Genomic serotyping, clinical manifestations, and antimicrobial resistance of non-typhoidal *Salmonella* gastroenteritis in hospitalized children in Ho Chi Minh City, Vietnam

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Running title

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Abstract

Nontyphoidal *Salmonella* (NTS) are among the most common aetiological agents of diarrhoeal diseases worldwide and have become the most commonly detected bacterial pathogen in children hospitalised with diarrhoea in Vietnam. Aiming to better understand the epidemiology, serovar distribution, antimicrobial resistance (AMR), and clinical manifestation of NTS gastroenteritis in Vietnam, we conducted a clinical genomics investigation of NTS isolated from diarrheal children admitted to one of three tertiary hospitals in Ho Chi Minh City. Between May 2014 and April 2016, 3,166 children hospitalized with dysentery were recruited into the study; 478 (~15%) children were found to be infected with NTS by stool culture. Molecular serotyping of the 450 generated genomes identified a diverse collection of serogroups (B, C1, C2-C3, D1, E1, G, I, K, N, O, Q); however, *S.* Typhimurium was the most predominant serovar, accounting for 41.8% (188/450) of NTS isolates. We observed a high prevalence of AMR to first line treatments recommended by WHO and more than half (53.8%, 242/450) of NTS isolates were multi-drug resistant (MDR; resistant to ≥3 antimicrobial classes). AMR gene detection positively correlated with phenotypic AMR testing, and resistance to empirical antimicrobials was associated with a significantly longer hospitalization (0.91 days, p=0.04). Our work shows that genome sequencing is a powerful epidemiological tool to characterize the serovar diversity and AMR profiles in NTS. We propose a revaluation of empirical antimicrobials for dysenteric diarrhoea and endorse the use of whole genome sequencing for sustained surveillance of NTS internationally.

Introduction

With an estimated 93.8 million cases (5^{th}-95^{th} percentile, 61.8-131.6 million) of gastroenteritis recorded globally per annum, Nontyphoidal *Salmonella* (NTS) are among the most common etiological agents of diarrheal diseases worldwide. This burden disproportionally affects young children in Asia and Africa, and results in ~155,000 deaths per year (5^{th}-95^{th} percentile, 39,000-303,000) (1, 2).

The *Salmonellae* are a genus of Gram-negative bacteria belonging to the Enterobacteriaceae family. The genus is classified into two species: *Salmonella enterica* and *Salmonella bongori*, with *S. enterica* comprised of a further six subspecies (3). *Salmonella enterica* subsp. *enterica* includes >2,500 serovars, which can cause a wide range of disease in humans and animals. This extensive diversity points to the ancestral origin of this subspecies, with *Salmonella* being recovered from human remnants dating back >6,500 years ago (4). Infections caused by different NTS serovars can present with differing pathology, epidemiology, clinical presentations, and antimicrobial resistance (AMR) profiles. Clinically, NTS infections are usually observed as acute gastroenteritis with the onset of fever, vomiting, abdominal cramp and diarrhea (5). These symptoms are typically self-limiting and resolve within 5-7 days. Consequently, antimicrobial treatment is deemed unnecessary and is generally not recommended (6, 7). However, salmonellosis can also result in invasive diseases (i.e. bloodstream infection) in immuno-compromised patients, which has a high mortality rate (8, 9). Therefore, antimicrobials remain crucial for the treatment of some *Salmonella* infections, especially in high-risk patients (7, 10). Currently, the WHO guidelines for treatment of paediatric diarrhoea recommend the use of low osmolarity oral rehydration solution (ORS), zinc, and antimicrobials for all patients with bloody diarrhea, irrespective of their age (11, 12). The drug of first choice is ciprofloxacin or one of the three second-line alternatives: pivmecillinam, azithromycin, or ceftriaxone.

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Vietnam has undergone rapid economic transition, with improved sanitation, accelerated urbanization and changes in the food production and supply chains. This development has been followed by a shift in the key causes of bacterial enteric infections; NTS has now become the most common bacterial etiology for children hospitalized with diarrheal illnesses (13, 14). This pattern now more closely resembles the distribution of diarrheal infections in children in high income countries (15, 16). However, despite these apparent changes in the dynamics of enteric bacteria, the epidemiology, serovar distribution, AMR, and clinical manifestation of NTS gastroenteritis have not been characterized at scale in Vietnam. The introduction of whole genome sequencing (WGS) and analysis as a routine methodology in Low- and Middle-Income Countries (LMICs), such as Vietnam, offers an opportunity for highly detailed molecular serotyping and genotyping to infer detailed epidemiological insights. In this study, we employed genomic analysis to describe some epidemiological features of the most common NTS serovars isolated from diarrheal children admitted to one of three tertiary hospitals in Ho Chi Minh City (HCMC), Vietnam.

