Nam? What transferable genetic elements mediated the transmission of these ESBL genes?

The occurrence of ESBL-producing *Shigella* strains in southern Viet Nam was discovered by chance. In HTD, ceftriaxone has been used as an alternative treatment for cases of acute bacterial diarrhoea which did not respond to oral antibiotic treatment or in patients in whom the clinical state was severe enough to require intravenous antibiotic treatment. As a result of this practice a ceftriaxone disc was placed on the susceptibility test plate for all *Shigella* strains from the year 2000. The first ceftriaxone-resistant *S.sonnei* was detected in May 2007 and further strains were identified at approximately one strain per month. I subsequently checked the susceptibility results of all strains in the period 1995-1996 and 2000-2002 and found only one *S.sonnei* strain isolated from a child in February 2001 which had a high MIC to ceftriaxone. This was the first reported ceftriaxone-resistant *Shigella* strain detected in southern Viet Nam. Approximately 60% of *Shigella* strains were ceftriaxone-resistant in the last 6 months of the the study (Oct. 2006- Mar. 2009. The combination disc test and double disc synergy test were used to confirm the ESBL production of these strains. The test was positive in 35/36 strains. The conjugation experiment was carried out to confirm the plasmid-transferability of *bla*ESBL genes. The conjugation experiment was successful in 35/36 strains. The plasmid extraction was performed using a modified Kado & Liu procedure. In the majority of cases there was only 1 plasmid transferred to the laboratory plasmid-less *E.coli* J53 AzR along with the ceftriaxone resistance. Among 35 strains the ESBL-producing genes were transferred by a plasmid of ~100kbp in 32 strains. In the other 3 strains the transferable plasmid was ~70kbp in size. The bacteria genomic DNA was subjected to PCR amplification to identify the molecular nature of the ESBL genes.

Firstly primers were selected to be specific for *bla*TEM, *bla*SHV and *bla*CTX-M classes of ESBL genes. Then primers specific for three major CTX clusters, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}2}$ were used to characterise the molecular nature of ESBL genes. All ESBL-positive strains (34 *S. sonnei* and 1 *S.flexneri*) strains produced CTX-M primer-specific amplicons.

Sequence analysis showed that there were two different types of *bla*CTX-M present in the *S. sonnei* population, *bla*CTX-M-24 (n=3, 8 %) and *bla*CTX-M-15 (n=32, 92 %). The only *S.flexneri* strain (EG0471) possessed *bla*CTX-M-15 genes. The *bla*CTX-M-24 was carried by the ~70kbp plasmid, while the *bla*CTX-M-15 gene was carried on the ~100kbp plasmid. The 3 *S.sonnei* strains containing the *bla*CTX-M-24 genes were isolated in February 2001, May 2007 and January 2008 consecutively. The *bla*CTX-M-15 gene occurred in June 2007 and from February 2008 has become the sole *bla*CTX-M gene in *Shigella* strains circulating in southern Viet Nam until the end of the study in March 2009.

Based on the scatter spatial distribution and variable plasmid profiles of ESBL-producing strains, the occurrence of ESBL-producing *Shigella* strains was unlikely to be the result of an outbreak of a single ceftriaxone resistant *Shigella* clone. This is the first time the *bla*CTX-M-15 was detected in Viet Nam. The presence of a *bla*CTX-M-15 carrying transferable plasmid in the *Shigellae* circulating in southern Viet Nam is of particular interest because this *bla*ESBL gene has been detected recently in all continents and lead to difficulties in the treatment of community acquired as well as health care associated infections caused by Enterobacteriaceae globally.

Question 4: Was gatifloxacin an effective treatment for *Shigella* infection in children?

The randomised controlled trial described in Chapter Six attempted to answer this question (registered at International Standard Randomised Controlled Trials Registry, ID number ISRCTN55945881). Five hundred patients age 3 months to 14 years were consented by their parents/care-givers to participate in the study. Six of them were withdrawn from the study soon after allocation before taking the study drug because the parents/care-givers changed their mind and decided to transfer them to other hospital. Among 494 children still in the study 245 were in the ciprofloxacin arm, 249 in gatifloxacin arm. After 5 days treatment in hospital, a total of 433 patients came back for follow-up (216 in ciprofloxacin arm and 217 in gatifloxacin arm). In this population, stool culture positive for *Shigella* spp. were 35 in ciprofloxacin arm and 47 in gatifloxacin arm. The primary outcome in this study was the failure rate (presence of diarrhoea or fever or *Shigella* in stool after 5 days from the start of drug treatment; or was relapse or had *Shigella* in stool culture on follow-up). In the intention-to-treat analysis, the failure rate was not different between the two treatment groups (7.4% in ciprofloxacin group versus 6.9% in gatifloxacin group, p=0.9). Time to become afebrile (median time were 24 hours in ciprofloxacin group versus 24 hours in gatifloxacin group, p=0.5) and time to first normal stool (median time were 67 hours in ciprofloxacin group versus 64 hours in gatifloxacin group, p=0.7) were also similar between the two groups.

In the per protocol analysis of the subset of children with *Shigella* cultured from the admission stool, the failure rate (8.5% in both treatment groups), fever clearance time and diarrhoea clearance time were not significant different in both treatment groups. The duration of faecal excretion of *Shigella* following antimicrobial treatment was a bit

longer in ciprofloxacin group (means 1.2 day in ciprofloxacin group versus 1.05 days in gatifloxacin group), but it was not significant statistically (p=0.11, log rank test) nor of clinical relevance. The dysglycaemic state seen in the elderly treated with gatifloxacin was not seen in our 3-day treatment course of gatifloxacin The results of this randomised controlled trial lead to the conclusion that gatifloxacin was comparable to ciprofloxacin for the treatment of acute bacillary dysentery in children, including those caused by *Shigella* spp.

7.2 Limitations of the studies and future directions:

There were limitations which may lead to bias need to be considered in the interpretation of studies included in this thesis.

The main limitation was the hospital-based design of the studies. This will inevitably miss mild (self treated or a self limiting illness) or the most severe patients who may have died before reaching the hospital for treatment. The failure rate in the in-hospital treatment may not reflect that of community health station where the diagnosis and management facilities may not as good as hospital-based treatment.

This thesis has described paediatric patients only (people under 15 years old who are defined as children in Viet Nam). That limited the generalisability of the conclusions to older populations in whom there is a second peak of *Shigella* infection in those older than 40 years of age (von Seidlein et al. 2006).

The diarrhoea aetiology study described in Chapter Three had limitations including the lack of *E.coli* identification, and of an appropriate control group. These lacking data may over-emphasise the magnitude of *Shigella* as well as other enteropathogens

infection. The clonality of ESBL-producing *Shigella* strains was not studied in sufficient detail and the molecular nature of *bla*ESBL-carrying plasmids was not described.

More works need to be done in the future to expand our understanding about *Shigella* infection in children. I would suggest that these include:

1. A comprehensive study on aetiology of diarrhoeal diseases in children using classic clinical microbiology techniques plus multiplex-PCR technique. Such a study will not only help to monitor the change of the magnitude of rotavirus, norovirus, *Shigella*, *Salmonella*, *Campylobacter* spp. as the cause of acute diarrhoea but also help to clarify the role of diarrhoeagenic *E.coli* in Vietnamese children. This study has already been started in my hospital and 2 children hospitals in Ho Chi Minh City. The control group included healthy children in baby day care centre and kindergarten.
2. The sero-group distribution and susceptibility of *Shigella* strains in different part of Viet Nam (Ha Noi in the northern part, Hue City and Nha Trang City in the central part, and Ho Chi Minh City of the southern part of Viet Nam) should be monitored.
3. The molecular nature of the plasmids carrying the *bla*CTX-M genes should be characterised. The ~70kbp plasmid which carried the *bla*CTX-M-24 has been characterised, the ~100kbp plasmid are being studied (Nhu et al. 2010). A study on the ESBL-producing *E.coli* and *K.pneumoniae* in community-acquired and hospital-acquired infection may define the magnitude of the *bla*CTX-M-15 and *bla*CTX-M-24 genes in southern Viet Nam.
4. Either a newer antimicrobial drug or a combination of an antimicrobial and an anti-diarrhoeal product may be compared with the current standard treatment in

a randomised controlled study in order to decide which treatment is the best for childhood shigellosis.

5. Lastly is the development of a simple, inexpensive, and reliable predictive score or algorithm which based on simple and easy available clinical and laboratory data that would assist clinicians in identifying patients with acute diarrhoea who to be treated with antibiotics and who not to.

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Appendices

Appendix A.

Publications Arising From This Thesis

Vinh H, Baker S, Campbell J, Hoang NV, Loan HT, Chinh MT, Anh VT, Diep TS, Phuong le T, Schultsz C, Farrar J. (2009), '**Rapid emergence of third generation cephalosporin resistant *Shigella* spp. in southern Viet Nam**', *J Med Microbiol.*, 58 (Pt2), 281-3.

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Rapid emergence of third generation cephalosporin resistant *Shigella* spp. in Southern Vietnam

Diarrhoeal disease caused by third generation cephalosporin resistant *Shigella* spp. has been described in the past, but it remains a relatively infrequent disease. Here, we report 11 cases of childhood shigellosis caused by ceftriaxone-resistant organisms isolated in Southern Vietnam between May 2007 and January 2008. We predict that the emergence of such strains may become more frequent and will hamper effective treatment. Improved microbiological surveillance and clinical investigations are required for an accurate assessment of the problem.

There are an estimated 165 million shigellosis episodes annually worldwide, of which 64 % of the patient burden is in children under the age of 5 years (Kotloff *et al.*, 1999). Transmission of *Shigella* follows the faecal–oral route; hence, *Shigella* infections are commonly associated with poor sanitation and limited access to clean water. Antimicrobial therapy of *Shigella* infections hastens the clinical recovery, prevents complications and stops the dissemination of the bacteria back into the community. The emergence of multiple-drug resistant (MDR) strains of *Shigella* spp. over the last two decades clearly highlights the problem of MDR pathogenic enteric bacteria and makes the selection of treatment for shigellosis more problematical. The World Health Organization (WHO) currently recommends ciprofloxacin (or other fluoroquinolones) as the drug of choice for the therapy of *Shigella* infections in both adults and children. In addition, ceftriaxone, pivmecillinam (amdinocillin pivoxil) and azithromycin are considered as alternative drugs suitable for *Shigella* treatment (WHO, 2005). However, similar to other pathogenic enteric bacteria like *Salmonella* (Chau *et al.*, 2007), strains of *Shigella* spp. that are resistant to ciprofloxacin have been described in Asia (Von Seidlein *et al.*, 2006). Additionally, strains of *Shigella* spp. that produce extended spectrum β-lactamases (ESBL), conferring resistance to third generation cephalosporins have also been reported (Fortineau *et al.*, 2001).

In Vietnam, MDR strains of *Shigella* spp. have been reported (Isenbarger *et al.*, 2002; Vinh *et al.*, 2000) but shigellosis caused by *Shigella* spp. that are resistant to third generation cephalosporins was, previously, unknown. During the course of a clinical shigellosis treatment trial conducted in Southern Vietnam from June 2006, we have identified several children infected with ceftriaxone-resistant *Shigella*. Here, we report the clinical, epidemiological and microbiological aspects of shigellosis in these patients.

The study was carried out at two locations in Southern Vietnam between June 2006 and January 2008. These locations were the Hospital for Tropical Diseases, Ho Chi Minh City, and Dong Thap Provincial Hospital, Dong Thap Province, in the Mekong Delta, approximately 180 km from Ho Chi Minh City. The criteria for enrolment in the clinical trial was: the patients had to be children under 15 years of age, of either sex, who were hospitalized because of acute dysentery (this was defined as passing bloody diarrhoea or mucoid stools with additional abdominal pain or tenesmus). Only those patients with written consent, given by parents or a guardian, were eligible to take part in the study. The clinical details were recorded in a study form.

Stool samples were taken from the patients on arrival in hospital, prior to the first administration of antimicrobials, then daily for the next 3 days, and additionally on a follow-up visit 1 week after being discharged from hospital. The stool samples were cultured by standard procedures in order to isolate *Shigella* spp. Antibiotic susceptibility testing was carried out using the disc diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The MIC was measured using Etest strips (AB Biodisk). The susceptibility of *Shigella* strains to ceftriaxone was used to ascertain strains that could generate ESBLs. Those strains that were identified as resistant to ceftriaxone using the disc diffusion susceptibility test were further subjected to the 'combination disc method' to confirm ESBL production. The combination disc method utilized discs containing cefotaxime (30 μg) alone, ceftazidime (30 μg) alone, and either antimicrobial combined with clavulanic acid (10 μg). All antimicrobial testing was performed on Mueller–Hinton agar according to CLSI guidelines (CLSI, 2007).

The clinical study was initiated in June 2006; in the period from June 2006 to January 2008 we isolated 72 *Shigella* strains in total, of which 11 (15.3 %), were ceftriaxone resistant. These strains were isolated on admission from the stools of 11 children (7 boys, 4 girls) aged between 17 and 48 months. Eight of the patients came from Ho Chi Minh City, one came from Long An (near Ho Chi Minh City) and two from Dong Thap Province in the Mekong delta. The first ESBL-producing ceftriaxone-resistant *Shigella sonnei* strain was isolated from a faecal sample taken from a child from Ho Chi Minh City, who had severe dysentery, in May 2007. We then identified new ceftriaxone-resistant strains at a rate of one per month, until January 2008, when we isolated three strains. *S. sonnei* accounted for the majority of the ceftriaxone-resistant strains isolated (10/11). All of the *S. sonnei* were confirmed to produce ESBL due to an increase of ⩾5 mm in zone diameter around cefotaxime/clavulanic acid disc compared to the zone around the cefotaxime disc, using the combination disc test. *Shigella flexneri* accounted for the remaining ceftriaxone-resistant strain (1/11). The *S. flexneri* isolate was found not to be ESBL producing as it only exhibited limited zone clearance with the combination disc test. This observation was corroborated by the lack of a

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characteristic bell-shaped zone of clearance between the two discs when the strain was grown on plates containing ceftriaxone and amoxicillin/clavulanic acid discs. All patients returned 1 week after discharge for a follow-up examination and were in good health. Faecal samples from 10 children were taken on the follow-up visit, all tested culture negative for *Shigella*.

Additionally, we retrospectively analysed a collection of 114 *Shigella* strains isolated between 2000 and 2002 for ceftriaxone resistance. We identified one strain of *S. sonnei* that displayed ESBL-mediated ceftriaxone resistance. This strain was isolated from a 10-month-old boy treated for acute watery diarrhoea in the Hospital for Tropical Diseases in February 2001. This, potentially, is the first recorded strain of third generation cephalosporin resistant *S. sonnei* in Vietnam.

Antimicrobial resistance is a well described phenomenon in the genus *Shigella*, but diarrhoeal disease caused by third generation cephalosporin resistant *Shigella* spp. is still uncommon. The first occurrence of third generation cephalosporin resistant *S. flexneri* was from the stool sample of a 16-month-old Algerian child hospitalized in Paris, France, in 1995; the microbiological details of this strain were published in 2001 (Fortineau *et al.*, 2001). Since that time many strains of *Shigella* spp. harbouring different types of ESBLs have been reported in developed and developing countries in Asia (Cheung *et al.*, 2005; Chuang *et al.*, 2006; Kim *et al.*, 2004; Pai *et al.*, 2001; Rahman *et al.*, 2004; Xiong *et al.*, 2007). However, to the best of our knowledge this is the first report of ESBL-producing *Shigella* spp. in Vietnam.

The clinical patterns of these cases were typical of dysentery caused by a *Shigella* infection. Despite 9/10 *S. sonnei* strains in this series demonstrating co-resistance to nalidixic acid, all patients responded to oral fluoroquinolones (ciprofloxacin, gatifloxacin or norfloxacin). None of the patients had any clinical signs or symptoms of *Shigella* infection within 3 days of the onset of treatment. Additionally, all faecal samples from the patients taken after treatment and on follow-up visits were negative for *Shigella*. These data show that resistance to third generation cephalosporins did not prolong the shedding of *Shigella* provided the appropriate antimicrobials were administered. The molecular characteristics of the ESBL-encoding genes harboured by the *S. sonnei* strains in these isolates are currently being investigated and will be reported elsewhere. However, preliminary data suggest that ceftriaxone resistance in these strains is mediated by a plasmid-borne CTX-M class ESBL-encoding gene. The molecular mechanism of the ESBL negative, ceftriaxone-resistant *S. flexneri* is also under investigation.

Our observations demonstrate that emergence of ceftriaxone-resistant shigellae in Southern Vietnam has not occurred by chance and the ability of these strains to produce ESBLs appears to be evolutionarily advantageous. The simple availability of antibiotics in Vietnam predicts that drug resistance in the community is rife. It is also known that ESBL-encoding genes can be carried on plasmids by non-pathogenic, commensal organisms, suggesting that spread of resistance genes to pathogenic bacteria may occur at a significant rate. The genetic transfer of drug resistance genes may not be of immediate concern for the treating clinicians, but will pose a potential problem in the future. We predict that the isolation of ceftriaxone-resistant shigellae and other *Enterobacteriaceae* will become increasingly common in Vietnam in the coming years. Currently, we have effective optional therapies; however, ceftriaxone is used in the hospital here in Vietnam as an alternative when the patient does not respond to fluoroquinolone treatment. A marked increase in resistance to nalidixic acid and newer fluoroquinolones, combined with ESBL-mediated third generation cephalosporin resistance would leave limited treatment options for those with life-threatening bacterial infections.

The majority of studies published in recent years on ESBL-producing *Shigella* have described mainly the transfer mechanisms and the molecular types of the β-lactamases produced. In order to have a comprehensive perspective on the clinical manifestations and outcomes of the illness caused by ESBL-producing shigellae more patient-oriented case series reports are required. Moreover, ESBL-producing strains of *Shigella* may be overlooked if screening for ESBLs is not routine in diagnostic microbiology laboratories. Therefore, more vigilance is required in detecting ESBL-producing *Shigella* strains and a worldwide rapid reporting system will allow the monitoring of the dissemination of such strains and whether diarrhoeal diseases caused by third generation cephalosporin resistant *Shigella* is an emerging trend worldwide.

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A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation

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Abstract

Background: Shigellosis remains considerable public health problem in some developing countries. The nature of *Shigellae* suggests that they are highly adaptable when placed under selective pressure in a human population. This is demonstrated by variation and fluctuations in serotypes and antimicrobial resistance profile of organisms circulating in differing setting in endemic locations. Antimicrobial resistance in the genus *Shigella* is a constant threat, with reports of organisms in Asia being resistant to multiple antimicrobials and new generation therapies.

Methods: Here we compare microbiological, clinical and epidemiological data from patients with shigellosis over three different periods in southern Vietnam spanning14 years.

