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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 IS$Ecp1$ element and demonstrated considerable sequence homology with other IncF1 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* 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.

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.
Shigella 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.

**Author Summary**

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 Enterobacteriaceae, 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 – sulpha-methoxazole (SXT), tetracycline (TET), nalidixic acid (NAL), ofloxaculin (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) (10 μg). 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
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× TBE 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, TEM, CTX-M, GES, NDM, IMP, OXA, and other class A, B, and C ESBLs. The resulting PCR products were separated and purified using QIAquick PCR purification kit (Qiagen, USA) and sequenced as described previously [31,32]. Further characterisation of the various sub-group of blaCTX ESBL genes was performed using primers, CTX-M-1; (F 5′ ATGGTTAAAAATCTACCTGGC, R 5′ TTACAGTTAGCGTGCAGTACG) and CTX-M-2; (F 5′ TGGAGGGCGTGGGAAAAAGT and R 5′ TTACATGCGTGGTCTGTT) and CTX-M-9; (F 5′ ATGGTGACAAGAAGAGTTCAAC, R 5′ TTACAGGCCGCGCAGTGGGC) using previously outlined PCR amplification conditions [31,32].

To identify an association with CTX-M genes and the adjacent ISεp1 transposase, all ESBL positive strains were subjected to PCR with primers forward primers Tnp24F 5′ CCAGTCGGCTGCGCATATAAACCGG, Tnp15F 5′ CGGGGCGTGAAGGGTTCGG. The Tnp24F and Tnp15F were located within the blaCTX-M-24 and blaCTX-M-15 genes respectively and TnpR was located within the ISεp1 transposase gene. The blaCTX-M in Shigella spp.