Materials and Methods

Ethics approval and consent to participate

This study was approved by Ethics Committees of participating local hospitals and the University of Oxford Tropical Research Ethics Committee (OxTREC No. 1045-13), as detailed previously (14). Written consent from parents or legal guardians of all participants was obtained prior to enrolment. Consent for publication was incorporated as a component of entrance into the study.

Study design

Fecal samples were collected from a prospective, observational, cross-sectional study in Children's Hospital No. 1, Children's Hospital No. 2 and the Hospital of Tropical Diseases in

HCMC, Vietnam from May 2014 to April 2016. Children (aged <16 years) hospitalized with diarrhea, defined as ≥3 passages of loose stools within 24 hours along with at least one loose stool containing blood and/or mucus were recruited into the study (12). Children were not eligible if they had suspected or confirmed intussusception at the time of enrolment. Following enrolment, a short questionnaire (requesting clinical and demographic information) was completed, and a fecal sample was collected and processed within 24 hours. All enrolled patients were provided with the routine standard of care practices, which may have included treatment with antimicrobials.

Microbiological culture and antimicrobial susceptibility

Fecal specimens were inoculated onto MacConkey agar (MC, Oxoid), xylose-lysine-deoxycholate agar (XLD, Oxoid), and into selenite broth (Oxoid) and incubated at 37°C for 18-24 hours. Presumptive *Salmonella* was detected based on colony morphology on XLD and MC agar, and confirmed using matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker) (12).

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid) for confirmed *Salmonella* isolates, interpreted using the updated CLSI Guidelines (17). The tested antimicrobial agents (Oxoid) included nalidixic acid (30µg), ciprofloxacin (5µg), trimethoprim-sulfamethoxazole (co-trimoxazole; 1.25/23.75µg), ceftriaxone (30µg), ceftazidime (30µg), ampicillin (10µg), amoxicillin-clavulanate (20/10µg), azithromycin (15µg), chloramphenicol (30µg), gentamicin (10µg), amikacin (30µg) and imipenem (10µg). For *Salmonella* spp., susceptibility to aminoglycosides *in vitro* does not translate into clinical effectiveness, and thus it was not reported (17). Multi-drug resistance (MDR) was defined as non-susceptibility to ≥1 agent in ≥3 antimicrobial categories (14).

Whole genome sequencing and in silico Salmonella serovar typing

Total genomic DNA was extracted from retrieved confirmed *Salmonella* specimen (N=460) using the Wizard genomic DNA extraction Kit (Promega, USA) and sent to the Wellcome Trust Sanger Institute (WTSI) for WGS using the Illumina HiSeq 2500 platform, generating paired end reads (125bp x2) (18). Raw sequences in FASTQ format were subjected to built-in quality checking pipeline at WTSI, as described previously (19), and input into Kraken (v0.10.6) for taxonomic identification by comparison to a pre-set database (20). We performed a *de novo* sequence assembly using Velvet v1.2.03 and VelvetOptimizer for each isolate (21), and each read set was mapped back to the corresponding assembly to improve assembly accuracy, as performed in the WTSI analysis pipeline (22). The median number of contigs and N50 statistic per assembly were 44 (IQR: 30 – 56) and 370,546 (IQR: 283,058 – 576,586), indicating that the assemblies were of sufficient quality to be used for downstream genomic analyses.

In silico molecular serotyping for *Salmonella* was performed for individual genome assembly using the *Salmonella* In Silico Typing Resource (SISTR) (23). The analysis was based on the Multi-Locus Sequence Typing (MLST) scheme for *Salmonella*, and serovar prediction was based on identification of genetic elements coding for the O (somatic) and H (flagellar) antigens. Additionally, we performed the read-based serotyping method for *Salmonella* (SeqSero2) for all sequenced NTS (24), and compare these outputs with those generated by SISTR.

Identification of antimicrobial resistance genes

AMR genes were predicted from the raw sequencing reads of each isolate using ARIBA (25) (version 0.4.1), which identifies AMR determinants by assembly and alignment. A manually curated input database of known resistance genes in FASTA format, taken from the CARD database (McMaster University; accessed on 21st March 2017), was used as the reference database. Resistance determinants were identified if they were predicted to be functional proteins

(no truncations or premature stop codons) and fit the criteria of ≥ 95% nucleotide identity and ≥50% sequence length matching. The output from ARIBA was manually curated to generate a list of high-confidence hits of acquired AMR genes. Chromosomal mutations in the quinolone resistance determining region (QRDR) were manually detected in *gyrA*, *gyrB*, *parC* and *parE*.