Results: Our data demonstrates a shift in dominant infecting species (*S. flexneri* to *S. sonnei*) and resistance profile of the organisms circulating in southern Vietnam. We find that there was no

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significant variation in the syndromes associated with either *S. sonnei* or *S. flexneri*, yet the clinical features of the disease are more severe in later observations.

Conclusions: Our findings show a change in clinical presentation of shigellosis in this setting, as the disease may be now more pronounced, this is concurrent with a change in antimicrobial resistance profile. These data highlight the socio-economic development of southern Vietnam and should guide future vaccine development and deployment strategies.

Trial Registration: Current Controlled Trials ISRCTN55945881

Background

Shigellosis is an ongoing global public health problem. Due to the fecal-oral transmission route of the organisms, the overwhelming burden of shigellosis is found in resource-poor settings with inadequate sanitation [1,2]. With an estimated number of episodes exceeding 90 million per annum in Asia alone, shigellosis represents a significant proportion of the total number of bacterial gastrointestinal infections worldwide [3]. Unlike other related bacteria which can cause a particular disease syndrome in specific locations (e.g. *Salmonella* Typhi) [4] it is a disease which "bridges the gap" between industrialized and developing countries. A report from the National Center for Infectious Diseases in the United States of America found the incidence of shigellosis to be 7.6 cases per 100,000 persons in 1993 [5].

The *Shigellae* are gram negative, non-motile bacilli of the larger bacterial family *Enterobacteriaceae*. *S. flexneri* are regarded to be the most abundant globally and are known to predominate in developing countries [3]. *S. sonnei* is the most commonly isolated species in developed countries, representing over 70% of the total isolates in the United States of America and Israel [5,6]. The disease syndrome associated with these organisms includes fever, headache, malaise, anorexia and occasionally vomiting, followed by excretion of profuse watery diarrhea proceeding bloody and/or mucoid diarrhea [7]. All the members of the genus *Shigella* are pathogens restricted to infecting humans and exert their effects on the gastrointestinal mucosa via the production of a multitude of virulence factors, including enterotoxins and effector proteins [8,9].

In a recent publication by von Seidlein *et al.* the authors found a change in dominant *Shigella* species related to the location in Asia (*S. sonnei* predominated in Thailand, *S. flexneri* was dominant in other Asian countries) and fluctuations in *S. flexneri* serotypes in the same location over the duration of the study [10]. The authors concluded that "*Shigella* appears to be more ubiquitous in Asian impoverished populations than previously thought and antibiotic-resistant strains of different species and serotypes have emerged" [10]. Such findings have important implications for treatment and prevention strategies of shigellosis.

On a larger scale, the *Shigellae* are a group of dynamic organisms, in which the overall bacterial population appears to be adaptable with a high recombination rate and a large amount of imported genetic material in the genome architecture [11]. These organisms are highly promiscuous regarding their ability to accept horizontally transferred genetic material. Like *E. coli* the *Shigellae* are successful recipients of numerous plasmids, which may be transferred from other enteric organisms in the gastrointestinal tract [12]. This is supported by evolutionary evidence that the *Shigellae* are a branch of the *E. coli* family, having developed a pathogenic phenotype by the acquisition of a virulence plasmid and other gene loci and genomic compensatory mechanisms [13,14].

It is known that the circulating species and serotypes may be considered a marker of the socio-economic climate in an individual setting [15]. It is clear that Vietnam has undergone rapid economic development since the early 1990's. To understand the nature of bacterial and clinical nature of shigellosis in southern Vietnam we have amassed and compared microbiological and epidemiological data on childhood shigellosis over three periods spanning 14 years, from 1995 to 2008.

Methods

Study sites and settings

The primary location was the pediatric gastrointestinal infections ward at the hospital for tropical diseases (HTD) in Ho Chi Minh City in southern Vietnam. The HTD is a 500 bed tertiary referral hospital treating patients from the surrounding provinces and from the districts within Ho Chi Minh City. The secondary location was Dong Thap provincial (DTP) hospital in Dong Thap province, approximately 120 km from the HTD in Ho Chi Minh City.

Studies contributing data for analysis

Data from three independent studies were combined and compared. All patients enrolled in the three studies were treated as inpatients and there were no fatalities. The initial period (referred to as period A from here onwards) was a study performed at the pediatric ward at HTD from January 1995 to August 1996. The enrollment and clinical observations for this randomized controlled trial are as

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described previously [16]. Briefly, children that were aged >3 months and <14 years, admitted to HTD with fever and bloody diarrhea (bloody diarrhea defined >3 loose stools with obvious blood) for <5 days were entered into the study provided that their parents or guardian gave fully informed consent. Additional strains for microbiological assessment only (nine in total) were collected for comparison within the same period of the study duration from DTP. Overall 80 strains were isolated from enrolled children over this period; clinical data was available for analysis on 63 patients with culture confirmed shigellosis.

The secondary period (referred to as period B from here onwards) was conducted only at the HTD, between March 2000 and December 2002. This period was a clinical and microbiological investigation of the etiology of diarrhea in the pediatric population admitted to the HTD in Ho Chi Minh City. Whilst the treatment criteria for this descriptive study were not controlled (> 90% of patients received treatment with fluroquinolones (norfloxacin or ofloxacin)), the remainder of the criteria for admission to the study were comparable, children were eligible for enrollment to the study if consent was given and they were aged less than 14 years. The obvious variation in the enrollment for this study was that children were enrolled on the basis of having any diarrheal syndrome, rather than specifically targeting those with dysentery and suspected shigellosis. One hundred and fourteen *Shigella* isolates were recovered during this period; clinical data was available for analysis on 113 patients.

The final period (referred to as period C from here onwards) in which data was combined was a trial conducted at the HTD and at DTP between June 2006 and December 2008. This was a randomized controlled trial for comparing the treatment of dysentery with ciprofloxacin and gatifloxacin in Vietnamese children (controlled trials number ISRCTN55945881) (HV and SB, *unpublished data*). The inclusion criteria were as period A. One hundred and three isolates were collected during this period and clinical data on all admitted children was available for analysis.

All three studies were approved ethical assessment by the Scientific and Ethical Committee of the hospital for tropical diseases and Oxford University tropical ethics committee (OXTREC) number 010-06 (2006).

Microbiological methods

From all studies, stool samples were collected from patients and cultured directly on the day of sampling. Initial isolation was as below, however, all bacterial isolates were stored in glycerol at -80°C and re-serotyped and checked for consistency with the original antimicrobial susceptibility profile for the purposes of this work. All specimens were processed and checked in the microbiology laboratory of the HTD.

Samples were cultured overnight in selenite F broth (Oxoid, Basingstoke, UK) and onto MacConkey and XLD agar (Oxoid) at 37°C. Colonies suggestive of *Salmonella* or *Shigella* (non-lactose fermenting) were sub-cultured on to nutrient agar and were identified using a 'short set' of sugar fermentation reactions (Kliger iron agar, urea agar, citrate agar, SIM motility-indole media (Oxoid)). After incubation for 18 - 24 h at 37°C, the test media were read for characteristic *Shigella* reactions and API 20E test strips of biochemical reactions (Biomerieux, Paris, France) were used to confirm the identity of *Shigella spp*. Serologic identification was performed by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed by testing with available monovalent antisera for specific serotype identification as per the manufacturers recommendations (Denka Seiken, Japan).

Antimicrobial susceptibility testing of all *Shigella* isolates against ampicillin (AMP), chloramphenicol (CHL), trimethoprim- sulfamethoxazole (SXT), tetracycline (TET), nalidixic acid (NAL), ofloxacin (OFX) and ceftriaxone (CRO) was performed by disk diffusion following standardized Clinical and Laboratory Standards Institute methods [17]. The minimum inhibitory concentrations (MICs) were additionally calculated for all isolates by E-test, according to manufacturer's recommendations (AB Biodisk, Solna, Sweden) and were compared to control strain *E. coli* ATCC 25922 and an in house fully sensitive *E. coli* control.

Clinical observations and statistical analysis

Clinical data was recorded on specialized clinical report forms for all three studies by clinical staff involved in the studies. The data collected was related to basic details of the patient, age (months), sex, location of residence and weight (kg). A history from all patients was also recorded, including; duration of illness prior to admission to hospital (days), fever (defined as a prolonged temperature > 37.5°C), abdominal discomfort, vomiting, watery diarrhea (defined as three or more loose bowel movements during a 24-h period), bloody or mucoid diarrhea (defined as >3 loose stools with obvious blood or mucus), estimated number of episodes of diarrhea before attending hospital, convulsions believed to be related to fever and/or infection and if there was any known pretreatment with antimicrobials. A white blood cell count was performed on all patients and stools were examined by microscopy (HPF (× 400)) to identify white and red blood cells, these observations were scored on scale from zero to three, scale 0 = 0 cells/HPF, scale 1 = 1 to 10 cells/HPF, scale 2 = 11 to 20 cells/HPF and scale 3 = >20 cells/HPF. Time in hours (from initial investigation in hospital) to

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the ceasing of bloody/mucoid and watery diarrhea was recorded. Duration of hospital stay was recorded in days post admission; patients were only discharged when all clinical symptoms had resolved completely.

Data were double entered into Microsoft Excel for storage and manipulation. Mapping data was entered, analyzed and draw in MapInfo software (Pitney Bowes MapInfo Corporation, USA). For intergroup comparisons, Chi-square tests were used for comparison of categorical variables. For the analysis of continuous variables, Wilcoxon rank sum, and Kruskal-Wallis test were used for non-normally distributed data. A *p*-value of less than 0.05 (two-tailed) was considered significant. Statistical analysis was performed in R http://www.r-project.org/

Results

Epidemiological findings

Over the duration of the three periods spanning 14 years, 228 *Shigellae* were isolated from children living within 13 districts that constitute Ho Chi Minh City (Figure 1). Whilst the distribution of the location of the residences of these patients is biased by referral patterns and people attending the local hospital (HTD is one of several hospi-

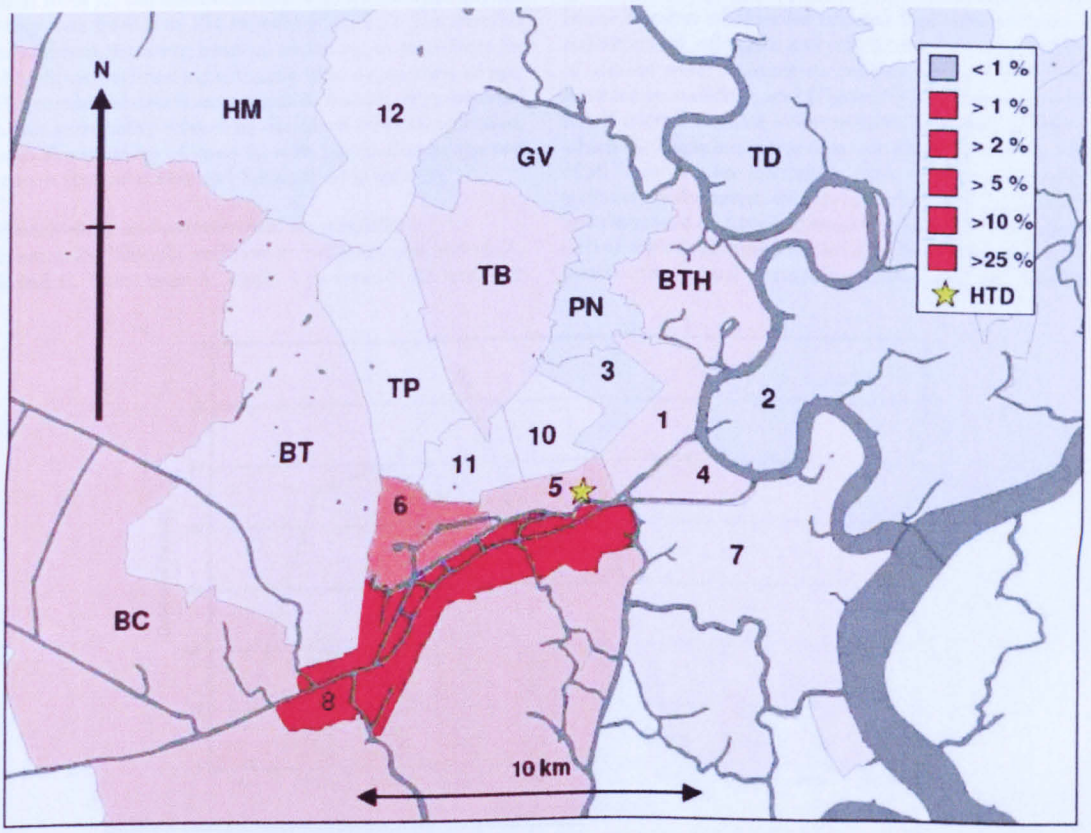

Figure 1
The distribution of the residences of cases of childhood shigellosis admitted to the hospital for tropical diseases in Ho Chi Minh City. Over the three periods we were able to positively identify infecting *Shigella* serotypes in the stools of 297 children with symptoms consistent with shigellosis. Of these patients, 228 (76.8%) children lived in the 23 districts that constitute Ho Chi Minh City. This figure represents the distribution of the homes of patients reporting to the hospital for tropical diseases with *Shigella* isolated from stool, by district. The percentage of cases reporting from each ward is distinguished by gradual shading. The location of the hospital for tropical diseases is shown by a yellow star. Large waterways (rivers and canals) are shown in dark grey shading.

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tals in the City where children may be treated for gastrointestinal infections), the majority of children attending HTD with culture confirmed shigellosis came from the three districts within the locality of the hospital (districts 5, 6 and 8), which constitutes a total population of over 800,000 people. In total, the majority of the patients resided in district eight (n = 88) within approximately 6 km of the hospital. There was no significant change in the locality of patients over the three periods, or any relationship between serotype and location of the residence of the patients.

The median age of children with culture confirmed shigellosis from all the combined data was 24 months; the age range was from 3 to 154 months (Figure 2). The number of children requiring hospital treatment as inpatients for shigellosis declined significantly after 36 months of age. The combined data from periods A, B and C demonstrated some seasonality related to the times of peak infection, with the majority of cases (> 60%) occurring in the wet season (between May and September) (Figure 3).

Microbiology and antimicrobial susceptibilities

In total, 297 *Shigella* strains were isolated from periods A, B and C. Three were *S. boydii*, 136 were *S. flexneri*, 149 were *S. sonnei* and nine were untypeable. There was a significant species shift from *S. flexneri* to *S. sonnei* between period A (29% *S. sonnei*) and period C (78% *S. sonnei*) with an approximate 1:1 ratio of *S. flexneri* to *S. sonnei* in the intermediate period (Figure 4). Apart from *S. flexneri* serotype one only being found in period A, there was no evident fluctuations in *S. flexneri* populations between the three periods. The most commonly isolated *S. flexneri* serotype was serotype 2a; representing 43% of all the *S. flexneri* strains (Table 1).

We identified a significant change in the profile of the proportions of organisms demonstrating resistance to seven antimicrobials (Figure 5). There was a sequential increase in the number of *Shigellae* isolated that were resistant to nalidixic acid, ofloxacin and ceftriaxone. In period C, 23% of strains were resistant to ceftriaxone and 68% were resistant to nalidixic acid (Figure 5). There was an additional overall increase in the number of antimicrobials to which the organisms were resistant. During period A, 62% of all *Shigellae* were resistant to three or more of the seven antimicrobials tested, this increased to 87% in period B and decreased to 83% in period C (Figure 5). The proportion of organisms that were resistant to trimethoprim-sulfamethoxazole and tetracycline was unchanged between

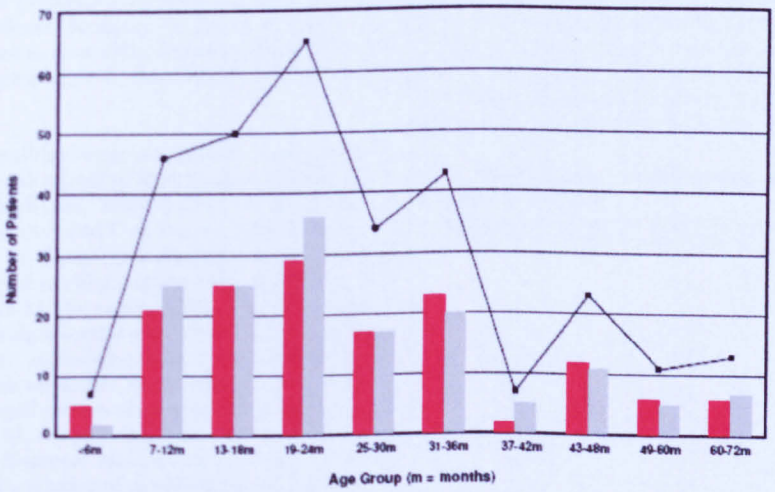

Figure 2
The combined sex and age distribution of childhood shigellosis patients in southern Vietnam. Graph depicts the combined age and sex distribution (female - red, male - grey) of 297 children with shigellosis. The black line with boxes represents the total number of cases per age group specified. The overall age range was from 3 months to 154 months, with a median of 24 months. There was no significant relationship of shigellosis with gender; in total, 152 patients were male (51.2%) and 145 were female (48.8%).

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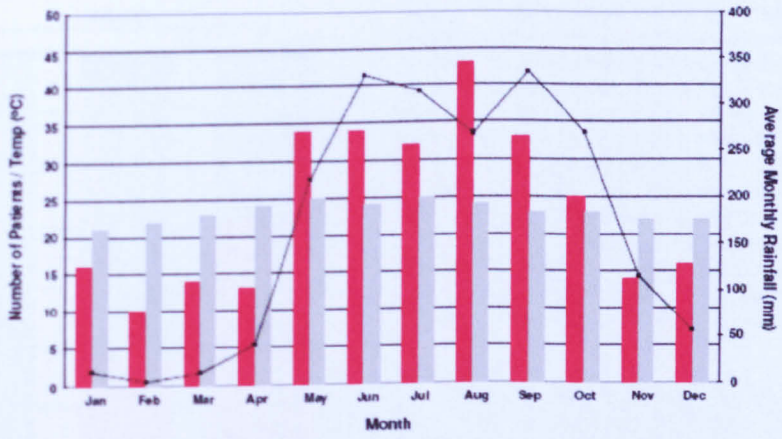

Figure 3
The seasonal distribution of shigellosis in southern Vietnam. southern Vietnam has two distinct seasons, wet and dry. The combined data were averaged by calculating the number of months represented to get an overall number of cases per month. Red bars; total number of cases, grey bars; average monthly temperature and black line with boxes; average monthly rainfall. The seasonal data represents the average rainfall and temperature per month for Ho Chi Minh City.

the three periods (Figure 5). Between period A and C, there were significant decreases in the proportions of organisms resistant to ampicillin, decreasing from 75% to 48%, and chloramphenicol, decreasing from 66% to 30%.