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 blaCTX 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. DNAPlotter [36] 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 [13]. The general antibiotic sensitivity patterns in these strains were variable, although resistance to trimethoprim – sulphonamethoxazole, 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 these strains.
| Strain ID | Serotype | Age (months) | Sex | Month | Year | Province | ESBL (+/-) | AMP | CHL | SXT | TET | NAL | OFX | CRO |
|-----------|----------|--------------|-----|-------|------|----------|------------|-----|-----|-----|-----|-----|-----|-----|
| DE611     | S. sonnei | 10           | M   | February | 2001 | HCMC     | +           | R              | >256 | R     | 8    | R    | >32 | 128 | S    | 2    | 0.06 | R    | >255 |
| EG0356    | S. sonnei | 48           | M   | May     | 2007 | HCMC     | +           | R              | >256 | S     | 6.0  | R    | >32 | 64  | R    | 64   | 0.38 | R    | >256 |
| EG0373    | S. sonnei | 30           | M   | June    | 2007 | HCMC     | +           | R              | >256 | S     | 6.0  | R    | >32 | 128 | S    | 1.5  | 0.064 | R    | >256 |
| EG0384    | S. sonnei | 36           | M   | July    | 2007 | HCMC     | +           | R              | >256 | S     | 6    | R    | >32 | 256 | R    | 32   | 0.38 | R    | >256 |
| EG0390    | S. sonnei | 17           | M   | August  | 2007 | VINH LONG | +           | R              | >256 | S     | 6    | R    | >32 | 128 | R    | >256 | 0.38 | R    | >256 |
| EG0395    | S. sonnei | 36           | F   | September | 2007 | HCMC    | +           | R              | >256 | S     | 12   | R    | >32 | 96  | R    | >256 | 0.5  | R    | >256 |
| EG0162    | S. sonnei | 28           | M   | October | 2007 | DONG THAP | +           | R              | >256 | S     | 8    | R    | >32 | 48  | R    | 64   | 0.38 | R    | >256 |
| EG0419    | S. flexneri | 23            | F   | December | 2007 | HCMC    | -           | R              | >256 | R     | >256 | R    | >32 | 48  | R    | >256 | 0.5  | R     | 128  |
| EG0187    | S. sonnei | 16           | M   | January | 2008 | DONG THAP | +           | R              | >256 | S     | 3    | R    | >32 | 192 | S    | 1.5  | 0.047 | R    | 24   |
| EG0421    | S. sonnei | 36           | F   | January | 2008 | HCMC     | +           | R              | >256 | S     | 4    | R    | >32 | 128 | R    | 64   | 0.38 | R    | >32  |
| EG0424    | S. sonnei | 48           | F   | January | 2008 | HCMC     | +           | R              | >256 | S     | 6    | R    | >32 | 64  | R    | >256 | 0.38 | R    | >256 |
| EG0204    | S. sonnei | 26           | F   | March   | 2008 | DONG THAP | +           | R              | >256 | S     | 6    | R    | >32 | 32  | R    | 64   | 0.38 | R    | >256 |
| EG0430    | S. sonnei | 36           | F   | March   | 2008 | HCMC     | +           | R              | >256 | S     | 6    | R    | >32 | >256 | R    | 48   | 0.25 | R    | 128  |
| EG1008    | S. sonnei | 18           | M   | May     | 2008 | LONG AN   | +           | R              | >256 | S     | 8    | R    | >32 | 96  | R    | 128  | 0.38 | R    | >256 |
| EG1009    | S. sonnei | 8            | M   | May     | 2008 | HCMC     | +           | R              | >256 | S     | 8    | R    | >32 | 96  | R    | 192  | 0.38 | R    | >256 |
| EG1010    | S. sonnei | 60           | F   | May     | 2008 | HCMC     | +           | R              | >256 | S     | 6    | R    | >32 | 96  | R    | >256 | 0.5  | R    | >256 |
| EG1013    | S. sonnei | 25           | M   | June    | 2008 | HCMC     | +           | R              | >256 | S     | 6    | R    | >32 | 96  | R    | >256 | 0.25 | R    | >256 |
| EG1012    | S. sonnei | 15           | F   | June    | 2008 | HCMC     | +           | R              | >256 | S     | 8    | R    | >32 | 96  | R    | 192  | 0.38 | R    | >256 |
| EG1011    | S. sonnei | 108          | F   | June    | 2008 | HCMC     | +           | R              | >256 | S     | 8    | R    | >32 | 96  | R    | 128  | 0.38 | R    | >256 |
| EG1007    | S. sonnei | 48           | M   | July    | 2008 | LONG AN   | +           | R              | >256 | S     | 6    | R    | >32 | 64  | R    | 48   | 0.38 | R    | 192  |
| EG0250    | S. sonnei | 35           | M   | August  | 2008 | DONG THAP | +           | R              | >256 | S     | 6    | R    | >32 | 48  | R    | 48   | 0.25 | R    | >256 |
| EG0250a   | S. sonnei | 36           | M   | September | 2008 | DONG THAP | +           | R              | >256 | S     | 6    | R    | >32 | 48  | R    | 48   | 0.25 | R    | >256 |
| EG0471    | S. flexneri | 49            | M   | September | 2008 | HCMC    | +           | R              | >256 | R     | >256 | R    | >32 | 128 | R    | >256 | 0.5  | R    | >256 |
| EG0472    | S. sonnei | 66           | M   | September | 2008 | HCMC     | +           | R              | >256 | S     | 4    | R    | >32 | 96  | R    | 48   | 0.38 | R    | >256 |
| EG1014    | S. sonnei | 29           | M   | January | 2009 | LONG AN   | +           | R              | >256 | S     | 6    | R    | >32 | >256 | R    | >256 | 0.25 | R    | >256 |
| EG1015    | S. sonnei | 72           | F   | January | 2009 | HCMC     | +           | R              | >256 | S     | 4    | R    | >32 | 32  | R    | 48   | 0.25 | R    | >256 |
| EG1016    | S. sonnei | 39           | M   | January | 2009 | HCMC     | +           | R              | >256 | S     | 6    | S    | 0.38 | S   | 1.5  | R    | 48   | 0.25 | R    | >256 |
| EG1017    | S. sonnei | 11           | F   | February | 2009 | HCMC     | +           | R              | >256 | S     | 5    | R    | >33 | 97  | R    | 49   | 1.38 | R    | >256 |
| EG1018    | S. sonnei | 29           | M   | February | 2009 | HCMC     | +           | R              | >256 | S     | 6    | R    | >32 | 48  | R    | >256 | 0.38 | R    | >256 |
| EG1019    | S. sonnei | 120          | F   | February | 2009 | HCMC     | +           | R              | >256 | S     | 6    | R    | >32 | >256 | R    | 48   | 0.25 | R    | >256 |
| EG1020    | S. sonnei | 48           | M   | March   | 2009 | HCMC     | +           | R              | >256 | S     | 8    | R    | >32 | 64  | R    | 192  | 0.38 | R    | >256 |
| EG1021    | S. sonnei | 20           | M   | March   | 2009 | HCMC     | +           | R              | >256 | S     | 8    | R    | >32 | 64  | R    | >256 | 0.25 | R    | >256 |
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 *Shigella* isolated that were resistant to ceftriaxone fluctuated over the following 18 months. However, there was an 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 *Shigella* 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 of 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