Correlation of susceptibility phenotypes and genotypes

The presence of AMR determinants, as identified by ARIBA, indicates a non-susceptible genotype to the corresponding antimicrobial. The phenotypic nonsusceptibility to all tested antimicrobials in our *Salmonella* collection was compiled, with intermediate phenotypes interpreted as non-susceptible. To determine the correlation of antimicrobial susceptibility phenotypes and genotypes, we calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the presence/absence of AMR genes, using the phenotypic data as the gold standard.

Demographic and clinical data analysis

Clinical and demographic data were collected from all anonymized participants and processed and analyzed using Stata v11 (StataCorp, College Station, TX, USA). The growth status of participants was assessed using the WHO global database on growth and nutrition, and Prevention and Management of Obesity for Children and Adolescents-Healthcare guidelines, using the "macro" package of Stata v11 developed by WHO (26, 27). Hemoglobin concentration cut-off for anemia diagnosis was assessed using the recommended WHO guidelines (28). The demographic (age, sex, nutritional status, anemia status), clinical (diarrhea type, number of diarrhea episodes per day, hospitalization duration, outcome) and laboratory data (neutrophil count, CRP concentration) were compared among the six most abundant ST (n ≥ 20), using the Kruskal-Wallis test (continuous variables) and Chi-squared/Fisher Exact tests (categorical

variables). Resulting p-values were corrected for multiple hypothesis testing (Bonferroni correction). Data analysis and visualization were performed using R v3.6.3 (29).

Availability of data and materials

The raw sequence data generated from this study are available in the European Nucleotide Archive (ENA) under the project number PRJEB9121 (ERR1764086 – ERR1764359; ERR1788605 – ERR1788707; ERR1821189 – ERR1821283; ERR1837087 – ERR1837088).

Results

Clinical manifestations of non-typhoidal Salmonella infections

Between May 2014 and April 2016, 3,166 children hospitalized with dysentery were recruited into the study (14); 478 (~15%) children were identified to be infected with NTS by stool culture. However, the bacteria were successfully retrieved and subjected to WGS for 460 cases. Three isolates failed during WGS due to low DNA yield. Subsequent quality control showed that additional seven isolates were contaminated with other bacteria (*Escherichia coli*, *Citrobacter*, *Pseudomonas*). Therefore, the downstream analyses were performed on 450 NTS organisms and associated metadata. More than half of these children were male (272/450; 60.3%), with the age ranging from 1 to 135 months (median 9 months, IQR 6.4-14.9 months); 22.4% (101/450) of children were <6 months of age. These children had a median of 2 days of symptoms (IQR: 2-4 days) before hospitalization. One third of this population had an abnormal growth status, with 22.2% (100/450) being overweighed/obese and 12.5% (56/450) being wasted/severely wasted (Table 1). Hemoglobin concentrations, according to WHO guidelines, showed that 32.9% of these children were anemic (148/450). Sixty percent (274/450) of these children were hospitalized for acute bloody diarrhea; the remainder had mucoid diarrhea without visible blood (Table 1). Profuse diarrhea was commonly recorded with an average of 10 episodes in 24-hour period (IQR: 6-10 episodes). Other symptoms, including fever (294/450, 65.3%) and vomiting (190/450,

42.2%), were also frequent. Most children (except for 25 cases) were assessed not to be dehydrated. No severe sequelae or death were recorded.

The temporal distribution of Salmonella sequence types

Molecular serotyping was performed on each of the 450 assembled genomes, all of which were assigned a serotype (Table 2). Serotyping results were largely consistent between the two employed methods (SISTR and SeqSero2), with mismatch in only 4 isolates (<1%). For consistency, the serotyping results presented herein were derived from the SISTR output. All but two (*houtenae* and *salamae*) NTS isolates were identified as subspecies *enterica*, and comprised of diverse collection of serogroups (B, C1, C2-C3, D1, E1, G, I, K, N, O, and Q). Accounting for 41.8% (188/450) of isolates, *S.* Typhimurium was the most predominant serovar. This serovar was comprised of three sequence types (STs). More than two thirds (133/188) of these *S.* Typhimurium were ST34, with the majority being monophasic (n=120) based on the presence of only one flagellar H antigen gene copy (antigenic formula: 4,[5],12:i:-) (30). The biphasic *S.* Typhimurium (ST36, ST19 and ST34) were associated with infection in 30, 25 and 13 cases, respectively. Other common serovars included *S.* Stanley ST29 (62/450, 13.8%), and *S.* Weltevreden ST365 (34/450, 7.6%). *S.* Newport (29/450, 6.4%) consisted of four different STs, including ST46 (n=22), ST31 (n=5), ST2366 (n=1) and ST2855 (n=1). Other serovars (with at most 10 cases) included Kentucky (ST198), Bovismorbificans, Rissen, Saintpaul, Virchow, etc. (Table 2). Furthermore, 34 serovars were detected and associated with a limited number of cases (1-5 cases).