There was a discernible change in sensitivity patterns over time, which was also related to *Shigella* species (Table 2). *S. flexneri* was significantly more likely to be resistant to ampicillin in periods A and C and when combined over all three studies. *S. flexneri* was also significantly more likely to be resistant to chloramphenicol in periods B, C and overall (Table 2). The combined data demonstrated that *S. sonnei* was significantly more likely to be resistant to trimethoprim- sulfamethoxazole and ceftriaxone, despite ceftriaxone resistance not becoming evident till period C. The overall pattern of reversion of sensitivity to ampicillin and chloramphenicol was mainly observed with respect to *S. sonnei* isolates. An increase in the number of organisms resistant to multiple antimicrobials over time was seen in both *Shigella* species. However, between period A and period C, *S. flexneri* was more likely to be resistant to more antimicrobials than *S. sonnei* (Figure 6). Resistance to multiple antimicrobials increased from two to three out of the seven tested from periods A to C for *S. sonnei* and from four to five from the seven antimicrobials tested from periods A to C for *S. flexneri* (Figure 6).

Clinical features associated with shigellosis

Clinical data was combined and analyzed from all three studies; this permitted a comparison of some of the features of the patients with confirmed shigellosis over the three studies. Data were available for analysis from 279 patients; 63 patients from period A, 113 patients from

Table 1: *Shigella flexneri* serotypes isolated in southern Vietnam between 1995 and 2008.

S. flexneri serotype	Number	Percentage (%)
1a	0	0
1b	0	0
1c	4	2.9
2a	59	43.4
2b	8	5.9
3a	13	9.6
3b	2	1.5
3c	16	11.8
4	7	5.1
4a	5	3.7
4b	1	0.7
4x	0	0
5a	0	0
6	13	9.6
x	0	0
y	0	0
Not typed	8	5.9
Total	136	100

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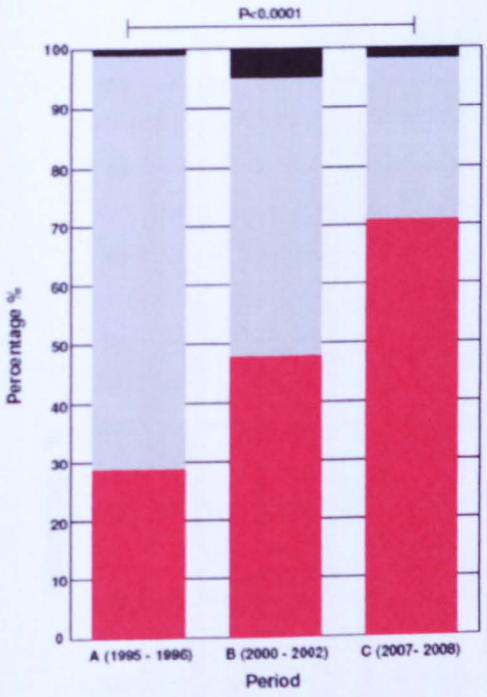

Figure 4
The distribution of *Shigella* species from three childhood shigellosis studies in southern Vietnam over fourteen years. The distribution of *Shigella* species from period A (n = 80), period B (n = 114) and period C (n = 103). The percentage of *S. sonnei* and *S. flexneri* are colored red and grey respectively, other *Shigella* species are colored black. The *p* value was calculated using the chi - squared test.

period B and 103 patients from period C (Table 3). These data demonstrated several changes in disease profile over the three periods. There was a statistically significant increase in age, which corresponded with an increase in weight of the children from period A to period C (Table 3). There was decrease in the number of days of history of the disease symptoms prior to admission to hospital. There was a statistically significant increase in the number of children with watery diarrhea, abdominal pain and febrile convulsions. These clinical features combined suggested a progressively more severe infection syndrome between 1995 and 2008. Additionally, patients in period C, had higher white blood cell counts. Over the 14 year period, patients had a higher density of white cells in their stool and had longer stays in hospital.

The increase in the severity of the disease was concurrent with a change in antimicrobial resistance profiles of the organisms and a change in the dominant *Shigella* species isolated. Therefore, these data suggested a more severe disease pattern may be related to infection with *S. sonnei*. To account for any variation in disease syndrome that may be species specific, the data were analyzed to compare the clinical syndromes related to species. The data presented in Table 4 demonstrates only subtle differences between the syndromes synonymous with the two differing species. *S. flexneri* shows an increase in the number of days of illness prior to admission in hospital, the number of episodes of diarrhea, an increase in the duration of mucoid/bloody diarrhea and the duration of stay in hospital.

Discussion

Our findings demonstrate that the epidemiology of shigellosis infection is similar in southern Vietnam to other locations in Asia. The main burden of infection in children is in those under three years of age [10,15,18,19]. The median age of patients in this investigation was 24 months, this is slightly less than a previous study in Nha Trang, Central Vietnam [10]. A discrepancy in age in the two settings may be related to the epidemiological study being performed with ongoing community surveillance, rather than those admitted to hospital for treatment. We also found a pattern of infection which correlated with the rainy season. The observation that *Shigella* infections generally coincide with the wet season in a tropical setting has been noted before in an urban setting in Jakarta, Indonesia [18]. Transmission of *Shigella* has been associated with wastewater and river water in Vietnam in two independent locations in Vietnam [20,21]. An increase in fecal contamination of the water supply due to increased ground water may account for this pattern as distance to a water source was found to be associated with higher risk of shigellosis in Nha Trang. The majority of patients enrolled in the studies combined here resided in District 8 of Ho Chi Minh City. Although we are unable to draw meaningful conclusions from the residences of these patients owning to referral and catchment areas of the HTD, district 8 represents the area of the city with the greatest density of canal networks and waterways.

In addition to a species shift over time, there was combined effect on antimicrobial resistance; there was a marked increase in resistance to ceftriaxone and nalidixic acid. We have previously reported an alarming increase in ceftriaxone resistant *Shigellae* in southern Vietnam [22]. Whilst nalidixic acid is no longer used therapeutically, resistance increases the MIC to fluoroquinolones, which are recommended for the treatment of *Shigella* infections [23]. Our theory that antimicrobial resistant organisms are under selective pressure in this population is sup-

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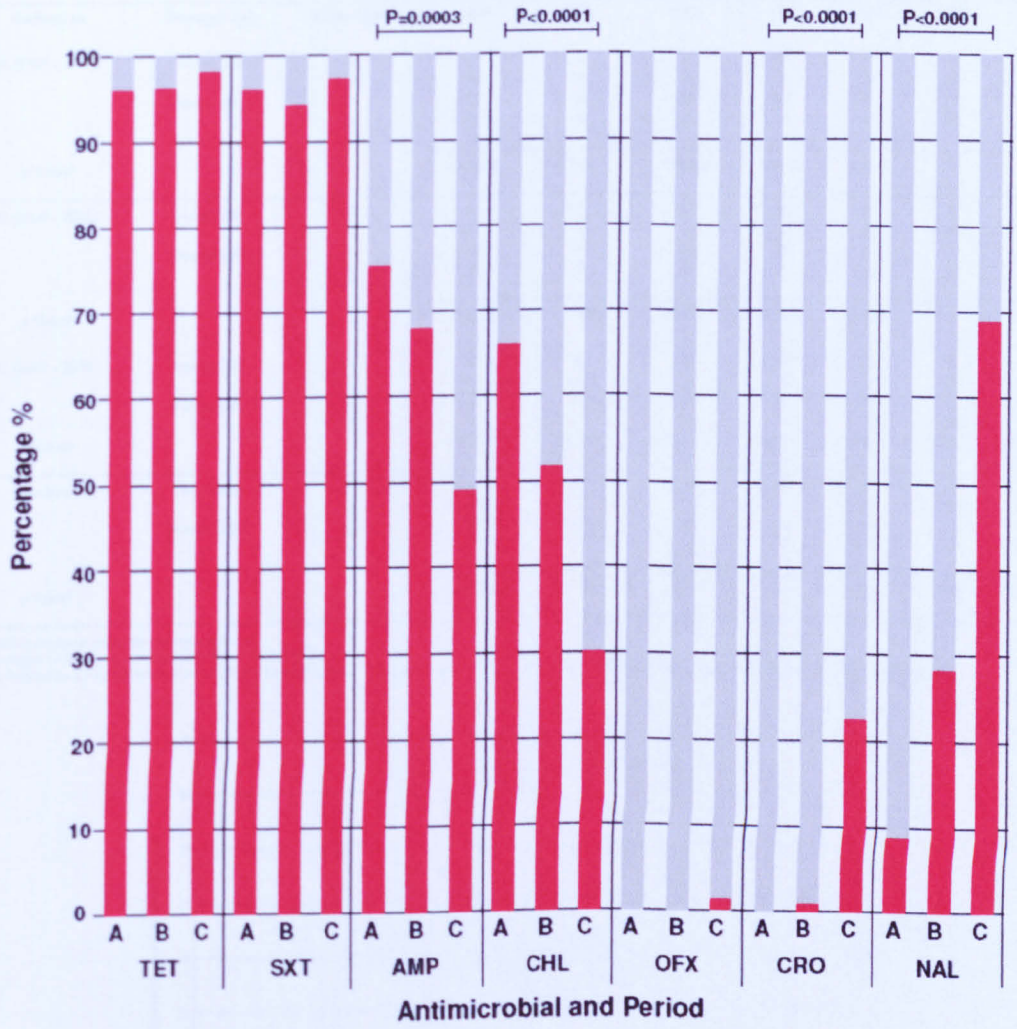

Figure 5
Changing antimicrobial resistance patterns of *Shigella spp*. All organisms were tested for susceptibility to seven antimicrobial agents by the disc diffusion and E-test methods. The antimicrobials tested were as follows, AMP; Ampicillin, CHL; Chloramphenicol, SXT; Trimethoprim- Sulfamethoxazole, TET; Tetracycline, NAL; Nalidixic Acid, OFX; Ofloxacin and CRO; Ceftriaxone. Graph shows the percentage of resistant (red) and sensitive (grey) organisms isolated from periods A, B and C. Statistical significance was calculated using a chi squared test.

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Table 2: Comparison of resistance patterns between *Shigella flexneri* and *Shigella sonnei* isolated in southern Vietnam between 1995 and 2008.

Collection	Serotype (n)	Phenotype[b]	AMP	CHL	SXT	TET	NAL	OFX	CRO
A (1995 - 1996)	*sonnei* (24)	R	11	8	23	23	0	0	0
		S	13	16	1	1	24	24	24
	flexneri (56)	R	54	10	53	53	9	0	0
		S	2	46	3	3	47	56	56
p Value[a]			< 0.0001	0.1287	0.8102	0.8102	0.0371	-	-
B (2000 - 2002)	*sonnei* (54)	R	50	10	53	52	9	0	1
		S	4	44	1	2	45	54	53
	flexneri (50)	R	46	38	44	49	21	0	0
		S	4	12	6	1	29	50	50
p Value[a]			0.9316	< 0.0001	0.0415	0.5577	0.0052	-	0.329
C (2007 - 2008)	*sonnei* (71)	R	17	5	71	69	51	0	12
		S	54	66	0	2	20	71	59
	flexneri (30)	R	25	28	28	30	19	1	1
		S	5	2	2	0	11	29	29
p Value[a]			< 0.0001	< 0.0001	< 0.0001	0.3696	0.4619	0.297	0.076
Combined	*sonnei* (148)	R	78	23	147	144	60	0	13
		S	70	125	1	4	88	148	135
	flexneri (136)	R	125	76	125	132	49	1	1
		S	11	60	11	4	87	135	135
p Value[a]			< 0.0001	< 0.0001	0.001	0.613	0.365	0.478	0.002

[a] p Value calculated using the chi-squared test
[b] Phenotype with respect to Resistant (R) or Sensitive (S)
AMP; Ampicillin, CHL; Chloramphenicol, SXT; Trimethoprim- Sulfamethoxazole, TET; Tetracycline, NAL; Nalidixic Acid, OFX; Ofloxacin, CRO; Ceftriaxone.

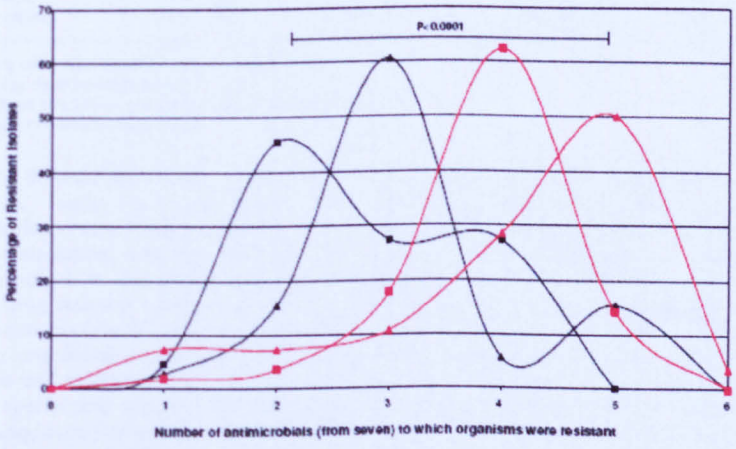

Figure 6
The increasing proportions of antimicrobial resistant *S. sonnei* and *S. flexneri* during a fourteen year transition. The distribution of the proportion of *S. sonnei* and *S. flexneri* isolates that were resistant to one or more of seven antimicrobials tested. *S flexneri* strains (red lines) were significantly more likely to be resistant to more antimicrobials that *S. sonnei* (black lines) over both collections compared. *S. sonnei* and *S. flexneri* were significantly more likely to be resistant to more antimicrobials when period C (2007 - 2008) (lines with triangles) was compared to period A (1995 - 1996) (lines with squares).

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Table 3: Clinical results of *Shigella* infections between 1995 and 2008.

	A (1995 - 1996)	B (2000 - 2002)	C (2007 - 2008)	Combined	p value[a]
Patients	*n* = 63	*n* = 113	*n* = 103	*n* = 279	
Age (months)[d]	23 (17 - 48)	21 (14 - 29)	30 (19 - 42)	24 (16 - 36)	< 0.001
Weight (kg)	10 (9 - 13)	10 (9 - 12)	11.5 (10 - 15)	10.5 (9 - 13)	0.004
Male sex (%)	31 (49)	50 (44)	61 (59)	184 (59%)	0.085
Patient history					
Days	2 (1 - 7)	2 (1 - 9)	1 (1 - 4)	2 (1 - 9)	< 0.001
Fever (%)	62 (98)	104 (92)	100 (97)	266 (95)	0.09
Abdominal pain (%)	33 (52)	41 (36)	79 (76)	153 (54)	< 0.001
Vomiting (%)	24 (38)	64 (56)	51 (49)	139 (50)	0.062
Watery diarrhea (%)	28 (44)	67 (59)	74 (71)	169 (60)	0.002
Bloody/mucoid diarrhea (%)	63 (100)	60 (53)	98 (95)	221 (79)	< 0.001
Diahorreal episodes per day	NA	8 (5-10)	8 (5-10)	8 (5-10)	0.595
Convulsions (%)	4 (6)	7 (6)	20 (19.4)	31 (11)	< 0.001
Known pretreatment (%)	3 (5)	8 (7)	4 (4)	14 (5)	0.543
Clinical details					
Serotype *sonnei* (%)	21/63 (33)	55/113 (49)	71/103 (69)	153/279 (55)	< 0.001
White cell count (× 10^3/mm^3)	10 (8.3 - 15)	10.1 (7.7 - 12.8)	13.1 (10.1 - 17.3)	11.3 (8.7 - 15.4)	< 0.001
Red cells in stool[b]	NA	1	1	1	0.715
White cells in stool[b]	NA	3	3	3	0.02
Mucoid duration (hrs)	31.5 (24 - 53.5)	36 (24 - 54)	28 (18 - 48)	30 (19.5 - 48)	0.113
Diarrhea duration (hrs)	48.5 (29.25 - 87)	48 (24 - 72)	48 (30 - 72)	48 (26.75 - 72)	0.402
Duration of illness					
Hospital stay (days)	3 (1 - 12)	4 (1 -15)	5 (2 - 14)	4 (1 -15)	< 0.001
Disease duration (days)[c]	4 (2 - 15)	6 (3 - 18)	6 (3 - 15)	6 (2 - 18)	< 0.001

[a] p Values calculated using either Chi-square test or the Kruskal-Wallis test
[b] Cells in Stools assessed as described in methods
[c] Disease duration calculated by addition of history of disease and stay in hospital
[d] Interquartile range values in brackets unless stated

ported by a sequential decrease in resistance to older antimicrobial therapies, such as ampicillin and chloramphenicol which are now rarely used in the community to treat gastrointestinal infections. The uncontrolled use of antimicrobials in this setting may fuel the spread of multiple drug resistant organisms. However, due to promiscuous nature of the *Shigellae* it is likely that resistance genes are transferred regularly to and from other enteric bacteria and maintained by selective pressure. The change is species and antimicrobial resistance pattern reflects a change occurring in the *Shigella* population over time in this setting. Locality and time of isolation data suggest that entrance to all studies was sporadic and there was no evidence of transient epidemics.

Currently there are several candidate *Shigella* vaccines in development, of which some have already been tested in initial clinical trials [24-27]. The development and deployment of *Shigella* vaccines may be hindered by the number of different species and serotypes circulating in one setting and in differing locations. For example, *S. flexneri* serotypes are known to fluctuate over time, this has been observed in India, Indonesia, Bangladesh, and Pakistan, [10,28]. Here, we have demonstrated a significant longitudinal transition of species from *S. flexneri* to *S. sonnei*. Vaccine development for shigellosis is challenging as primary infection offers only serotype specific immunity [29]. A study concerning a cohort of Chilean children found infection conferred 76% protective efficacy against re-infection with the same serotype [30]. An option for controlling shigellosis would be the development of a series of single serotype vaccines which could be implemented in individual locations with a known serotype profile. Alternatively, the most cost effective method of control would be the development of a polyvalent vaccine offering cross protection to a number of known dominant

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Table 4: The clinical presentation of *Shigella flexneri* and *Shigella sonnei* infections.