| Antimicrobial Tested | AMP | CHL | SXT | TET | NAL | OFX | CRO |
|----------------------|-----|-----|-----|-----|-----|-----|-----|
| Strain ID Serotype   |     |     |     |     |     |     |     |
| Age (months)         |     |     |     |     |     |     |     |
| Sex                  |     |     |     |     |     |     |     |
| Month                |     |     |     |     |     |     |     |
| Year                 |     |     |     |     |     |     |     |
| Province             |     |     |     |     |     |     |     |

*bla* _CTX-M_ in *Shigella* spp.
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 blaTEM, blaSHV and blaCTX-M genes. Further PCR amplifications were performed on DNA from all strains that produced amplicons with the blaCTX-M primers.
blaCTX-M in Shigella spp.
Primers that were specific for the three major CTX-M clusters, \(\text{bla}_{\text{CTX-M-9}}\), \(\text{bla}_{\text{CTX-M-1}}\) and \(\text{bla}_{\text{CTX-M-2}}\) were selected [40]. Three strains (DE0611, EG0187 and EG0356) produced amplicons with the \(\text{bla}_{\text{CTX-M-9}}\) primers and the remaining 32 isolates produced amplicons with the \(\text{bla}_{\text{CTX-M-1}}\) primers (Table 2). All 35 PCR amplicons were sequenced. Sequence analysis of the PCR amplicons demonstrated that there were two differing \(\text{bla}_{\text{CTX-M}}\) genes present in the \(\text{Shigella}\) population, these were, \(\text{bla}_{\text{CTX-M-24}}\) (n = 3, 8%) and \(\text{bla}_{\text{CTX-M-15}}\) (n = 32, 92%) (Table 2). Both genes (\(\text{bla}_{\text{CTX-M-24}}\) and \(\text{bla}_{\text{CTX-M-15}}\)) share 74% DNA homology with each other; \(\text{bla}_{\text{CTX-M-15}}\) and \(\text{bla}_{\text{CTX-M-24}}\) differ by 12 and 6 nucleotides from the precursor.

Table 2: Characterisation of \(\text{bla}_{\text{CTX-M}}\) genes and the corresponding plasmids of ESBL expressing \(\text{Shigella spp.}\)

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

*Estimated plasmid size by agarose gel electrophoresis with known markers.

bConjugation frequency calculated per donor cell from the mean of two replicates.
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genes within their respective parent groups, (blaCTX-M-1 and blaCTX-M-5).