The eight most common STs (≥ 10 cases) accounted for ~73% of all NTS and were comprised of ST34, ST29, ST36, ST365, ST19, ST46, ST11, and ST198. The temporal distribution of the diarrheal cases caused by these eight STs is shown in Figure 1. In 2015, the absolute number of NTS cases increased from March and peaked in May and September, followed by a rapid decline.

This pattern coincides with the duration of the rainy season in Southern Vietnam (from April to October). Over time, there was no apparent change in the proportional distribution of NTS genotypes, with *S*. Typhimurium (ST34) dominating the epidemiological landscape during our investigational period. The eight most common STs could be detected throughout the study, with *S*. Enteritidis (ST11) and *S*. Kentucky (ST198) more apparent during periods with more cases.

High concordance between antimicrobial resistance genotype and phenotype

The majority of NTS were susceptible to imipenem (445/450) and amikacin (447/450); whereas over half of the collection exhibited resistance to ampicillin and chloramphenicol. Resistance to ciprofloxacin was comparatively low (39/450), but 221 isolates exhibited reduced susceptibility to this antimicrobial. Correspondingly, ~58% of the NTS were non-susceptible to ciprofloxacin. Resistance against trimethoprim-sulfamethoxazole (38%), 3rd generation cephalosporins (13%), and azithromycin (18%) were comparatively low. Consistent with a high prevalence of resistance, more than half (53.8%, 242/450) of NTS isolates were determined to be MDR.

We exploited *in silico* methods to detect the AMR determinants (acquired genes and mutations) in all the NTS genomes. The prevalence of all detected AMR genes alongside with their corresponding antimicrobial classes is shown in Table S1. Subsequently, we assessed how detected AMR genes (classified by antimicrobial class) could predict phenotypic resistance (Table 3).

Quinolone resistance is mediated by mutations in the quinolone resistance determining region (QRDR; *gyrA*-83, *gyrA*-87 and *parC*-80) and/or the acquisition of plasmid-mediated quinolone resistance genes (PMQR) (31). At least one QRDR mutation was detected in 38 isolates (8.4%) (Table S2), leading to nalidixic acid resistance. PMQR were present in around half the collection, with *qnrS1* identified in 47.8% of the isolates (215/450), followed by *aac(6')-lb-cr*, *qnrS2*, *qnrB6*,

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253 *qnrD1*, *oqxAB* and *patA*. The carriage of QRDR mutations and/or PMQR predicted nalidixic acid
254 resistance with a sensitivity of 96.2% and the specificity of 73.4%. Seven different QRDR
255 mutations were identified; 11 isolates harbored triple mutations associated with resistance to
256 ciprofloxacin (Table S2). These organisms included 10 *S.* Kentucky ST198 (*gyrA*-S83F, *gyrA*-
257 D87N, and *parC*-S80I) and one *S.* Indiana ST17 (*gyrA*-S83F, *gyrA*-D87G, and *parC*-S80R).
258 Additionally, ciprofloxacin resistance was associated with the presence of PMQR in either a
259 single QRDR mutation (n=7) or no mutation (n=20) background (Table S2). The presence of
260 QRDR mutations and/or PMQR could explain non-susceptibility to ciprofloxacin with 90.8%
261 sensitivity and 93.2% specificity.
262
263 The most commonly identified β-lactamases were $bla_{\text{TEM-95}}$ (58.7%, 264/450) and $bla_{\text{CTX-M-55}}$
264 (11.6%, 52/450). The presence of β-lactamases corresponded with resistance to different β-
265 lactams, including ampicillin (97.2% sensitivity, 99.4% specificity), and 3rd generation
266 cephalosporins (91.2% sensitivity, 99.2% specificity). Resistance to the latter was associated with
267 $bla_{\text{CTX-M}}$ extended-spectrum beta-lactamases (ESBLs) (present in 55/450 isolates). The
268 carbapenemase $bla_{\text{NDM-1}}$ was present in one *S.* Typhimurium ST34, but phenotypic testing found
269 it susceptible to imipenem. Resistance to azithromycin has been reported previously in
270 *Salmonella*, and is chiefly attributed to the presence of the macrolide inactivation gene cluster
271 (*mphA*-*mrx*-*mphR*) within a *Salmonella* genomic island (SARGI) (32). Herein, 58 NTS isolates
272 carried the *mphA*-*mrx* construct, followed by other less common macrolide resistance genes such
273 as *ermF*, *ermT*, *ermB* and *mefB*. A genotype-phenotype comparison demonstrated that the
274 presence of these genes was in high concordance with macrolide resistance (98.1% specificity).
275
276 Sulphonamide resistance was conferred by *sul2*, *sul3* and *sul1*, which were present in 237, 116
277 and 33 of 450 NTS isolates respectively. Resistance to trimethoprim could be explained by