	S. flexneri	*S. sonnei*	*p* value[a]
Patients	*n* = 123	*n* = 147	
Age (months)[d]	25 (12 - 42)	23 (14 - 36)	0.105
Weight (kg)	11 (8.5 - 14)	10 (9.9 -13)	0.558
Male sex (%)	55 (44.7)	83 (56.5)	0.055
Patient history			
Days	2 (2 - 3)	1 (1 - 2)	< 0.001
Fever (%)	117 (95)	141 (96)	0.761
Abdominal pain (%)	64 (52)	84 (57.1)	0.48
Vomiting (%)	60 (48.8)	74 (50.3)	0.78
Watery diarrhea (%)	78 (63.4)	86 (58.5)	0.41
Bloody/mucoid diarrhea (%)	97 (78.9)	117 (80)	0.88
Diarrhea episodes per day	10 (5 - 10)	8 (5 - 10)	0.051
Convulsions (%)	9 (7.3)	21 (14.3)	0.07
Known pretreatment (%)	7 (5.7)	7 (4.8)	0.585
Clinical details			
White cell count (× 10^3/mm^3)	10 (8 - 13.6)	12 (10.5 - 15.5)	0.029
Red cells in stool[b]	1	1	0.056
White cells in stool[b]	3	3	0.173
Mucoid duration (hrs)	36 (24 - 53.5)	25 (18 - 48)	0.054
Diarrhea duration (hrs)	48 (39 - 72)	48 (27 - 72)	0.088
Duration of illness			
Hospital stay (days)	5 (4 - 5)	4 (3 - 5)	0.276
Disease duration (days)[c]	7 (6 - 8)	5 (4 - 7)	0.009

[a] *p* Values calculated using either the Chi-square test or the Kruskal-Wallis test
[b] Cells in Stool assessed as described in methods
[c] Disease duration calculated by addition of history of disease and stay in hospital
[d] Interquartile range values in brackets unless stated

serotypes, this approach may aid in tackling the global burden of shigellosis. The transition of dominant *Shigella* species in southern Vietnam has occurred on a background of economic development and may predict a continuing cycle in other areas under going similar rapid economic changes.

Conclusions

What we are unable to specifically ascertain from this study is the overall incidence and greater epidemiological picture of shigellosis in this setting. On the basis of these data a thorough epidemiological assessment of burden is warranted to calculate the financial and health implications of any potential future routine vaccination against shigellosis that may become available. However, here we have shown a significant transition in *Shigella* species and antimicrobial resistance dominance overtime and a concurrent change in the clinical disease presentation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NTKN, TVTN, PHD, JIC, NVMH, TTTN, PVM, CTT, PVBB and TSD performed the microbiological culturing, sensitivity testing and serotyping. MFB, PVTM provided critical analysis related to this work. HV, CP, LTP, MNL, BLM, VTCA, PVBB, HTL, MTC, NTHT, NVVC and JF conducted the clinical work providing the data for analysis. HV, JF, MFB and SB conceived the study, analyzed and interpreted the data and prepared the manuscript. All authors have read and approved the final version of this manuscript.

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PLoS NEGLECTED TROPICAL DISEASES

The Sudden Dominance of *bla*$_{CTX-M}$ Harbouring Plasmids in *Shigella* spp. Circulating in Southern Vietnam

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Abstract

Background: Plasmid mediated antimicrobial resistance in the *Enterobacteriaceae* is a global problem. The rise of CTX-M class extended spectrum beta lactamases (ESBLs) has been well documented in industrialized countries. Vietnam is representative of a typical transitional middle income country where the spectrum of infectious diseases combined with the spread of drug resistance is shifting and bringing new healthcare challenges.

Methodology: We collected hospital admission data from the pediatric population attending the hospital for tropical diseases in Ho Chi Minh City with *Shigella* infections. Organisms were cultured from all enrolled patients and subjected to antimicrobial susceptibility testing. Those that were ESBL positive were subjected to further investigation. These investigations included PCR amplification for common ESBL genes, plasmid investigation, conjugation, microarray hybridization and DNA sequencing of a *bla*$_{CTX-M}$ encoding plasmid.

Principal Findings: We show that two different *bla*$_{CTX-M}$ genes are circulating in this bacterial population in this location. Sequence of one of the ESBL plasmids shows that rather than the gene being integrated into a preexisting MDR plasmid, the *bla*$_{CTX-M}$ gene is located on relatively simple conjugative plasmid. The sequenced plasmid (pEG356) carried the *bla*$_{CTX-M-24}$ gene on an *ISEcp1* element and demonstrated considerable sequence homology with other *IncFI* plasmids.

Significance: The rapid dissemination, spread of antimicrobial resistance and changing population of *Shigella spp.* concurrent with economic growth are pertinent to many other countries undergoing similar development. Third generation cephalosporins are commonly used empiric antibiotics in Ho Chi Minh City. We recommend that these agents should not be considered for therapy of dysentery in this setting.

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Introduction

Enterobacteriaceae that have the capability to express CTX-M (so named because of their hydrolytic activity against cefotaxime) family extended spectrum beta lactamases (ESBLs) have emerged as a major health threat worldwide [1,2]. Most of the research in this area is conducted in industrialized countries, where organisms, such as *Escherichia coli* and *Klebsiella spp.*, mostly from urinary tract infections are the commonest source [3,4,5]. Relatively little is known about the distribution of such genes in organisms found developing or countries undergoing an economic transition, where the circulating pathogens may differ.

Enterobacteriaceae capable of producing ESBLs have been described previously in South East Asia [6,7]. Ho Chi Minh City in southern Vietnam is typical of many cities where patterns of infectious diseases are changing due to rapid economic growth, better access to health care and improving infrastructure. We recently showed that 42% of healthy people carried ESBL producing bacteria as part of their regular intestinal flora [8]. This previous work suggested that commensal organisms play a role in the dissemination and maintenance of such antimicrobial resistance genes in the population. Furthermore, the uncontrolled use of antimicrobials in the human population and in livestock rearing may lead to further problems with drug resistance and even more limited therapeutic options.

Author Summary

Shigellosis is a disease caused by bacteria belonging to *Shigella spp.* and is a leading cause of bacterial gastrointestinal infections in infants in unindustrialized countries. The *Shigellae* are dynamic and capable of rapid change when placed under selective pressure in a human population. Extended spectrum beta lactamases (ESBLs) are enzymes capable of degrading cephalosporins (a group of antimicrobial agents) and the genes that encode them are common in pathogenic *E. coli* and other related organisms in industrialized countries. In southern Vietnam, we have isolated multiple cephalosporin-resistant *Shigella* that express ESBLs. Furthermore, over two years these strains have replaced strains isolated from patients with shigellosis that cannot express ESBLs. Our work describes the genes responsible for this characteristic and we investigate one of the elements carrying one of these genes. These finding have implications for treatment of shigellosis and support the growing necessity for vaccine development. Our findings also may be pertinent for other countries undergoing a similar economic transition to Vietnam's and the corresponding effect on bacterial populations.

Shigellosis is a gastrointestinal infection caused by members by *Shigella spp.* Due to the faecal oral route of transmission of the *Shigellae*, children less than five years old and living in developing countries have the highest incidence [9,10]. In our hospital in Ho Chi Minh City, shigellosis is the leading cause of paediatric diarrhoeal admission with bacterial aetiology. The infection is typically self limiting, although antimicrobial treatment is necessary for the young and those that are severely ill as it ensures fewer complications and curtails the duration of the disease [11].

Fluoroquinolones are the drugs of choice to treat *Shigella* infections in both adults and children [12]. However, as with many other members of the *Enterobacteraceae*, mutations in the genes encoding the target proteins for fluoroquinolones are common in *Shigella* [13,14]. Our recent findings show that patients with shigellosis are staying in hospital for longer periods compared with 5 and 10 years ago and the disease severity has concurrently increased [15]. Interestingly, at the same time there has been a significant species shift from *S. flexneri* to *S. sonnei* isolated from patients [15]. Patients here are treated with fluoroquinolones, however, those patients that do not respond to the standard therapy are treated with third generation cephalosporins (mainly ceftriaxone). The intravenous third generation cephalosporins are amongst the most commonly used antimicrobials in hospitals in Ho Chi Minh City and the oral second and third generation cephalosporins are also widely available in the community.

Antimicrobial resistance in the *Shigellae* is common; these organisms are closely related to *E. coli* and are readily transformed by exogenous DNA [16,17,18]. The distribution of antimicrobial resistance is, however, often different depending on the species. A multi-centre study across Asia demonstrated that *S. flexneri* were more likely to be resistant to ampicillin, whilst *S. sonnei* were more likely to be resistant to co-trimoxazole [19]. Resistance patterns and species dominance are variable depending on the specific location [20,21,22].

We have previously reported the rapid emergence of third generation cephalosporin resistant *Shigella* in Vietnam, where we noted the routine isolation of a number of ESBL producing microorganisms [15]. Here, we present data suggesting that ESBL negative organisms have been replaced with ESBL positive organisms.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. This study was approved by the scientific and ethical committee of the HTD and Oxford tropical research ethics committee (OXTREC) number 010-06 (2006). All parents of the subject children were required to provide written informed consent for the collection of samples and subsequent analysis.

Patient criteria

The work was conducted on the paediatric gastrointestinal infections ward at the hospital for tropical diseases (HTD) in Ho Chi Minh City in Vietnam. The HTD is a 500 bed tertiary referral hospital treating patients from the surrounding provinces and from the districts within Ho Chi Minh City. All patients from which *Shigella spp.* were isolated were enrolled into a randomized controlled trial comparing treatment with ciprofloxacin and gatifloxicin as described previously [15] (trial number ISRCTN55945881). Briefly, all children (aged 0–14 years) with dysentery (defined as passing bloody diarrhoea or mucoid stools with additional abdominal pain or tenesmus) whose parent or guardian gave fully informed written consent were eligible for admission to the study. The primary outcome of the trial was treatment failure, defined as the patient not clearing symptoms after five days of antimicrobial treatment.

Microbiological culture and antimicrobial testing

Stool samples were collected from patients and cultured directly on the day of sampling. Samples were cultured overnight in selenite F broth (Oxoid, Basingstoke, UK) and plated onto MacConkey and XLD agar (Oxoid) at 37°C. Colonies suggestive of *Shigella* were sub-cultured on to nutrient agar and were identified using a 'short set' of sugar fermentation reactions (Kliger iron agar, urea agar, citrate agar, SIM motility-indole media (Oxoid, United Kingdom)). Serologic identification was performed by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed by testing with available monovalent antisera for specific serotype identification as per the manufacturer's recommendations (Denka Seiken, Japan).

Antimicrobial susceptibility testing of all *Shigella* isolates against ampicillin (AMP), chloramphenicol (CHL), trimethoprim – sulfamethoxazole (SXT), tetracycline (TET), nalidixic acid (NAL), ofloxacin (OFX) and ceftriaxone (CRO) was performed by disk diffusion (Oxoid, United Kingdom). The minimum inhibitory concentrations (MICs) were additionally calculated for all isolates by E-test, according to manufacturer's recommendations (AB Biodisk, Sweden).

Those strains that were identified as resistant to ceftriaxone using the disk diffusion susceptibility test were further subjected to the combination disc method to confirm ESBL production [23,24]. The combination disc method utilizes discs containing only cefotaxime (CTX) (30 μg) and ceftazidime (CAZ) (30 μg) and both antimicrobials combined with clavulanic acid (CLA) (10μg). ESBL producing strains were identified as those with a greater than 5 mm increase in zone with the single antimicrobial compared to the combined antimicrobial, i.e. demonstrating ESBL inhibition [25]. All antimicrobial testing was performed on Mueller-Hinton agar, data was interpreted according to the Clinical and Laboratory Standards Institute guidelines [26].

Genomic DNA isolation and DNA microarray hybridisation

Genomic DNA was isolated from strains that were subjected to PCR and DNA microarray hybridisation from 1 ml of a 5 ml

overnight bacterial culture using the wizard genomic DNA extraction kit (Promega, USA), as per the manufacturer's recommendations.

For characterization of gene content of isolated *Shigella* strains, genomic DNA was hybridized to an active surveillance of pathogens (ASP) oligonucleotide microarray [27,28]. The ASP array contains over 6,000 gene markers, including species signature genes, virulence genes and antimicrobial resistance genes from over a hundred bacterial species. Thus the ASP array provides data for assessing horizontally transferred genes, such data is helpful for diagnosis and for guiding antimicrobial therapy.

The ASP array used in this study was version 6.2 and was designed and constructed as described previously [28]. Test samples were labelled and hybridised as described previously [29]. Briefly, 5 µg genomic DNA was labelled with Cy5 and hybridised with a formamide based hybridisation buffer solution in a final volume of 48 µl at 50°C for 16–20 hours. The ASP arrays were washed as described previously but with the initial wash at 50°C [29]. The ASP arrays were scanned using a 418 microarray Scanner (Affymetrix, USA) and intensity fluorescence data acquired using ImaGene 7.5 (BioDiscovery, USA). Data was analysed as described previously by Stabler *et al.* [28]. Briefly, a reporter was considered positive if the background corrected mean reporter signal from duplicate spots was both greater than one standard deviation of reporter signal (reporter variation) and the mean reporter signal was greater than the whole background corrected microarray mean plus one standard deviation, as shown for *S. sonnei* EG1007 in Dataset S1 in supporting information. The raw microarray data for all isolates is presented in Dataset S2 in supporting information.

Plasmid extraction and visualisation

Plasmid DNA was isolated from ESBL positive and ESBL negative *Shigella* isolates using a modified version of the methodology previously described by Kado and Liu [30]. The resulting plasmid DNA was separated by electrophoresis in 0.7% agarose gels made with 1× E buffer. Gels were run at 90 V for 3 h, stained with ethidium bromide and photographed. For DNA sequencing plasmid DNA containing an ESBL gene was extracted from an *E. coli* transconjugant using a NucleoBond® Xtra Midi kit as per the manufacturers recommendations (Clontech, USA).

ESBL gene PCR amplification and characterisation

Genomic DNA was subjected to PCR amplification targeting known classes of *bla* genes using, initially, primers that would recognise sequences encoding SHV, (F; 5′ TCTCCCTGTTAGCCACCCTG, R; 5′; CCACTGCAGCAGCTGC) TEM (F; 5′ TGCGGTATTATCCCGTGTTG, R; 5′ TCGTCGTTTGGTATGGCTTC) and CTX-M (F; 5′ CGATGTGCAGTACCAGTAA, R; 5′ TTAGTGACCAGAATCAGCGG) class ESBLs [31,32]. Further characterisation of the various sub-group of bla_{CTX} ESBL genes was performed using primers, CTX-M-1; (F 5′ ATGGTTAAAAAATCACTGCG, R 5′ TTACAAACCGTCGGTGAC), CTX-M-2; (F 5′ TGGAAGCCCTGGAGAAAAGT and R 5′ CTTATCGCTCTCGCTCTGT) and CTX-M-9; (F 5′ATGGTGACAAAGAGAGTGCAAC, R 5′ TTACAGCCCTTCGGCGATG) using previously outlined PCR amplification conditions [31,32].

To identify an association with CTX-M genes and the adjacent IS*Ecp1* transposase, all ESBL positive strains were subjected to PCR with primers forward primers Tnp24F 5′ CACTCGTCTGCGCATAAAGCGG, Tnp15F 5′ CCGCCGTTTGCGCATA CAGCGG (for $bla_{CTX-M-24}$ and $bla_{CTX-M-15}$ respectively) and reverse primer TnpR 5′ AGATATGTAATCATGAAGTTGTCGG. The Tnp24F and Tnp15F were located within the $bla_{CTX-M-24}$ and $bla_{CTX-M-15}$ genes respectively and TnpR was located within the IS*Ecp1* transposase gene. The *bla*-transposase PCR was performed under the following conditions; 95°C for 1 minute, 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute 30 seconds and 72°C for 2 minutes. All PCRs were performed using Taq DNA polymerase and appropriate recommended concentrations of reagents (Bioline, UK).

Positive PCR amplicons were cloned into cloning vector pCR 2.1 (Invitrogen, USA) and sequencing reactions were carried out as recommended by the manufacturer using big dye terminators in forward and reverse orientation on an ABI 3700 sequencing machine (ABI, USA). All sequencing reactions were performed twice to ensure correct sequencing and sequences were verified, aligned and manipulated using Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All ESBL gene sequences were compared to other ESBL sequences by BLASTn at NCBI. The DNA sequence of various classes of bla_{CTX} were downloaded and aligned with the produced sequences.

Bacterial conjugation

Bacterial conjugation experiments were performed by combining equal volumes (3 ml) of overnight Luria-Bertani cultures of donor and recipient strains. The donor strains were *Shigella* clinical isolates carrying bla_{CTX} genes and the recipient was *E. coli* J53 (sodium azide resistant). Bacteria were conjugated for 12 hours at 37°C and transconjugants were selected on Luria-Bertani media containing sodium azide (100 µg/ml) and ceftriaxone (6 µg/ml). Potential transconjugants were verified by serotyping and plasmid extraction.

Plasmid sequencing and annotation

Plasmid pEG356 was selected for DNA sequencing and annotation as previously described [33]. The DNA sequence was annotated to identify coding sequences and repeat sequences in Artemis. To identify plasmids with similar sequences, pEG356 was compared by BLASTn at NCBI. pAPEC-01-ColBM (Ac. DQ381420) [34] was downloaded and aligned with pEG356 and viewed in Artemis Comparison Tool (ACT) [35]. Schematic drawing of the sequence of pEG356 was constructed using DNAplotter [36]. Artemis, ACT and DNAplotter are freely available at (http://www.sanger.ac.uk/Software). The full sequence and annotation of pEG356 was submitted to EMBL with the accession number FN594520.

Results

The escalating isolation rate of ESBL positive *Shigella spp.* in Ho Chi Minh City

During a 24 month period between April 2007 and March 2009 we isolated 94 *Shigella* strains from the stools of children admitted with dysentery. Of these 94 strains, 24 were *S. flexneri* and 70 were *S. sonnei*, confirming the species substitution previously noted from isolates in this region [15]. The general antibiotic sensitivity patterns in these strains were variable, although resistance to trimethoprim – sulfamethoxazole, tetracycline and latterly nalidixic acid were ubiquitous and there was an overall propensity of sensitivity towards older generation antimicrobials such as chloramphenicol (Table 1). A reversion of sensitivity to older therapies highlights how antimicrobial resistance genes can be maintained (or otherwise) by selective antimicrobial pressure in the population.

Table 1. Resistance profiles and isolation date of ceftriaxone resistance *Shigella* from southern Vietnam.