Plasmid sizing, by visualisation of the previous agarose gel electrophoresis demonstrated that the estimated plasmid size corresponded with either the blaCTX-M gene (Table 2); blaCTX-M-15 was consistently located on a plasmid larger than that associated with blaCTX-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; 29mm, CTX-M-15; 20mm) (Table 2).

All blaCTX-M harboursing plasmids with the exception of the plasmid in strain EG1020 were transmissible with high conjuga
tion frequencies, ranging from 4.84x10^7 to 4.88x10^9 (median 1.55x10^8) per donor cell (Table 2). The mobilisation of one of these blaCTX-M harboursing 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, naïve S. sonnei strain at a similarly high frequency.

DNA sequence analysis of the pEG356 plasmid

The ESBL encoding gene blaCTX-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 blaCTX-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 sequencing.
applicable to this location, for further characterisation by DNA conjugative pilus with high sequence similarity to the transfer main structural features, a replication region, the ESBL gene premature stop codons, frameshifts or missing start codons. The 14 were considered to be pseudogenes on the basis of apparent the DNA sequence homology to the replication region) (Figure 3).

restricted to the plasmid from isolate EG0356, carrying a pEK499 (Ac. EU935739) isolated from an ESBL carrying plasmids, pEG356 does not carry multiple segregational killing genes are missing from within the plasmid genes of unknown function, genes involved in conjugative transfer [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 (sbd). 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 ColI plasmid pAPEC-O1 (Ac. DQ301420), isolated from an avian pathogenic E. coli strain [34] (Figure 4). pEG356 shared around 80% of the gene content with pAPEC-O1, including the conjagation (tra), replication (rep) and a putative ATP iron transport system (irp). The tra region consisted of four coding sequences, which include, a putative permease, an iron binding protein and an export associated protein.

The blaCTX-M-24 was located on an IS911 element. The overall sequence of the IS911 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 blaCTX-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 blaCTX-M-24 gene (Figure 4) [44].The IS911 element contains a IS911 transposase and a small hypothetical coding sequence of unknown function which is spanned by two IS1380 elements. The blaCTX-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 IS911 transposase. The location of the PCR primers TraP24F and TraR are highlighted in Figure 4 and were designed to produce an amplicon if the bla gene and the adjacent IS911 transposase were in the same location and orientation in strains with a blaCTX-M-24. A secondary forward primer was designed in equivalent location for those strains with a blaCTX-M-15 (Tra15F). Therefore, if blaCTX-M-24 or the blaCTX-M-15 was consistently adjacent to the IS911 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 blaCTX-M-15 and the blaCTX-M-24 gene were associated with an IS911 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 [9]. 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 Shigella 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,
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\textit{CTX-M-15} and \textit{bla\textit{CTX-M-24}} genes. Current data suggests that \textit{bla\textit{CTX-M-24}} is found mainly in Asia [41,42], yet may have been transferred to other locations [43]. MDR CTX-M-15 producing \textit{E. coli} is emerging worldwide as an important pathogen causing hospital-acquired infections [2]. The potential impact of MDR \\textit{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 \textit{bla\textit{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 \textit{bla\textit{CTX-M-24}} gene has been located on a relatively uncompli-

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Figure 3. A schematic representation of the \textit{bla\textit{CTX-M-24}} encoding plasmid, pEG356. pEG356 is a 70,255bp IncF I 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 \textit{ISEcp1} type element carrying the \textit{bla\textit{CTX-M-24}} is distinguished by grey shading.

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cated plasmid in Asia, however, pKP96 only demonstrates limited homology to pEG356 [44].

All ESBL gene were located adjacent to a IS\textit{Ecp1} transposase (as identified by PCR). We are currently unable to substantiate if it is the IS\textit{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. \textit{S. sonnei} is a...
monophyletic bacterial pathogens, 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 sufficiently [data not shown]. Further epidemiological investigation of CTX-M containing strains combined with a more sensitive sequence-based methodology, such as is attested for Shigella Typhimurium 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 cepodoxime 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 PTD LTMV. 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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