dihydrofolate reductase (*dfrA*) variants, with *dfrA12* (97/450) and *dfrA14* (69/450) being the most common. Genotypic prediction for trimethoprim-sulfamethoxazole resistance was calculated by combining the presence of both *sul* and *dfr* genes in each isolate, resulting in high sensitivity (94.2%) and specificity (98.6%). A large number of aminoglycoside resistance genes was prevalent, including the *aac(3)*, *aac(6)*, *aph* and *aad* families. Each of these is known to confer resistance to a distinct class of aminoglycosides (Table S1). We observed a high concordance in genotype-phenotype prediction for gentamicin (Table 3). However, in some instances, the presence of these genes did not translate to phenotypic resistance. For example, *aac(6')-Iy* is chromosomally encoded and specific to *Salmonella*, but it is not usually transcribed due to the absence of an upstream promoter (33). This resulted in the observed genotype-phenotype mismatch in resistance to amikacin.

Antimicrobial resistance differs between the major Salmonella sequence types

We proceeded to compare the AMR profiles of the eight most common STs (Figure 2A) in order to gain insights into potential broader treatment strategies. There was significant variation in the AMR profile of these major STs. Nearly 80% (149/188) of *S.* Typhimurium were MDR, with the majority of the biphasic ST19 and ST36 exhibiting non-susceptibility to four antimicrobial classes (ampicillin/amoxicillin-clavulanate, chloramphenicol, ciprofloxacin, and co-trimoxazole). This profile was due to the acquisition of the corresponding $bla_{\text{TEM-95}}$, *floR*, *qnrS1*, and *dfrA12-sul* (Figure 2B). *S.* Typhimurium ST34 displayed a more variable AMR profile, with 33.8%, 30.8% and 14.3% of these organisms non-susceptible to three, four and five antimicrobial classes, respectively. Noticeably, the presence of $bla_{\text{CTX-M}}$ or *mphA-mrx* was more frequent in ST34 (~25%), resulting to marked increase in the resistance to ceftriaxone and azithromycin, respectively. A high proportion of MDR was also evident in *S.* Newport ST46 (59.1%, 13/22) and *S.* Kentucky ST198 (70%, 7/10). Half of ST46 (n=11) were non-susceptible to all five classes of tested antimicrobials, owing to the presence of $bla_{\text{TEM-95}}$, $bla_{\text{CTX-M-55}}$, *aac(3)-IIa*, *aph(6)-Id*,

aph(3')-Ia, *aadA22/24*, *mphA-mrx*, *qnrS1*, *dfrA14-sul3*, *floR*, *linG*, and *arr2/arr3* (Figure 2). *S.* Stanley ST29 and *S.* Weltevreden ST365 were generally susceptible to all classes of antimicrobials. No AMR determinants were detected in *S.* Weltevreden ST365, except for two isolates carrying the chromosomally encoded *aac(6)-Iy* and *qnrS1*.

To explore some additional features of the major NTS genotypes, we sought to compare the demographic and clinical data associated within the six most prevalent STs. Age was the only variable with a significant difference among these STs (Kruskal-Wallis test, adjusted p=0.0057), the median age of children infected with *S.* Weltevreden ST365 was 4.52 months [IQR 3.48 – 9.4], the lowest among the compared STs. Disease severity factors such as duration of hospitalization, frequency of diarrhea, and blood neutrophil count were not significantly different between these six STs (Figure S1). However, we identified two cases in which imipenem was given as the last resort after unsuccessful recovery with other antimicrobials, and both infections were caused by MDR *S.* Newport ST46. The first case was a 7.5-month-old male, who was treated initially with macrolides and then fluoroquinolones. Imipenem was later administered, and the patient was hospitalized for 19 days. The second case was a 3-month-old male who was treated initially with intravenous ceftriaxone and hospitalized for nine days.