							Antimicrobial Tested															
							AMP		CHL		SXT		TET		NAL		OFX		CRO	
Strain ID	Serotype	Age (months)	Sex	Month	Year	Province	ESBL (+/−)	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC
DE0611	*S. sonnei*	10	M	February	2001	HCMC	+	R	>256	R	8	R	>32	R	128	S	2	S	0.06	R	>256
EG0356	*S. sonnei*	48	M	May	2007	HCMC	+	R	>256	S	6.0	R	>32	R	64	R	64	S	0.38	R	>256
EG0373	*S. sonnei*	30	M	June	2007	HCMC	+	R	>256	S	6.0	R	>32	R	128	S	1.5	S	0.064	R	>256
EG0384	*S. sonnei*	36	M	July	2007	HCMC	+	R	>256	S	6	R	>32	R	256	R	32	S	0.38	R	>256
EG0390	*S. sonnei*	17	M	August	2007	VINH LONG	+	R	>256	S	6	R	>32	R	128	R	>256	S	0.38	R	>256
EG0395	*S. sonnei*	36	F	September	2007	HCMC	+	R	>256	S	12	R	>32	R	96	R	>256	S	0.5	R	>256
EG0162	*S. sonnei*	28	M	October	2007	DONG THAP	+	R	>256	S	8	R	>32	R	48	R	64	S	0.38	R	>256
EG0419	*S. flexneri*	23	F	December	2007	HCMC	−	R	>256	R	>256	R	>32	R	48	R	>256	S	0.5	R	128
EG0187	*S. sonnei*	16	M	January	2008	DONG THAP	+	R	>256	S	3	R	>32	R	192	S	1.5	S	0.047	R	24
EG0421	*S. sonnei*	36	F	January	2008	HCMC	+	R	>256	S	4	R	>32	R	>256	R	128	S	0.38	R	>32
EG0424	*S. sonnei*	48	F	January	2008	HCMC	+	R	>256	S	6	R	>32	R	64	R	>256	S	0.38	R	>256
EG0204	*S. sonnei*	26	F	March	2008	DONG THAP	+	R	>256	S	6	R	>32	R	32	R	64	S	0.38	R	>256
EG0430	*S. sonnei*	36	F	March	2008	HCMC	+	R	>256	S	6	R	>32	R	>256	R	48	S	0.25	R	128
EG1008	*S. sonnei*	18	M	May	2008	LONG AN	+	R	>256	S	8	R	>32	R	96	R	128	S	0.38	R	>256
EG1009	*S. sonnei*	8	M	May	2008	HCMC	+	R	>256	S	8	R	>32	R	96	R	192	S	0.38	R	>256
EG1010	*S. sonnei*	60	F	May	2008	HCMC	+	R	>256	S	6	R	>32	R	96	R	>256	S	0.5	R	>256
EG1013	*S. sonnei*	25	M	June	2008	HCMC	+	R	>256	S	6	R	>32	R	96	R	>256	S	0.25	R	>256
EG1012	*S. sonnei*	15	F	June	2008	HCMC	+	R	>256	S	8	R	>32	R	96	R	192	S	0.38	R	>256
EG1011	*S. sonnei*	108	F	June	2008	HCMC	+	R	>256	S	8	R	>32	R	96	R	128	S	0.38	R	>256
EG1007	*S. sonnei*	48	M	July	2008	LONG AN	+	R	>256	S	6	R	>32	R	64	R	48	S	0.38	R	192
EG0250	*S. sonnei*	35	M	August	2008	DONG THAP	+	R	>256	S	6	R	>32	R	48	R	48	S	0.25	R	>256
EG0250a	*S. sonnei*	36	M	September	2008	DONG THAP	+	R	>256	S	6	R	>32	R	48	R	48	S	0.25	R	>256
EG0471	*S. flexneri*	49	M	September	2008	HCMC	+	R	>256	R	>256	R	>32	R	128	R	>256	S	0.5	R	>256
EG0472	*S. sonnei*	66	M	September	2008	HCMC	+	R	>256	S	4	R	>32	R	96	R	48	S	0.38	R	>256
EG1014	*S. sonnei*	29	M	January	2009	LONG AN	+	R	>256	S	6	R	>32	R	>256	R	>256	S	0.25	R	>256
EG1015	*S. sonnei*	72	F	January	2009	HCMC	+	R	>256	S	4	R	>32	R	32	R	48	S	0.25	R	>256
EG1016	*S. sonnei*	39	M	January	2009	HCMC	+	R	>256	S	6	S	0.38	S	1.5	R	48	S	0.25	R	>256
EG1017	*S. sonnei*	11	F	February	2009	HCMC	+	R	>256	S	5	R	>33	R	97	R	49	S	1.38	R	>256
EG1018	*S. sonnei*	29	M	February	2009	HCMC	+	R	>256	S	6	R	>32	R	48	R	>256	S	0.38	R	>256
EG1019	*S. sonnei*	120	F	February	2009	HCMC	+	R	>256	S	6	R	>32	R	>256	R	48	S	0.25	R	>256
EG1020	*S. sonnei*	48	M	March	2009	HCMC	+	R	>256	S	8	R	>32	R	64	R	192	S	0.38	R	>256
EG1021	*S. sonnei*	20	M	March	2009	HCMC	+	R	>256	S	8	R	>32	R	64	R	>256	S	0.25	R	>256

Table 1. Cont.

								Antimicrobial Tested															
								AMP		CHL		SXT		TET		NAL		OFX		CRO		
Strain ID	Serotype	Age (months)	Sex	Month	Year	Province	ESBL (+/−)	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC	Disc	MIC
EG1022	*S. sonnei*	29	M	March	2009	HCMC	+	R	>256	S	8	R	>32	R	48	R	>256	S	0.25	R	>256		
EG1023	*S. sonnei*	9	F	March	2009	LONG AN	+	R	>256	S	6	R	>32	R	48	R	96	S	0.38	R	>256		
EG1024	*S. sonnei*	84	M	March	2009	LONG AN	+	R	>256	S	6	R	>32	R	64	R	96	S	0.25	R	>256		
EG1025	*S. sonnei*	30	M	March	2009	LONG AN	+	R	>256	S	6	R	>32	R	48	R	96	S	0.25	R	>256		

Ampicillin (AMP), chloramphenicol (CHL), trimethoprim – sulfamethoxazole (SXT), tetracycline (TET), nalidixic acid (NAL), ofloxacin (OFX) and ceftriaxone (CRO).

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The first isolation of a ceftriaxone resistant organism during the transitional period occurred in May 2007 and similar strains were isolated in low numbers for the following months (Figure 1). The numbers of *Shigellae* isolated that were resistant to ceftriaxone fluctuated over the following 18 months. However, there was increase in the proportion of resistant to sensitive isolates 19% to 41% (5 to 11) between the periods from April 2007–September 2007 and April 2008–September 2008, respectively. This trend peaked in March 2009, with six out of seven *Shigella* strains isolated resistant to ceftriaxone (MIC>256). The overall rate of resistance to ceftriaxone between September 2008 and March 2009 was 75%.

The combined resistance patterns of ESBL producing *Shigella spp.*

We initially cultured a ceftriaxone resistant *S. sonnei* strain in 2001 (DE 0611) (Table 1), however, this strain was a single, isolated organism and a secondary ceftriaxone resistant *Shigella* was not isolated again until 2007. Between 2007 and 2009, 35 (34%) *Shigella* isolates cultured were resistant to ceftriaxone (Table 1). Of these strains, 33 were *S. sonnei* and the other two isolates were *S. flexneri*. In total, we isolated 36 ceftriaxone resistant organisms between 2001 and 2009.

The mechanism of ceftriaxone resistance was examined by the double disc inhibition method to identify ESBL producing organisms. All the *S. sonnei* and one *S. flexneri* strain (35 from 36 ceftriaxone resistant *Shigella*) produced the characteristic ESBL pattern on investigation, whereas the hydrolysing activity of the other *S. flexneri* organism was not inhibited by clavulanic acid [23,24] (Table 1).

The median age of patients harbouring third generation cephalosporin resistant *Shigellae* was 32 months (range; 8 to 120 months), the median age of shigellosis patients during the same period was 30 months [15]. Owing to the rapid increase in the rate isolation of such organisms we hypothesised that an individual dominant strain had began circulating in one area of Ho Chi Minh City. However, residence data procured on the time of admission showed that such strains were circulating over a wide area of the city and not purely limited to an isolated outbreak (Table 1). 12 patients were resident in surrounding provinces, some 150 km from the hospital.

In conjunction with ceftriaxone, all strains were examined for resistance to an additional five antimicrobials by disc diffusion and MIC (Table 1). As predicted, all strains demonstrated co-resistance to ampicillin. Thirty five of the 36 strains (97%) were resistant to trimethoprim – sulfamethoxazole and tetracycline, whilst 33/36 were resistant to nalidixic acid. Only three isolates; DE0611, EG0419 and EG0471 were co-resistant to chloramphenicol, of which two, EG0419 and EG0471 (6%), were resistant to five of the six antimicrobials tested (Table 1).

Identifying the genetic nature of ceftriaxone resistance in *Shigella spp.*

The most common mechanism of dissemination of ESBL genes in the *Enterobacteriaceae* is plasmid mediated transfer. Our previous studies have suggested that Vietnam (and other parts of South East Asia) may be hotspot for the origin and further transmission of antimicrobial resistant organisms [8,13,37,38]. *Enterobacteriaceae* which carry MDR plasmids are common in Vietnam and the isolation of MDR *Shigella* strains has been repeatedly reported [19,20,39].

We hypothesised that the ESBL phenotype was related to the insertion of a transposon carried on an MDR plasmid that had

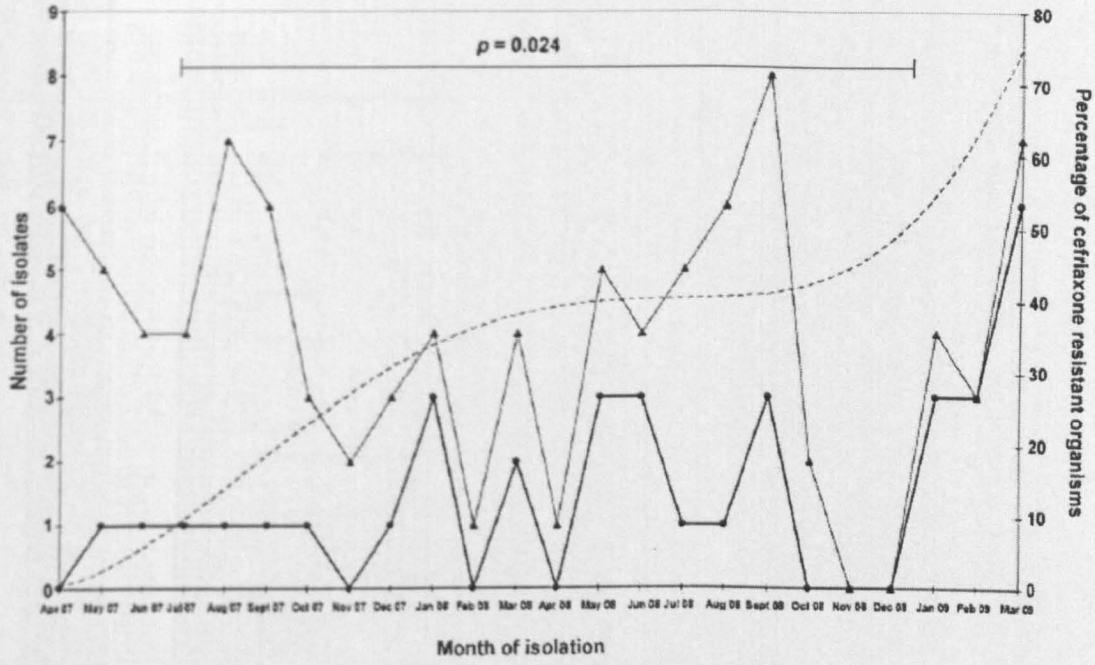

Figure 1. Graph depicting an increase in number and proportion of ceftriaxone resistant *Shigella spp.* isolated between April 2007 and March 2009 at the hospital for tropical diseases in Ho Chi Minh City. The thick black line with circles represents the number of ceftriaxone resistant *Shigella* isolates per month (*x* axis); the thin black line with triangles represents the total number of *Shigella* isolates per month (both related to the left *y* axis). The broken line represents the proportion of strains isolated in six month periods resistant to ceftriaxone (right *y* axis). The increasing proportion of ceftriaxone resistant organisms over six month periods is statistically significant ($p = 0.024$) as calculated using the chi-squared test.
doi:10.1371/journal.pntd.0000702.g001

permeated into and was circulating within the *Shigella* population. To investigate the genetic nature of the ESBL positive isolates compared to the ESBL negative isolates we hybridised genomic DNA to an active surveillance of pathogens (ASP) DNA microarray. In total, 15 isolates (seven ESBL positive and eight ESBL negative) were compared. The ASP array is designed to monitor gene flux, genetic content and the nature of horizontally transferred DNA in a bacterial population. The resulting hybridisation is shown in Figure 2. Concurrently, plasmid DNA was isolated and compared from the same bacterial isolates to assess plasmid content.

Figure 2 is a heatmap representation of the 142 ASP microarray reporters which demonstrated positive hybridisation to DNA in two or more of the *S. sonnei* samples and the 11 reporters representing the *S. sonnei* Ss046 plasmid pSS_046. The overall hybridisation data and the names and predicted functions of the genes are presented in Dataset S2 (supporting information).

The pattern of relative hybridisation across all strains was remarkably homogenous, with only 30% (42/142+11 pSS_046) of the total proportion of the positive coding sequences demonstrating variable hybridisation patterns. The coding sequences demonstrating common hybridisation patterns across all 15 strains included a number of signature *E. coli*, *Shigella spp.* regions and sequences corresponding to virulence and antimicrobial resistance (Figure 2 and Supporting information Datasets S1 and S2).

The common antimicrobial resistance genes identified between isolates included genes conferring resistance to streptomycin, macrolides, tetracycline, beta lactams and also some unspecific antimicrobial resistance efflux genes. The homogenous nature of hybridisation suggests that variation between isolates is limited and dependent on plasmid content. All the ESBL producing strains demonstrated significant hybridisation to sequences corresponding to *bla* genes, highlighted in Figure 2. DNA from the ESBL negative strains failed to hybridise to these targets.

Plasmid visualisation of plasmid DNA by agarose gel electrophoresis with all hybridised strains revealed that in contrast to the ESBL negative isolates, all the ESBL producing isolates had a large plasmid, we roughly estimated to be greater than 63 Kbp in size (according to the marker plasmid). Despite the ESBL negative isolates lacking a large plasmid; these strains demonstrated similar resistance profiles, with the obvious exception of ceftriaxone (data not shown). These data suggested that the ESBL genes may be located on simple (none MDR) extrachromosomal elements. This hypothesis was supported by evidence of *in vivo* horizontal plasmid transfer; two strains cultured two days apart from the same patient were identical in serotype, plasmid content and MIC resistance profile, with the exception of the secondary strain carrying a large plasmid and displaying resistance to ceftriaxone (data not shown). Furthermore, sequencing of a conjugative, ESBL encoding plasmid confirmed our suggestion of a simple extrachromosomal element.

Characterisation of *bla* genes

PCR was performed to detect the bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. Further PCR amplifications were performed on DNA from all strains that produced amplicons with the bla_{CTX-M} primers.

Figure 2. Demonstration of the absence and presence of genes from DNA isolated from ceftriaxone resistant and ceftriaxone sensitive *S. sonnei* isolates using the ASParray. Red boxes indicate presence of genes; green boxes indicate absence of genes. BLAST indicates reporter DNA identity (%) to the *S. sonnei* Ss046 genome. DNA was hybridized from isolates (left to right) DE0115, DE0477, DE0685, DE0891, DE1150, DE1198, DE1256, DE0611, EG0204, EG0373, EG0395, EG0430, EG1007, EG1008 and EG1009.
doi:10.1371/journal.pntd.0000702.g002

Primers that were specific for the three major CTX-M clusters, $bla_{CTX-M-9}$, $bla_{CTX-M-1}$ and $bla_{CTX-M-2}$ were selected [40]. Three strains (DE0611, EG0187 and EG0356) produced amplicons with the $bla_{CTX-M-9}$ primers and the remaining 32 isolates produced amplicons with the $bla_{CTX-M-1}$ primers (Table 2). All 35 PCR amplicon were sequenced.

Sequence analysis of the PCR amplicons demonstrated that there were two differing bla_{CTX-M} genes present in the *Shigella* population, these were, $bla_{CTX-M-24}$ (n = 3, 8%) and $bla_{CTX-M-15}$ (n = 32, 92%) (Table 2). Both genes ($bla_{CTX-M-24}$ and $bla_{CTX-M-15}$) share 74% DNA homology with each other; $bla_{CTX-M-15}$ and $bla_{CTX-M-24}$ differ by 12 and 6 nucleotides from the precursor

Table 2. Characterisation of bla_{CTX-M} genes and the corresponding plasmids of ESBL expressing *Shigella spp.*

Strain ID	Ceftazidime zone (mm)	bla_{CTX-M}	Plasmid size (kbp)[a]	Conjugation frequency[b]	*bla* -transposon PCR (+/−)
DE0611	28	CTX-M-24	70	4.43×10^{-2}	+
EG0162	18	CTX-M-15	100	2.73×10^{-2}	+
EG0187	27	CTX-M-24	70	2.58×10^{-2}	+
EG0204	19	CTX-M-15	100	1.93×10^{-2}	+
EG0250	19	CTX-M-15	100	4.43×84^{-2}	+
EG0250a	19	CTX-M-15	100	4.00×84^{-2}	+
EG0356	28	CTX-M-24	70	2.41×10^{-3}	+
EG0373	18	CTX-M-15	100	1.50×10^{-2}	+
EG0384	20	CTX-M-15	100	2.92×10^{-2}	+
EG0390	22	CTX-M-15	100	1.38×10^{-2}	+
EG0395	20	CTX-M-15	100	2.33×10^{-2}	+
EG0421	20	CTX-M-15	100	1.83×10^{-4}	+
EG0424	21	CTX-M-15	100	3.77×10^{-3}	+
EG0430	21	CTX-M-15	100	2.00×10^{-4}	+
EG0471	20	CTX-M-15	100	1.38×10^{-3}	+
EG0472	20	CTX-M-15	100	3.59×10^{-3}	+
EG1007	22	CTX-M-15	100	1.60×10^{-2}	+
EG1008	20	CTX-M-15	100	1.43×10^{-2}	+
EG1009	21	CTX-M-15	100	3.11×10^{-5}	+
EG1010	21	CTX-M-15	100	1.82×10^{-2}	+
EG1011	21	CTX-M-15	100	5.68×10^{-6}	+
EG1012	20	CTX-M-15	100	2.37×10^{-2}	+
EG1013	19	CTX-M-15	100	4.88×10^{-6}	+
EG1014	19	CTX-M-15	100	2.50×10^{-3}	+
EG1015	22	CTX-M-15	100	2.75×10^{-3}	+
EG1016	20	CTX-M-15	100	3.00×10^{-4}	+
EG1017	20	CTX-M-15	100	3.20×10^{-3}	+
EG1018	20	CTX-M-15	100	1.45×10^{-2}	+
EG1019	20	CTX-M-15	100	2.00×10^{-3}	+
EG1020	20	CTX-M-15	100	0	+
EG1021	21	CTX-M-15	100	1.85×10^{-3}	+
EG1022	21	CTX-M-15	100	3.75×10^{-2}	+
EG1023	21	CTX-M-15	100	8.57×10^{-4}	+
EG1024	20	CTX-M-15	100	3.43×10^{-2}	+
EG1025	20	CTX-M-15	100	2.36×10^{-2}	+

[a]Estimated plasmid size by agarose gel electrophoresis with known markers.
[b]Conjugation frequency calculated per donor cell from the mean of two replicates.
doi:10.1371/journal.pntd.0000702.t002

genes within their respective parent groups, ($bla_{CTX-M-1}$ and $bla_{CTX-M-9}$).

Plasmid sizing, by visualisation of the previous agarose gel electrophoresis demonstrated that the estimated plasmid size corresponded with either the bla_{CTX-M} gene (Table 2); $bla_{CTX-M-15}$ was consistently located on a plasmid larger than that associated with $bla_{CTX-M-24}$. These observations were confirmed by Southern blotting hybridisation of plasmid DNA extractions (data not shown). The differing plasmid sizes and ESBL genes correlated precisely with two distinct zone clearance areas when strains were susceptibility tested with ceftazidime. The strains expressing CTX-M-24 demonstrated less activity against ceftazidime when compared to CTX-M-15 (median zone size, CTX-M-24; 28mm, CTX-M-15; 20mm) (Table 2).

All bla_{CTX-M} harbouring plasmids with the exception of the plasmid in strain EG1020 were transmissible with high conjugation frequencies, ranging from 4.84×10^{-2} to 4.88×10^{-6} (median 1.55×10^{-2}) per donor cell (Table 2). The mobilisation of one of these bla_{CTX} harbouring plasmids was further demonstrated by conjugative transfer of the plasmid originally from *S. sonnei* EG356 from an *E.coli* transconjugant back into a fully susceptible, naive *S. sonnei* strain at a similarly high frequency.

DNA sequence analysis of the pEG356 plasmid

The ESBL encoding gene $bla_{CTX-M-24}$ appears to be generally restricted to *Enterobacteriaceae* in Asia [41,42], with only sporadic reports of this gene in other locations [43]. Therefore, we selected the plasmid from isolate EG0356, carrying a $bla_{CTX-M-24}$, as it is applicable to this location, for further characterisation by DNA sequencing.

Plasmid pEG356 was found to be a circular replicon consisting of 70,275 nucleotides, similar in size to another $bla_{CTX-M-24}$ encoding plasmid from Asia; pKP96. pKP96 was isolated from a *Klebsiella pneumoniae* strain from China in 2002, yet demonstrates limited DNA homology to pEG356, with exception to the ESBL encoding region [44]. pEG356 was comparatively GC neutral (52.26%) and belonged to incompatibility group *FI* (on the basis of the DNA sequence homology to the replication region) (Figure 3). pEG356 was predicted to contain 104 coding sequences, of which 14 were considered to be pseudogenes on the basis of apparent premature stop codons, frameshifts or missing start codons. The density of coding sequencing approached 95% and contained four main structural features, a replication region, the ESBL gene encoding region with predicted homology to an IS*Ecp1* element, an iron ABC transport system and a DNA transfer region (labelled red, pink, dark blue and light blue, respectively in Figure 3).

pEG356 encoded the complete *tra* gene-set encoding a conjugative pilus with high sequence similarity to the transfer region from the F plasmid sequence from *E. coli* K12 [45] (Ac. AP001918). This is consistent with the *in vitro* data demonstrating that this plasmid is transmissible into an *E. coli* recipient. The *IncFI* replication region was highly similar to other *IncF* plasmids, including the recently described CTX-M-15 encoding plasmid pEK499 (Ac. EU935739) isolated from an *E. coli* O25:H4-ST131 epidemic strain circulating in the United Kingdom [46]. Additionally, pEG356 shared another 30 Kbp (position 15,152 to 44,255 in pEG356) of high sequence similarity with pEK499 [46]. This region contains multiple common hypothetical plasmid genes of unknown function, genes involved in conjugative transfer (*traM* to *traC*), plasmid partitioning and a predicted single stranded DNA binding protein (*ssb*). Unlike pEK499 the *mok* and *hok* post segregational killing genes are missing from within the plasmid maintenance region [46]. With respect to pEK499 and other ESBL carrying plasmids, pEG356 does not carry multiple antimicrobial resistance genes, transposons, insertion sequences or any additional virulence associated genes [44,46,47](Chen et al. 2007; Shen et al. 2008; Woodford et al. 2009)(Chen et al. 2007; Shen et al. 2008; Woodford et al. 2009).

In overall structure, but not size, pEG356 shared the most DNA sequence similarity with the ColBM plasmid pAPEC-O1 (Ac. DQ381420), isolated from an avian pathogenic *E. coli* strain [34] (Figure 4). pEG356 shared around 80% of the gene content with pAPEC-O1, including the conjugation (*tra*), replication (*rep*) and a putative ATP iron transport system (*iro*). The *iro* region consisted of four coding sequences, which include, a putative permease, an iron binding protein and an export associated protein.

The $bla_{CTX-M-24}$ was located on an IS*Ecp1* like element. The overall sequence of the IS*Ecp1* variant on pEG356 is 4,725 bp and 3,000 bp shares 99% DNA homology with an ESBL gene encoding element from an *E. coli* strain that was isolated in China; pOZ174 (AF252622) [48]. The $bla_{CTX-M-24}$ carrying region is also highly similar (99% DNA homology) to the equivalent region in the previously described plasmid, pKP96, including the IS903D downstream of the $bla_{CTX-M-24}$ gene (Figure 4) [44].The IS*Ecp1* element contains two pairs of inverted repeat (Figure 4); the larger inverted repeat (31 bp) flanks the complete element, inclusive of six coding sequences. The 3'end of the IS*Ecp1* element contained a IS*Ecp1* transposase and a small hypothetical coding sequence of unknown function which is spanned by two IS1380 elements. The $bla_{CTX-M-24}$ isadjacent to two pseudogenes, which were understood to have encoded a conserved hypothetical transposon protein and a maltose-inducible porin precursor, it is not clear what significance, if any, these genes are to the overall functionality of the element or the plasmid.

All ESBL producing *Shigella* were subjected to PCR to demonstrate if all *bla* genes were associated with the IS*Ecp1* transposase. The location of the PCR primers Tnp24F and TnpR are highlighted in Figure 4 and were designed to produce an amplicon if the *bla* gene and the adjacent IS*Ecp1* transposase were in the same location and orientation in strains with a $bla_{CTX-M-24}$. A secondary forward primer was designed in equivalent location for those strains with a $bla_{CTX-M-15}$ (Tnp15F). Therefore, if $bla_{CTX-M-24}$ or the $bla_{CTX-M-15}$ was consistently adjacent to the IS*Ecp1* transposase it would produce an amplicon of 414 bp in all strains. All ESBL positive strains (CTX-M-15 and CTX-M-24) generated a PCR amplicon of the predicted size (Table 2). Sequencing of all PCR products demonstrated that all the $bla_{CTX-M-15}$ and the $bla_{CTX-M-24}$ gene were associated with an IS*Ecp1* transposase, The DNA sequence from all PCR products was identical from within the transposase gene up to and including the IS1380.

Discussion

Members of the *Enterobacteriaceae* that carry CTX-M family ESBLs have been isolated from many parts of the world since the mid 1990s [40]. CTX-M genes have been previously identified from pathogenic *Enterobacteriaceae* circulating in South East Asia; such as Vietnam, Thailand, Cambodia and Singapore [6,7,49,50]. Additionally, our work has shown that ESBLs are commonly found in organisms which constitute the "normal" gastrointestinal flora in the general population living in Ho Chi Minh City [8]. Such data predicts that intestinal flora may be a considerable reservoir of ESBL encoding genes and the genetic elements they circulate on, permitting potential transmission to their pathogenic counterparts.

CTX-M genes in the *Shigellae* have been previously reported in Argentina, (CTX-M-2) [51], Korea (CTX-M-14) [52] and from a traveler returning from India (CTX-M-15) [53]. More recently,

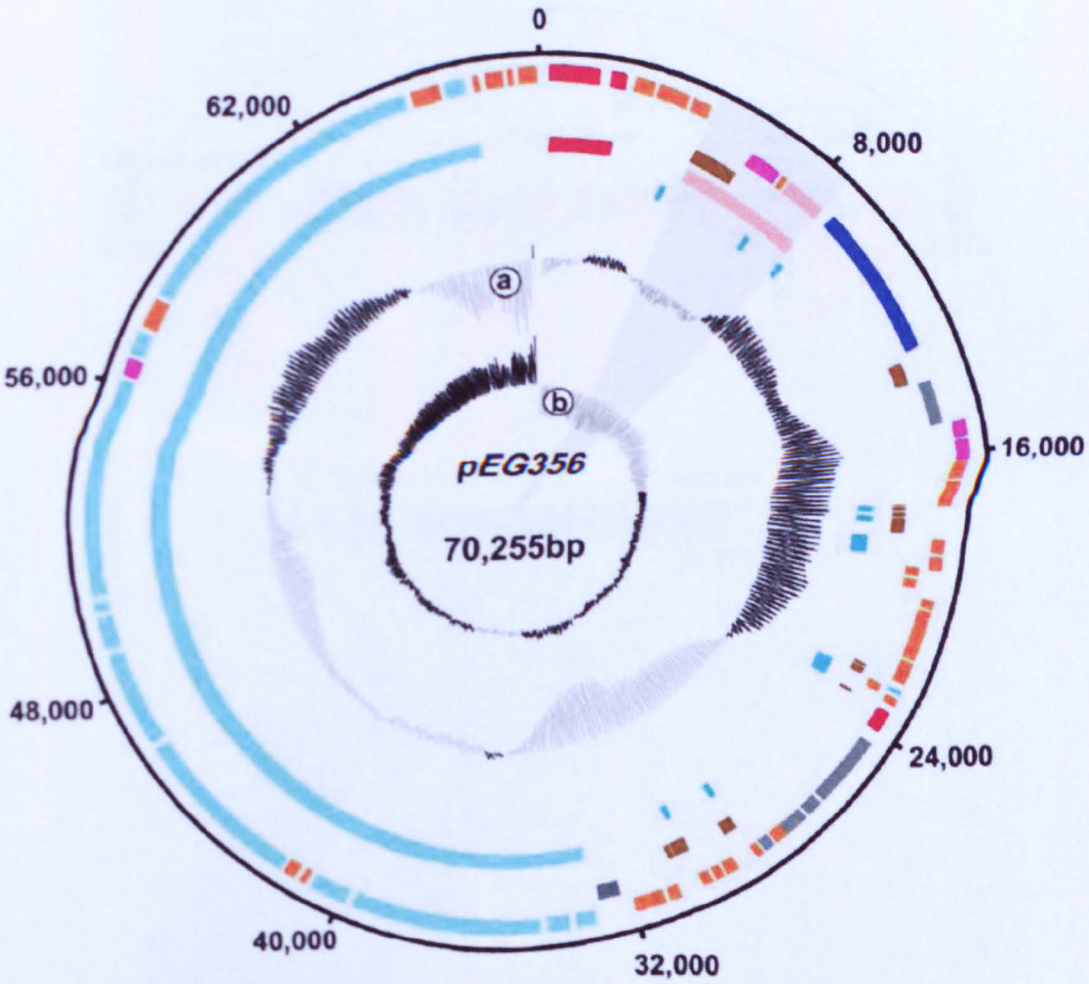

Figure 3. A schematic representation of the $bla_{CTX-M-24}$ encoding plasmid, pEG356. pEG356 is a 70,275bp *IncFI* plasmid containing 104 coding sequences. The various features are highlighted by the various concentric circles according to the annotation of the of the plasmid (ac. FN594520). The outer colored circle represents coding sequences on the forward strand, the second circle represents coding sequences on the reverse strand. The coding sequences are coded by colour, red; plasmid replication, orange; conserved hypothetical, brown; pseudogene, dark blue; adaptation, grey; segregation, light blue; conjugation/transfer, light pink; transposition, dark pink; degradation/resistance and yellow; metabolism. The third concentric circle represents the location of pseudogenes and the fourth circle represents the four main modules of predicted function, red; replication, pink; transposition, dark blue; iron transport and light blue; conjugational transfer. The fifth and final coloured circle represents the location of the repeat sequences. The primary central graph (a) represents GC content, ranging from high (black) to low (grey) (mean 52%) and the secondary central graph (b) represents G/C coding bias ranging from high (black) to low (grey). The IS*Ecp1* type element carrying the $bla_{CTX-M-24}$ is distinguished by grey shading.
doi:10.1371/journal.pntd.0000702.g003

Nagano *et al.* described a novel CTX-M-64 hybrid from a shigellosis patient infected with *S. sonnei* after returning to Japan from China [54]. The *S. sonnei* strains isolated here in Ho Chi Minh City harbored the $bla_{CTX-M-15}$ and $bla_{CTX-M-24}$ genes. Current data suggests that $bla_{CTX-M-24}$ is found mainly in Asia [41,42], yet may have been transferred to other locations [43]. MDR CTX-M-15 producing *E. coli* is emerging worldwide as an important pathogen causing hospital-acquired infections [2]. The potential impact of MDR *Shigella* combined with CTX-M-15/24 carrying plasmids is substantial, with implications for local treatment policy and the transportation of such plasmids into other countries as has been implicated in Canada [43,55].

The structure of pEG356 as a vector for transferring $bla_{CTX-M-24}$ implies that such plasmids may be common. The streamlined nature of pEG356, remarkably high conjugation frequency may ensure onward circulation of the genetic cargo as it becomes stable in the bacterial population. The simplistic nature of pEG356, with a lack of additional resistance genes suggests that this is a contemporary element, with the $bla_{CTX-M-24}$ a recent acquisition. The $bla_{CTX-M-24}$ gene has been located on a relatively uncompli-

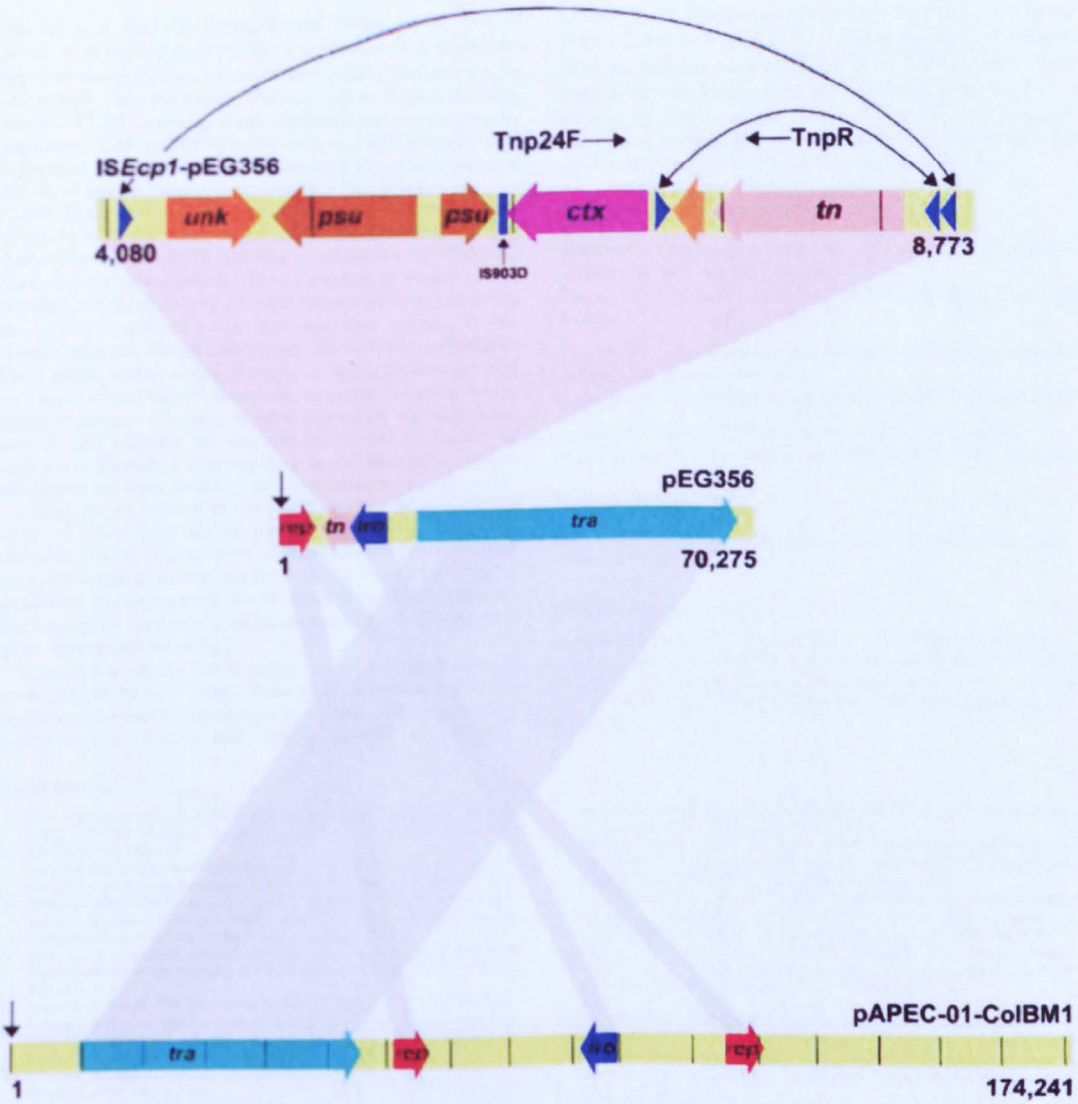

Figure 4. A schematic representation of *ISEcp1*-pEG356 and DNA sequence alignment highlighting corresponding DNA homology between pEG356 and pAPEC-O1-ColBM. The DNA sequences for pEG356 and pAPEC-O1-ColBM were aligned and compared in Artemis comparison tool (ACT). The numbers on the diagram correspond to the respective plasmid sizes and the black integers highlight 10 Kbp intervals. The genetic backbone of the pEG356 and pAPEC-O1-ColBM is shown in yellow along with the various modules, red; *rep* (replication), pink; *tn* (transposition), dark blue; *iro* (iron uptake) and light blue; *tra* (DNA transfer/conjugation). Areas with high DNA homology between pEG356 and pAPEC-O1-ColBM are shown with grey shading and the pink shading corresponds to a magnified view of *ISEcp1*-pEG356. The numbers on *ISEcp1*-pEG356 correspond to the location of the element on the host plasmid, with integers representing 1 Kb intervals. The genes are functionally coded, pink; *tn* (transposition), orange; *unk* (unknown function), brown; *psu* (pseudogene) and dark pink; $bla_{CTX-M-24}$. primer locations for the transposon PCR are highlight by Tnp24F and TnpR. The location of the IS*903D* downstream of the $bla_{CTX-M-24}$ is highlighted. The region is flanked by an inverted repeat (blue triangles) and contains an additional inverted repeat sequence flanking the transposase gene. Corresponding inverted repeats are linked by arrows.
doi:10.1371/journal.pntd.0000702.g004

cated plasmid in Asia, however, pKP596 only demonstrates limited homology to pEG356 [44].