Resistance to first line antimicrobials correlates with disease severity

Treatment regimens was recorded for all enrolled patients, which included ORS, intravenous rehydration, zinc, probiotics, and antimicrobials. More than 90% of patients were administered with ORS and zinc supplementation (Table 1). Antimicrobials were also frequently prescribed, with 92.4% (423/450) of patients receiving empirical antimicrobial treatment following admission to the hospital and prior to obtaining a microbiological susceptibility test result. Most of the infected children recovered or had their symptoms improved after three days of enrolment; the children were discharged from the hospital after a median of five days (IQR 3-7 days) (Table

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1). Fluoroquinolones were most commonly used for primary treatment (299/423; 70.7%), followed by 3rd generation cephalosporins (85/423), and macrolides (32/423). These initial treatments may be changed to a different (secondary or tertiary) antimicrobial, dependent on the patient's clinical progression. Such changes occurred in 12.3%, 11.8% and 25% of the corresponding fluoroquinolone, 3rd generation cephalosporin, and macrolide initial treatments. For all three scenarios, a change in antimicrobial therapy was significantly associated with a prolonged hospital stay (Wilcoxon signed-ranked test, $p<0.05$), possibly reflecting the patients' worsening illness or as required for parenterally administered antimicrobial (i.e. ceftriaxone).

We have previously reported that the NTS MDR status was not associated with hospitalization duration or clinical outcome (14). As salmonellosis is frequently treated with either fluoroquinolones, 3rd generation cephalosporins, or macrolides, we stratified the NTS cases by resistance to these antimicrobials only. For all patients, cases caused by NTS non-susceptible to more than one of these drug classes had a small but significantly longer hospitalization (pairwise mean difference: 0.91 day; Wilcoxon signed-rank test, $p=0.04$) (Figure 3A). This effect was observed throughout the major NTS STs (Figure 3B). We next sought to understand how the non-susceptibility to the treating agent influences disease recovery (Figure 3C). For patients receiving initial fluoroquinolone treatment, non-susceptibility to ciprofloxacin was not associated with a difference in hospitalization time. However, non-susceptibility to ceftriaxone was significantly associated to a longer hospital stay in children treated with 3rd generation cephalosporins (Wilcoxon signed-rank test, $p=0.028$). This effect was also observed with macrolide treatment but was not significantly different. These observations were not confounded by age as there was no significant difference in the age of patients receiving these different antimicrobial treatments (Kruskal-Wallis test, $p>0.05$). Additionally, we performed the same statistical analyses on the original full dataset (478 NTS diarrhea cases), which produced analogous results, demonstrating the robustness of our observations despite the sample size reduction.

356

357 **Discussion**

358 Our study combined a wealth of clinical, microbiological and genomic data to uniquely detail the
359 characteristics of NTS infections in Southern Vietnam. We found that *Salmonella* gastroenteritis
360 exhibits clear seasonality, with the prevalence of disease increasing in May-September. This
361 pattern has also been recapitulated in NTS surveillance studies in Guangdong, China (34) and
362 Bangkok, Thailand (35). The incidence rate of dysentery was previously found to be significantly
363 higher between May and October in Vietnam, particularly for the Southeast region where our
364 study was conducted (36). This period coincides with the rainy season, with higher precipitation
365 and humidity, which could grant higher survival and transmissibility of bacterial pathogens in the
366 environment. This stands in contrast with the seasonal pattern of viral diarrhea, of which the
367 burden is highest in January to March in Southern Vietnam (13, 37).

368

369 We observed a great diversity of circulating *Salmonella*, with the monophasic *S.* Typhimurium
370 ST34 dominating the NTS epidemiological landscape. This observation resonates with recent
371 findings elsewhere in Asia, including China (34). The monophasic ST34 is strongly associated
372 with swine food production (38) and has risen to prominence in Europe and globally during the
373 last two decades (39). Genomic and phenotypic investigations have suggested that this variant is
374 ecologically successful due to its extensive repertoire of antimicrobial and heavy metal resistance
375 genes (30, 40). Indeed, the ST34 isolated from our study show elevated resistance to several
376 antimicrobials of first line treatments, especially ceftriaxone and azithromycin. We also recently
377 discovered a novel biphasic, MDR ST34 clone causing invasive diseases in HIV-infected patients
378 in Vietnam (9, 41). It is therefore of epidemiological interest how this invasive clone is
379 genetically related to the diarrheagenic ST34 described herein, and such genomic analysis is
380 being conducted. Other major STs, such as *S.* Weltevreden ST365, *S.* Stanley ST29 and *S.*
381 Kentucky ST198, have been frequently reported in Asia (34, 42). Similarly to previous reports,

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the majority of *S.* Stanley (ST29) and *S.* Weltevreden (ST365) were susceptible to all tested antimicrobials (19, 34). In contrast, all *S.* Kentucky (ST198) isolated displayed resistance to ciprofloxacin and are likely to belong to the Asian expansion of the internationally disseminating ST198 (43). While *S.* Enteritidis is predominant in previous surveillances in Greece (44), China (45), Tunisia (46), USA, and is emerging in Australia, we only documented 17 cases attributed to this serovar. This discrepancy is explicable due to the younger age of our cohort (median of 9 months-old), as *S.* Enteritidis has been mainly reported in infections of children > 3 years-old (34).

More than half of the isolated NTS were classified as MDR, with particular high non-susceptibility occurrence to commonly prescribed antimicrobials, such as ciprofloxacin. The major genetic mechanism for resistance was the widespread carriage of *qnrS1*, which could be maintained by the sustained use of the antimicrobial locally. Nevertheless, this does not preclude the importation and local propagation of a pandemic resistant clone, as likely in the case of ciprofloxacin-resistant ST198 *S.* Kentucky, or as proven previously for *S. sonnei* in Vietnam (47). The high concordance between AMR genotype and phenotype was observed in our collection, with sensitivity and specificity surpassing 90% for all classes of antimicrobials tested, except for imipenem, nalidixic acid and azithromycin. Such high genotype-phenotype agreement has been noted in the *Salmonella* collection housed in Public Health England, UK (48). However, this study did not report testing for the aforementioned three antimicrobials. The low sensitivity in azithromycin non-susceptibility prediction indicates that other resistance mechanisms, such as the recently determined mutations in *acrB* (49), remained uncharacterized. Alternatively, the low specificity in the case of nalidixic acid is likely due to the non-functionality or insufficient expression of the genetic determinants. These discrepancies warrant further research to improve the accuracy in inferring AMR phenotypes using sequencing data, which is applicable in public health surveillances or culture-independent multiplex molecular assay (50).

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Differing *Salmonella* STs were not associated with the clinical outcome in our patient cohort. Clinical severity also did not differ among patients infected with different NTS serogroups in Thailand (35). However, two patients infected with MDR *S.* Newport ST46 had to be treated with the last resort antimicrobial imipenem, showing that tailored antimicrobial treatment remains crucial in certain scenarios. *S.* Newport ST46 is infrequently described in Asia and has been linked to reservoir in reptiles (51). Its pan-resistance and epidemiological ambiguity require further investigations. Additionally, our analysis indicated that disease recovery may take longer if the NTS organism was non-susceptible to the treating antimicrobial, particularly for ceftriaxone and likely azithromycin. Here, NTS infections most commonly occurred in young age group, particularly children under 1 years-old. These patients presented with many severe clinical symptoms, including high bloody fecal output, fever, and vomiting, suggesting the highly infectious and severe nature of this disease. Updated guidelines advise that antimicrobials should not be used routinely in NTS infections, except for the immunocompromised, the neonates and the elderly (6, 7, 10). However, antimicrobial treatment should be considered carefully to both benefit patients with moderate-severe symptoms and to limit the chances of developing resistance.

Our observational study was limited to the description of hospitalized cases with mucoid/bloody diarrhea, NTS was the most common pathogen confirmed by microbiological culturing. Therefore, other pathogens or cases of co-infection were not analyzed. Also, the burden of NTS causing milder or acute watery diarrhea was not accounted. As we did not record detailed epidemiological data (i.e. diet, animal contact, household cases), it was not possible to identify the possible source of infection. NTS are widely distributed in humans, animals and environmental reservoirs, so the transmission route may differ case by case. The infections reported in our study could be either associated with zoonotic transmissions (through the food

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chain/contact with animals) or via sustained human-to-human transmissions distinct from the animal NTS reservoirs (52, 53). For instance, NTS isolated from asymptomatic close contacts have been shown to be closely related to ones causing invasive diseases in children in Africa (54). Our study relied on molecular serotyping (SISTR), without conducting phenotypic serotyping due to the shortage of trained personnel and dedicated resources. This approach has become attractive for its high throughput and accurate performance, and produces high concordance between genotyping and phenotypic serotyping in *Shigella flexneri* (55). Recently, the web-based application EnteroBase has independently employed serotype prediction from *Salmonella* genomes, which largely produces congruent results with the SISTR outputs (56).