All ESBL gene were located adjacent to a IS*Ecp1* transposase (as identified by PCR). We are currently unable to substantiate if it is the IS*Ecp1*-like element, the plasmids or the circulation of bacterial clone is responsible for the increasing rate of isolation. However, the geographical spread of these strains suggests that they are widely disseminated throughout southern Vietnam. *S. sonnei* is a

monophyletic bacterial pathogen, and owing to the lack of sensitivity of existing sequence based methods such as multi locus sequence typing [56], we are currently unable to confirm clonality satisfactorily (data not shown). Further epidemiological investigation of CTX-M containing strains combined with a more sensitive sequenced based methodology, such as is used for *Salmonella* Typhi is required [57]. We are currently assessing the genetic nature of the strain and the plasmids carrying the ESBL genes.

Our findings show a transfer from 0% to 75% ceftriaxone resistance in *S. sonnei* over just two years in the key age group (1 to 3 years) for this disease. By sampling across the Ho Chi Minh City area, covering approximately 150 sq kilometres of Vietnam and a population of approximately 15 million people we have shown that the genetic explanation for this resistance pattern is the dissemination two distinct ESBL genes, of which one is dominant. These are the leading source of ESBLs in clinical *Shigella* cases and their rapid spread suggests that these organisms are under strong selection pressure. The use of third generation cephalosporins, such as oral cefpodoxime and cefixime in the community is common in Vietnam, and places the even the short term usage of ceftriaxone and other broad-spectrum cephalosporins in jeopardy.

Shigella spp. are capable of carrying multiple plasmids with an array of phenotypes including virulence and antimicrobial resistance [16,18]. The presence of *Shigella* in the gastrointestinal tract of humans is an ideal environment to acquire horizontally transferred genetic material. Small highly transmissible plasmids that impinge on the fitness of the host may be rapidly disseminated under appropriate conditions.

Vietnam is a country that in many respects is representative of many parts of the world. The Vietnamese economy is developing rapidly and the country is undergoing transition with an increasing population, urbanisation and shifting patterns of infectious diseases. In the past decade there has been a transition in species from *S. flexneri* to *S. sonnei* in the Southern provinces of Vietnam. With this shift has come the emergence of ESBL *S. sonnei*. These findings from the Vietnamese population should perhaps serve as a warning for other countries encountering the same economic transition. The progressive evolution of pan-resistant *Shigella* makes vaccine development an increasingly important objective.

Supporting Information

Alternative Language Abstract S1 Translation of abstract into Vietnamese by Tran Vu Thieu Nga.
Found at: doi:10.1371/journal.pntd.0000702.s001 (0.04 MB DOC)

Dataset S1 Corrected microarray data mean plus one standard deviation for *S. sonnei* EG1007.
Found at: doi:10.1371/journal.pntd.0000702.s002 (1.03 MB XLS)

Dataset S2 Raw microarray data for all *S. sonnei* isolates.
Found at: doi:10.1371/journal.pntd.0000702.s003 (1.03 MB XLS)

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Author Contributions

Conceived and designed the experiments: SB. Performed the experiments: NTKN TVTN RS PTD LTMV. Analyzed the data: TVTN RS ACT NT SB. Contributed reagents/materials/analysis tools: HV RS HRvD ACT NT JC NVMH TTTN PVM CTT BW. Wrote the paper: BW JF SB.

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Characterization of rotaviruses causing diarrhoea in Vietnamese children

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Faecal samples from 123 children admitted to the Centre for Tropical Diseases in Ho Chi Minh city, Vietnam, with acute watery diarrhoea were screened by negative-stain electron microscopy for viral enteropathogens. In addition to the 48 children who were found to be infected with rotavirus only, one had both rotavirus and astrovirus, two had adenovirus 40/41, and one had astrovirus only. The rotaviruses were subjected to molecular analysis by electropherotyping, G- and P-genotyping (by reverse-transcriptase PCR), and amplicon sequencing. By use of newly designed PCR primers, all 49 isolates could be G-genotyped and all but one P-genotyped. Novel variants of G1 — G1* — were the most commonly detected G-genotype and such variants of P[8] — P[8*] — were the second commonest P-genotype. The P[8*] and G1* amplicons were, respectively, only 92%–93.4% and 88.1%–89% similar to the corresponding sequences from the prototype P[8] G1 rotavirus, Wa. Several unusual P- and G-genotype combinations were detected. Four (8%) of the children investigated were each found to be co-infected with two different rotaviruses. These data add to our knowledge of the continuing evolution and diversity of human rotaviruses, and should help in the rational design of vaccines.

Diarrhoeal disease is the fourth commonest cause of death world-wide (Murray and Lopez, 1997). Of the more than 50 pathogens able to cause such disease, rotavirus is arguably the most important, and is certainly so in infants (Hart *et al.*, 2002). Globally, rotavirus causes an estimated 418,000–870,000 deaths/year, rotavirus-attributable mortality among children being markedly greater in developing countries than in the developed (Anon., 1986; Miller and McCann, 2000).

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Rotavirus is a small unenveloped virus with an 11-segmented, double-stranded, RNA genome. Two viral proteins (VP) are expressed on the surface of the virion. VP4 is encoded by genome segment 4 and must be cleaved proteolytically, to VP5* and VP8*, for full viral infectivity. VP7 is encoded on genome segment 9 and is a glycoprotein. VP4 and VP7 are both involved in attachment to, and entry into enterocytes, and are the major rotavirus-neutralizing antigens; antibodies to them confer immunity to infection. Epitopes on VP7 define the G (for glycoprotein) types whereas those on VP4 define the P (for protease) types. So far, 14

G-types and 20 P-types have been described. Infection with one rotavirus G-type does not usually confer protection against the other G-types (and, similarly, infection with one P-type only usually induces immunity to that P-type). Originally, rotaviruses were categorized, by use of hyper-immune sera and monoclonal antibodies, into G- and P-serotypes but increasing knowledge of the rotavirus genome permitted rotavirus genotyping (Gentsch *et al.*, 1996). The G-genotypes fortunately coincide with the G-serotypes but there are now 20 P-genotypes and only 12–15 P-serotypes. By convention, P-genotypes are designated by a squared bracket, and thus the antigenic formula of a rotavirus is described as, for example, P[8] G1 1A. A global survey of rotavirus G-types indicated that just four G-types (G1–G4) accounted for most infections in humans (Gentsch *et al.*, 1996). However, since then it has become apparent that things are not so clear-cut. G8 rotaviruses have emerged in Africa [now being responsible for over a third of cases of gastro-enteritis in Malawian infants (Cunliffe *et al.*, 2001*b*)], G5 has become more common in South America (Gouvea *et al.*, 1994), and G9 has emerged as a new global serotype (Ramachandran *et al.*, 2000; Cunliffe *et al.*, 2001*a*). Rotaviruses show tremendous diversity (Cunliffe *et al.*, 2002) and alter their genomic and antigenic structure by mutation (antigenic drift), re-assortment (antigenic shift), and internal re-arrangement (Desselberger, 1996; Palombo, 2002). This can result in the emergence of novel and variant P- and G-types, as well as new P- and G-type combinations. The live rhesus re-assortant vaccine that was recently introduced for routine use against rotavirus in the U.S.A. expresses G1–G4 (Lynch *et al.*, 2000). If novel or variant G-types emerge, the efficacy of this vaccine (and others) might suffer. The aim of the present study — part of a 1-year investigation of the aetiology of acute diarrhoea in Vietnamese children — was to explore rotavirus diversity in Vietnam, by characterizing the rotaviruses causing diarrhoeal disease in the environs of Ho Chi Minh city.

SUBJECTS AND METHODS

On each weekday between 27 September and 14 December in the year 2000, samples of faeces were collected from the first two, three or four young children (aged <5 years) who were admitted to the Centre for Tropical Diseases (CTD) in Ho Chi Minh city because they had acute, watery diarrhoea. (The CTD is a 500-bed hospital serving all those who develop infectious disease in the local community.) Each stool sample was frozen at $-20\,^{\circ}C$ and transported to the University of Liverpool's Department of Medical Microbiology (Liverpool, U.K.), for analysis. Approval for the study was granted by the CTD's Scientific and Ethical Committee.

Electron Microscopy

Faecal suspensions (10%–20%, v/v) were prepared in phosphate-buffered saline (pH 7.2). Copper, electron-microscopy grids (3 mm in diameter; 400 mesh) that had been precoated in polyvinyl formal resin (Formvar®) were dipped into the suspension and air-dried. The grids were then negatively stained with 2% (v/v) phosphotungstic acid and then viewed on a Philip's 301 electron microscope at an initial screen magnification of $\times 45{,}000$.

Extraction of Rotavirus dsRNA

The faecal suspensions were centrifuged in a bench-top microcentrifuge ($13{,}000 \times g$ for 10 min). The double-stranded RNA (dsRNA) of any rotavirus present was then extracted from the supernatant solutions, using a guanidine-isothiocyanate–silica-glass-powder method (Boom *et al.*, 1990; Gentsch *et al.*, 1992). The dsRNA was analysed by PAGE and also used for G- and P-typing, by reverse-transcriptase PCR (see below).

PAGE

PAGE was undertaken in pre-formed, polyacrylamide gels (10%, v/v), each lane being loaded with 30 μl of a dsRNA extract which had previously been heated to 65°C for 10 min. Gels were run at 150 V for 120 min, then stained with silver nitrate (Herring *et al.*, 1982) and finally photographed, for later analysis.

P- and G-genotyping

A semi-nested, multiplex, reverse-transcriptase PCR (RT-PCR) was used to identify the P- and G-genotypes represented in each dsRNA sample. The primers used enable the detection not only of P[4], P[6], P[8], P[9], P[10], G1, G2, G3, G4, G8 and G9, but also of a novel variant of G1 (G1*) that has been detected in Malawi and a novel variant of P[8] — P[8*] — that has been detected in Malawi and the U.K. (Gentsch *et al.*, 1992; Das *et al.*, 1994; Gouvea *et al.*, 1999; Iturizza-Gomara *et al.*, 2000; Cunliffe *et al.*, 2001*b*). Amplicons were subjected to electrophoresis in 2% (w/v) agarose, stained with ethidium bromide and then visualized by ultra-violet trans-illumination. Genotypes were assigned by comparison of the banding patterns with those produced with reference samples of known genotype.

Sequencing

The variant G1* and P[8*] amplicons were cut from the agarose gels, eluted and purified using a commercial gel purification kit (Qiagen, Crawley, U.K.), cloned into pGEM-T (Promega, Madison, WI) and then transformed into *Escherichia coli* TG2. Plasmid DNA containing the insert was isolated from the *E. coli*, using another commercial kit (Qiagen), and then sequenced, using MI2 primers, by Lark Technologies (Saffron Walden, U.K.). The sequences were compared using the CLUSTAL X software package (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg; Thompson *et al.*, 1994) and weighted residue tables.

RESULTS

Over the 3-month sampling period, faeces were collected from 123 children with acute, watery diarrhoea. Electron microscopy revealed that 48 (39%) of these children were infected with rotavirus alone, two (1.6%) with adenovirus 40/41, one (0.8%) only with astrovirus, and one (0.8%) with both rotavirus and astrovirus. Thus, 49 (40%) of the children investigated were excreting rotavirus in their stools. When subjected to PAGE, clear electrophoretic patterns were produced from the dsRNA extracted from 46 of the 49 rotavirus isolates (Fig.): 17 (37%) were considered to be of the short electropherotype, 27 (59%) of the long, and two (4%) shared a mixed pattern. However, the 'long' and 'short' electropherotypes

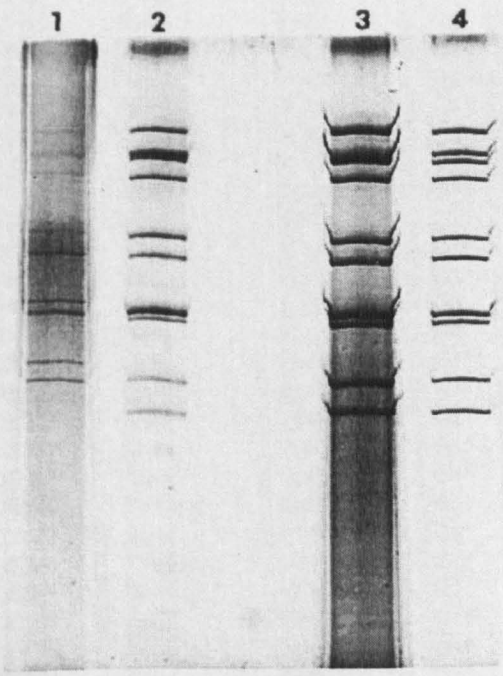

FIG. The results of the PAGE of the double-stranded RNA from some of the rotavirus isolates, showing the short (lane 1) and long (lanes 2, 3 and 4) electrophoretic patterns. Lane 4 contained a sample from an isolate of the P[8] G1* genotype whereas lanes 2 and 3 contained samples from P[8] G1 isolates.

each formed a broad category, with considerable inter-isolate variation within each category.

The RT-PCR was successful in G-typing all 49 isolates and in P-typing all but one of them (see Table). In addition to the two mixed infections detected by PAGE, a further two cases of co-infection (each with two rotaviruses) were detected by RT-PCR, making a total of four mixed infections (8%) and 53 rotaviruses. Overall, the novel G1* variant (represented by 25, or 47% of the viruses) was the commonest G-type, followed by G2 (20 viruses; 38%), and G1 (four viruses; 7.5%) and G4 (four viruses; 7.5%). No G3, G8, G9 or G10 rotaviruses were detected. In the P-typing, 24 (45%) of the viruses were found to be P[4], 17 (32%) to be the novel variant P[8*], eight (15%) to be P[8], three (6%) to be P[6], and one (2%) to be non-typeable. As expected, almost all of the short-electropherotype rotaviruses were P[4] G2, but one was found to be P[6] G2 and another to be P[non-typeable] G2. Most of the long-electropherotype rotavirus strains were P[8] (or P[8*]) and G1 (or G1*). However, some unusual combinations — P[6] G1 and P[6] G4 — were detected. Finally, it is of note that the G1 strain shared a slightly different but reproducible electrophoretic pattern from the novel variant G1* (see Figure).

Two of the novel P[8*] amplicons (of 301 bp) were sequenced and compared with the same regions in both the prototype Wa P[8] and Malawian P[8*] variant. The two Vietnamese P[8*] amplicons were 97.7% and 98.7% similar to the Malawian P[8*] and 92% and 93.4% similar to Wa P[8]. Two novel G1* amplicons (of 118 bp) were sequenced and compared with the same region of Wa G1 and a novel Malawian G1*. The Vietnamese strains were 99.2% similar to each other and 92.4% and 91.5% similar to the Malawian G1*. They were very different from the Wa G1, however, being only 89.0% and 88.1% similar over this small region.

TABLE *Molecular typing of the Vietnamese rotaviruses*

Isolate	Electropherotype	P-type	G-type
DE408	Unclear	P[4]	G2
DE409	Short	P[4]	G2
DE410	Long	P[8*]	G1*
DE411	Long	P[8]	G1
DE412	Long	P[8*]	G1*
DE416	Long	P[8]	G1*
DE420	Long	P[8*]	G1*
DE421	Short	P[4]	G2
DE422	Long	P[8]	G1*
DE426	Short	P[4]	G2
DE428	Long	P[8*]	G4
DE441	Long	P[8]	G1*
DE442	Long	P[8*]	G1*
DE447	Long	P[8*]	G1*
DE448	Long	P[8*]	G1*
DE454	Long	P[8*]	G1*
DE464	Short	P[4]	G2
DE469	Short	P[4]	G2
DE473	Long	P[8*]	G1*
DE474	Short	P[4]	G2
DE476	Short	P[4]	G2
DE487	Long	P[8*]	G4
DE491	Short	P[4]	G2
DE492	Long	P[8]	G1
DE495	Long	P[4]/P[8*]	G2/G1
DE496	Long	P[4]/P[6]	G1*
DE497	Long × 2	P[6]/P[4]	G4
DE500	Short	P[4]	G2
DE506	Long	P[8*]	G1*
DE507	Long	P[8]	G1*
DE514	Long	P[8]	G1*
DE515	Long	P[8*]	G1*
DE516	Short	P[6]	G2
DE517	Short	P[4]	G2
DE519	Long	P[8*]	G1*
DE520	Short	P[4]	G2
DE527	Short	P[4]	G2
DE531	Long	P[8*]	G1*
DE532	Long	P[4]	G1*
DE540	Unclear	P[8*]	G2
DE542	Short	P[4]	G2
DE544	Unclear	P[8*]	G1*
DE546	Short	P[4]	G2
DE547	Long	P[4]	G1*
DE548	Long	P[4]	G1*
DE550	Long	P[4]	G1*
DE551	Short	P[4]	G2
DE552	Short	Not typeable	G2
DE553	Long × 2	P[8]/P[4]	G1/G1*

Long × 2, Two different long electropherotypes were observed.

DISCUSSION

Rotavirus was the most important viral enteropathogen in this survey of the aetiology of childhood gastro-enteritis in Ho Chi Minh city, Vietnam, being responsible for 40% of the cases admitted to hospital. This frequency lies within the world-wide range reported for hospitalized infants by Hart and Cunliffe (1999), and is close to that, of 50%, reported both for a rural area close to Ho Chi Minh city (for the period 1994–1996; Nishio *et al.*, 2000) and from four other cities in Vietnam (for the period 1995–2000; Nguyen *et al.*, 2001). In a global collection of rotaviruses, Gentsch *et al.* (1996) found that four strains — P[8] G1, P[4] G2, P[8] G3 and P[8] G4 — predominated, although some of their samples were non-typeable. In the present study from Vietnam, every strain was G-typeable and only one strain was not P-typeable. Although P[8] G1 (and variants on it) formed the commonest 'strain' detected in the present study (represented by at least 20 isolates), the many G1* and/or P[8*] viruses would not have been typeable had not the newly designed PCR primers, which detect these variants (Cunliffe *et al.*, 2001*b*), been employed. The next commonest strains were P[4] G2 (confirmed for 16 isolates) and then, much more rarely, P[4] G1* (at least four isolates) and P[8*] G4 (two isolates); no G3 strains were detected. In previous studies of Vietnamese rotaviruses (which took no account of the P[8] and G1 variants), the results have been slightly different. Although Nishio *et al.* (2000), working in a rural area on the outskirts of Ho Chi Minh city, also found G1 to be the most common G-type (29%) and G3 to be absent, they observed more G4 isolates (27%) than G2 (16%). However, 28% of their isolates were not G-typeable. When Nguyen *et al.* (2001) analysed a subset of the rotaviruses they had collected in four Vietnamese cities, they found that G2 predominated (53%), with significantly fewer isolates typed as G1 (13%), G4 (17%) or G3 (3.6%); only a small proportion of their rotaviruses were not G-typeable using the conventional PCR primers but 10% were not P-typeable.

It appears that the G1* and/or P[8]* variants detected previously in Malawi in 1999 (both G1* and P[8*]) and the U.K. in 1995–1996 (P[8*] only) are also circulating in Vietnam. Full confirmation would require nucleotide sequence analysis of the VP4 and VP7 genes. Four of the patients investigated in the present study were each found to be co-infected with two different rotaviruses. Such co-infection has been observed before, predominantly in developing countries such as Bangladesh (Tabassum *et al.*, 1994), India (Jain *et al.*, 2001) and Vietnam, where 9% of the infections investigated by Nguyen *et al.* (2001) were with two different rotaviruses. In co-infected individuals, two different rotaviruses can infect the same enterocyte, allowing novel viruses to arise by re-assortment (Desselberger, 1996; Palombo, 2002).

By demonstrating a high prevalence of variant G1 and P8 rotavirus genotypes and the occurrence of unusual G- and P-genotype combinations, the present results again reveal the tremendous diversity of human rotaviruses (Cunliffe *et al.*, 2002). Knowledge of this variation is important to those designing and using the next generation of rotavirus vaccines, since such vaccines may need to encompass the recently detected G- and P-types, including the variants. At the nucleotide level, the Vietnamese P[8*] variants were >98% similar to the Malawian P[8*]. However the Vietnamese G1* were very different from the prototype Wa G1 and only 92% similar to the Malawian G1*. Whether the variant P[8*] and G1* genotypes are sufficiently different from the progenitor P[8] and G1 strains to allow them to cause disease in those immunized with P[8] G1 virus is not clear. However, it is noteworthy that the Malawian P[8*] G4 strains formed a cluster, with only 90% homology, at the amino-acid level, to other P[8] rotaviruses (Cunliffe *et al.*, 2001*b*).

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Appendix B:

Clinical Record Form of The RCT in Chapter Six

CHILDHOOD SHIGELLOSIS STUDY EG _____,

Name: _____ , Hospital ID [SHS]:_____

Address: _____, Ward/Village: _____,

District: _____, Province :_____

Age (m)[Tháng]: _____, Boy/Girl, Weight:_____kg, Height:_____cm

Date of Admission to Hospital [Ngày vào viện]: _____

Date of Study [Ngày vào NC]: _____ Time: _____

Duration of Present Illness [Đã bệnh bao lâu]: _____ hours

PAST HISTORY [TI ỀN S Ử]:

Febrile convulsion [Sốt làm kinh]: Có , Không

Bloody Diarrhea [Tiêu đàm máu]: Có , Không

Breast feeding [Bé có đã/đang bú mẹ chút ít nào không?]: Có , Không

FAMILY HISTORY :

Học lực Mẹ [Mom education]: Mù chữ , TiểuH , TrungH , CĐ_ĐH

Kinh tế [Econo. Status]: rất nghèo , nghèo , trung bình , khá giả

Số người cùng ở trong nhà [Total people living together] : _____

Nước sinh hoạt [Running water]: sông-ao , giếng , có đồng hồ nước

Đi tiêu trên sông/ao [Public toilet] , nhà có cầu tiêu riêng [Private toilet]:

HISTORY OF PRESENT ILLNESS [BỆNH SỬ]:

	Day 1	Day 2	Day 3
Fever (+/–) [Sốt]			
Convulsion (No.) [Co giật – Số lần]			
Vomitting (No.) [Ói mửa – số lần]			
Abdo.Pain (+/–) [Đaubụng]			
Tenesmus (+/–) [Mót rặn]			
WateryDia(No.)[Tiêulỏng- Số lần/ngày]			
MucoidDia(No.)[Tiêu nhớt -Số lần/ngày]			
Bloody Dia(No.) [Tiêu có máu- Số lần/ngày]			
Abd.Distention (+/–)[Chướng bụng]			
Rect.Prolapse (+/–) [Sa trực tràng]			
Antibiotics used: (Tên & liều KS đã dùng/ Không có/Không biết) AB/N/U			
Others			

OBSERVATION SHEET EG _____

Name: _____, Hospital ID: _____

Date																				
Time																				
Study Time	0	6	12	18	24	30	36	42	48	54	60	66	72	78	84	90	96	102	108	114
Drug C / G																				
41																				
40																				
C° 39																				
38																				
37																				
36																				
T° (Number)																				
Pulse [Mạch]																				
BP [HA]																				
RR [Nhịp thở]																				
Dehydration [không/vừa/nặng]																				
Vomitting [ói]																				
Seizure [co giật]																				
Ab.Pain [Đaubụng]																				
Tenesmus [rặn]																				
RectalProlapse +/-																				
Total stools /24 h																				
Time of 1st non-bloody stool [tiêu không máu lần đầu]																				
Time of 1st normal stool [phân tốt lần đầu]																				
Drug Rash																				
Stool WBC																				
Stool RBC																				
WBC [BC]																				
Plt [TC]																				
Hct																				
Glucose																				
Bacter [VT]																				

OBSERVATION SHEET EG _____

Name: _____, Hospital ID: _____

Date																
Time																
Study Time	120	126	132	138	144	150	156	162	168	174	180	186	192	198	204	210
Drug C / G																
41																
40																
39																
C° 38																
37																
36																
T° (Number)																
Pulse [Mạch]																
BP [HA]																
RR [Nhịp thở]																
Dehydration [không/vừa/nặng]																
Vomitting [ói]																
Seizure [co giật]																
Abd.Pain [Đaubụng]																
Tenesmus [rặn]																
Rectal Prolapse [satrựctrảg] +/-																
Total stools /24 h																
Time of 1st non-bloody stool [tiêu khộg máu lần đầu]																
1st normal stool [phân tốt lần đầu]																
Drug Rash																
Stool WBC																
Stool RBC																
WBC [BC]																
Plt [TC]																
Hct																
Glucose																
Bacter [VT]																

CLINICAL SUMMARY EG _____

Name: _____, Hospital ID: _____

Nutrition Status: Overweight / Normal / Malnutrition I / II / III

Duration of illness before study:_____hours

Fever Clearance Time: _____hours

Bloody Diarrhea Stopping Time: _____hours

Diarrhea Stopping Time: _____hours

Bacterial Clearance Time: _____days

Complications during Study:

_____

Pathogen: _____

Fully sensitive / NalS / NalR / MDR / others

Adverse drug Reaction: rash / drug fever / anaphylaxis

Outcome: Cure / Clinical Failure / Microbio.Failure / Clinico-microFailure / Relapse

Doctor: _____

FOLLOW-UP 1-2 WEEKS AFTER DISCHARGE

Date: _____/_____/ 200___

Healthy: Y / N

Diarrhea: Y / N ; Others: _____

_____

Stool / Rectal swap taken: Y / N

Doctor: _____

EG Shigellosis in Children Study CHECK LIST

[BẢNG KIỂM NHỮNG VIỆC ĐÃ LÀM XONG √]

1. CLINICAL DIAGNOSIS OF INVASIVE DIARRHEA:
   - Mucoid-bloody diarrhea [Tiêu đàm máu]
   - Mucoid diarrhea+AbdPain/tenesm. [Tiêu lỏng có nhớt + đbụg/rặn]
2. CONSENT TO TRIAL [Cha mẹ BN ký đồng ý vào NC]
3. RANDOMISED [Mở bao thư đúng thứ tự NC]
4. STOOL/RECTAL SWAB TAKEN FOR CULTURE & EXAM (Day1)

   [Lấy mẫu phân gửi phòng XN khi vào nghiên cứu]
5. BLOOD FOR CBC, BG [Thử CTM, ĐH] (Day1)
6. EVERY 6-HOURS TEMPERATURE AND ASSESSMENT

   [Ghi mạch nhiệt, số lần đi tiêu và tính chất phân mỗi 6 giờ]
7. STOOLS/RECTAL SWAB FOR CULTURE [Lấy mẫu cấy phân mỗi ngày]:
   - Day 2
   - Day 3
   - Day 4
   - Day 5 [nếu cần]
   - Day 6 [nếu cần]
8. CLINICAL SUMMARY [Ghi bản tổng kết lâm sàng]
9. ONE WEEK FOLLOW-UP & STOOL/R.SWAB CULTURE
   - [Tái khám và Cấy phân sau xuất viện 1 tuần]

Doctor: _____

Appendix C:

Study Information Sheet and Consent Form

SỞ Y TẾ TP HỒ CHÍ MINH
BỆNH VIỆN BỆNH NHIỆT ĐỚI
(Hospital for Tropical Diseases)
190 Hàm Tử, F.1 Q.5

ĐT: (84 – 8) 8353704 – 8353804
Fax: (84 – 8) 8353943 – 8353904

CỘNG HÒA XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập – Tự do – Hạnh phúc

Ngày 15 tháng 6 năm 2006

Dr Hà Vinh 0913 195507

INFORMATION SHEET

OXTREC No: 010-06

An open randomized comparison of Ciprofloxacin versus Gatifloxacin for the treatment of shigellosis in children

Introduction to the study

Your child is being asked to be in a research study on shigellosis. Shigellosis is a serious infection and in Viet Nam is now becoming difficult to treat. This study is designed to see if we can improve the treatment of shigellosis.

Stools Tests:

Whether or not your child takes part in the study your child will be given the same standard of care for her/his illness. If you wish your child to take part in the study we will randomize the treatment your child receives to one of two alternatives. We do not know which one your child will receive or which is the best treatment. We believe both treatments to be effective. We will ask to take stool samples during your child stay in hospital and for the next week after your child are discharged from hospital to ensure that your child have cleared the infection. This is very important to ensure that your family and friends are not at risk of infection. Further tests on these stored samples may be undertaken in the future to further the understanding of this disease.

Confidentiality

We will keep the information we get from you as private. Your child name will not be on your test results–we will use a number instead of your name. These results will be under the authority and supervision of the doctor responsible for your child inpatient care. The doctor will discuss these results with you. All this information will be kept confidential in your child medical records. Your child name will not be mentioned in any papers or speeches about the study.

Risks

There are very few risks to your child from being in our study. All the drugs being used in this study are routinely used in Viet Nam.

Costs

There will be no cost to your child.

Refusal to participate

Your child may refuse to be in any parts of the study. If you do not want your child to be in the study that decision will not in any way interfere with your child ability to receive proper medical care or attention.

Questions:

If you have any other questions about the study please ask the doctor on the ward or the doctors named at the top of this sheet: Dr. Hà Vinh 0913195507 (This is the doctor you see every day. Please ask any nurse or doctor who will help you)

TRANG THÔNG TIN

OXTREC No: 010-06

Nghiên cứu so sánh ngẫu nhiên tác dụng giữa 2 loại thuốc Ciprofloxacin và Gatifloxacin trong điều trị bệnh lỵ trực trùng trẻ em

BS Hà Vinh 0913 195507

Giới thiệu nghiên cứu

Con (hay cháu) của Ông/Bà được đề nghị tham gia vào nghiên cứu bệh lỵ trực trùng.Lị trực trùng là một loại bệnh nhiễm trùng nặng ở Việt Nam, và hiện nay đang trở nên rất khó điều trị. Nghiên cứu này được thực hiện nhằm cải tiến việc điều trị bệnh lỵ trực trùng.

Các xét nghiệm

Cho dù bệnh nhân có tham gia vào nghiên cứu hay không thì đều nhận được tiêu chuẩn điều trị và chăm sóc như nhau. Nếu tham gia vào nghiên cứu Con (cháu)Ông/Bà sẽ nhận được điều trị ngẫu nhiên theo một trong 2 cách. Chúng tôi sẽ không biết con/cháu bạn sẽ được điều trị theo phương cách nào cũng như cách nào là tốt nhất. Tuy nhiên chúng tôi tin rằng cả 2 cách điều trị đó đều có hiệu quả. Trong suốt thời gian con/cháu Ông /Bà nằm viện, chúng tôi sẽ tiến hành lấy mẫu phân để xét nghiện tìm vi trùng gây bệnh. Đồng thời sau 1-2 tuần sau khi xuất viện, chúng tôi lại lấy mẫu phân như trên một lần nữa để đảm bảo con/cháu Ông/Bà hoàn toàn không còn bị nhiễm trùng nữ. Một điều rất quan trọng là phải chắc chắn rằng người thân trong gia đình và bạn bè của con/cháu Ông/Bà không có nguy cơ bị nhiễm bệnh.

Bảo mật thông tin

Chúng tôi sẽ bảo mật những thông tin thu được từ bệnh nhân. Chúng tôi sẽ ghi mã số thay vì ghi tên của bệnh nhân trên các mẫu xét nghiệm, do đó tên của con/cháu Ông/Bà sẽ không thể hiện trên các kết quả xét nghiệm. Các kết quả này sẽ được kiểm soát chặt chẽ bởi Bác sĩ, người duy nhất được biết kết quả và chịu trách nhiệm điều trị bệnh nhân, cũng như thông báo kết quả đến họ. Tất cả thông tin trong quá trình điều trị đều được ghi nhận và bảo mật trong hồ sơ bệnh án. Tên của con/cháu Ông/Bà sẽ không được đề cập đến trong bất kỳ bài báo hay thảo luận nào về nghiên cứu này.

Các nguy cơ

Sẽ có rất ít nguy cơ khi con/cháu Ông/Bà tham gia vào nghiên cứu này. Các loại thuốc điều trị trong nghiên cứu là những loại thuốc được dung phổ biến ở Việt Nam. Lấy phân bằng que bông gòn có thể gây khó chịu chút đỉnh.

Chi phí

Con cháu Ông/Bà tham gia vào nghiên cứu sẽ không phải tốn bất kỳ chi phí nào.

Từ chối tham gia nghiên cứu

Bệnh nhân có thể từ chối tham gia nghiên cứu bất cứ phần nào của nghiên cứu. Cho dù con/cháu Ông/Bà không tham gia nghiên cứu cũng được điều trị phù hợp với bệnh lý của mình.

Các thắc mắc về nghiên cứu

Nếu Ông/Bà có bất cứ thắc mắc nào khác về nghiên cứu này xin vui lòng liên hệ với Bác sĩ tại khoa điều trị hay Bác sĩ có tên trên đầu trang thông tin này: BS Hà Vinh 0913195507. (Đây là Bác sĩ mà Ông/Bà/Cháu gặp hàng ngày tại khoa. Ông / Bà cũng có thể hỏi bất cứ Bác sĩ hay Điều dưỡng nào trong khoa để được giúp đỡ).

CONSENT FORM

OXTREC No: 010-06:

An open randomized comparison of Ciprofloxacin versus Gatifloxacin for the treatment of shigellosis in children

Consent from patient's parents/caregivers:

I have been fully informed of the possible risks and benefits of taking part in this study and agree to let my child take part. I agree that the samples may be stored and that further tests may be undertaken on these samples in the future to further the understanding of this disease.

Name of patient parents _____ Signature: _____

Relationship with patient:_____ Date:_____

Name of physician _____ Signature: _____

Date_____

If the patient's relative give verbal consent to take part in the trial but is unable to sign, the physician can record the consent here:

Name of physician:_____ Signature:_____

Date:_____

GIẤY ĐỒNG Ý:

OXTREC No: 010-06:

Nghiên cứu so sánh ngẫu nhiên tác dụng giữa 2 loại thuốc Ciprofloxacin và Gatifloxacin trong điều trị bệnh lỵ trực trùng trẻ em

Đồng ý của cha mẹ hoặc người nuôi dưỡng bệnh nhân:

Tôi đã được nghe Bác sỹ/ Điều dưỡng cho biết điều thuận lợi và không thuận lợi có thể xảy ra khi tham gia vào nghiên cứu này, và tôi đồng ý để con tôi được tham gia. Tôi đồng ý những bệnh phẩm có thể được trữ lại để về sau làm các xét nghiệm cần thiết cho sự hiểu biết về bệnh này .

Tên của Cha mẹ:_____ Ký tên: _____

Quan hệ với bệnh nhân:_____ Ngày:_____

Tên thầy thuốc _____ Ký tên: _____

Ngày:_____

Nếu thân nhân bệnh nhân nói đồng ý nhưng không viết chữ được thì thầy thuốc ghi lời đồng ý và ký tên dưới đây:

Tên thầy thuốc _____ Ký tên: _____

Ngày:_____

Appendix D:

International Standard Randomised Controlled Trial Number Registration

A research study to compare 2 antibacterial drugs, gatifloxacin with ciprofloxacin, for the treatment of dysentery in children	
ISRCTN	ISRCTN55945881
ClinicalTrials.gov identifier	
Public title	A research study to compare 2 antibacterial drugs, gatifloxacin with ciprofloxacin, for the treatment of dysentery in children
Scientific title	A randomised controlled trial of gatifloxacin versus ciprofloxacin for the treatment of bacillary dysentery in children
Acronym	EG
Serial number at source	061330
Study hypothesis	In 2004, The World Health Organization (WHO) organised a meeting of experts around the world and recommended that ciprofloxacin or other fluoroquinolones should be used to treat shigellosis in children as well as adults. However after being used for some years to treat shigellosis, the clinical response to ciprofloxacin treatment has decreased, strains of Shigella dysenteriae type 1 resistant to ciprofloxacin were also detected. This is why searching for alternative regimens is obviously needed. This study will compare the currently recommended WHO regimen with a newer, affordable and potentially more active fluoroquinolone, i.e., gatifloxacin.
Ethics approval	Ethics approval received from Oxford Tropical Research Ethics Committee on the 20th June 2006 (ref: OXTREC 010-06).
Study design	Open-label randomised controlled trial
Countries of recruitment	Viet Nam
Disease condition study domain	Shigella dysentery
Participants - inclusion criteria	1. Patients aged 0 - 14, either sex 2. Symptomatic uncomplicated dysentery 3. Gives consent
Participants - exclusion criteria	No consent given.
Anticipated start date	01/06/2006
Anticipated end date	30/03/2009
Status of trial	Completed
Patient information material	
Target number of participants	366
Interventions	Patients are randomised to: 1. Ciprofloxacin 15 mg/kg body weight taken orally every 12 hours for a total of 6 doses in 3 days, or 2. Gatifloxacin 10 mg/kg body weight taken orally every 24 hours for a total of 3 doses in 3 days Follow-up will occur for 7 - 10 days after discharge from the hospital.
Primary outcome measure(s)	Failure of treatment: 1. Persistent fever at Day 5 2. Failure to clear completely the admission symptoms at Day 5 3. Stool culture positive at Day 3 of treatment 4. Need for 'rescue' treatment with ceftriaxone 5. The development on treatment of any complication
Secondary outcome measure(s)	1. Fever clearance time: from the start of treatment until axillary temperature falls to 37.5°C and remains at or below this value for greater than 48 hours 2. Bloody diarrhoea clearance time: the time to the last stool containing visible blood passed 3. Diarrhoea clearance time: the time to the first formed stool 4. Bacterial clearance time: time to the last positive stools culture for Shigella
Sources of funding	The Wellcome Trust (UK) (grant ref: 061330)

Trial website	
Publications	
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