Conclusions

NTS has become the most common bacterial pathogen detected in children hospitalized with bloody or mucoid diarrheal diseases in HCMC. *S*. Typhimurium was the predominant serovar in this setting and associated with a variety of AMR genes leading to a high rate of MDR in this serovar. We observed an increasing trend of AMR, especially against the first- and second-line antimicrobial classes recommended by WHO, i.e. ciprofloxacin, ceftriaxone, azithromycin. Multi-resistance could lead to prolonged hospitalized duration and the difficulty of choosing empirical antimicrobials for dysenteric diarrhea. Our work shows that WGS is a powerful method to characterize the serovar diversity and AMR profiles in NTS. A phylogenetic investigation of these NTS isolates in the context of global and/or invasive collections is the next step to better understand transmission dynamics and the evolutionary processes underpinning the circulation of these organisms.

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Competing interests

The authors declare no competing interests.

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678

679 **Figure legends**

680 **Figure 1.** Monthly hospitalization incidence of non-typhoidal *Salmonella*
681 Only the eight most common sequence types are visualized. Sequence types that caused fewer
682 than 10 incidences are grouped together as 'Other'.

683

684 **Figure 2.** Antimicrobial resistance in major non-typhoidal *Salmonella* sequence types
685 (A) Heat map of antimicrobial resistance phenotype of the eight most common Sequence types.
686 The left panel displays the proportion of non-susceptibility to 7 classes of tested antimicrobial
687 agents, including ampicillin/amoxicillin-clavulanate (AMP/AMC); ceftriaxone/ceftazidime
688 (CRO/CAZ); imipenem (IMP); azithromycin (AZM); ciprofloxacin (CIP); trimethoprim-
689 sulfamethoxazole (SXT) and chloramphenicol (CHL). The right panel displays to proportion of
690 non-susceptibility to the number of tested antimicrobial classes, including β-lactam
691 (AMP/AMC/CRO/CAZ); IMP; AZM; CIP; SXT and CHL. Isolates were classified as non-
692 susceptible to an antimicrobial class if they were non-susceptible to any agent of that class. The
693 colour intensity in a cell is proportional to the percentage of non-susceptible NTS isolates to the
694 tested antimicrobial class. The STs are arranged in decreasing order of prevalence (from top to
695 bottom). (B) The prevalence of antimicrobial resistance determinants in the eight most common
696 Sequence types. These determinants are classified by antimicrobial classes, including 3rd
697 generation cephalosporin (*bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-55}}$), quinolone (*qnrS1*, *qnrS2*, *qnrB6*,
698 *qnrD1*, *oqxAB*, *patA*, *aac(6)-Ib-cr*, QRDR mutations), azithromycin (*ermB*, *ermF*, *ermT*, *mefB*,

699 *mphA*), and co-trimoxazole (*sul1*/*sul2*/*sul3* + *dfrA1*/*dfrA12*/*dfrA14*/*dfrA17*/*dfrA5*). Each bar graph
700 denotes the proportion of isolates carrying any of these determinants, classified by antimicrobial
701 class and sequence type.
702
703 **Figure 3.** Resistance to first line antimicrobials correlated with clinical severity
704 (A) The hospitalization duration (in days) of all 450 children infected with non-typhoidal
705 *Salmonella*, classified by the number of first- and second-line antimicrobials (ciprofloxacin,
706 ceftriaxone, azithromycin) to which the pathogen was phenotypically non-susceptible, and (B)
707 further classified by the eight most common sequence types. (C) The hospitalization duration of
708 NTS infected children who received initial treatment of fluoroquinolone (CIP), 3rd generation
709 cephalosporin (CRO), or macrolide (AZM), stratified by the pathogen's non-susceptibility to the
710 corresponding treating agent (ciprofloxacin, ceftriaxone, azithromycin). The asterisk indicates
711 statistical significance in the pairwise comparison (Wilcoxon signed-rank test, $p < 0.05$).
712

Table 1. Demographic and clinical manifestations of diarrheal pediatric patients infected with non-typhoidal *Salmonella* (N=450).

Characteristics		n	%
Socio-demographic			
	Male	271	60.2
	Age in months, *median* [IQR]	9	6.4-14.9
Growth [a]			
	Obese / Overweight / Risk of overweight	100	22.2
	Normal	271	63.5
	Wasted / Severely wasted	56	12.5
Clinical symptoms			
	Bloody diarrhea	274	60.9
	Mucoid diarrhea	167	37.1
	Number of episodes per day, *median* [IQR]	10	6-10
	Dehydration, [b]	25	5.6
	Abdominal pain	115	25.6
	Fever (≥37.5°C) at enrolment	294	65.3
	Vomit	190	42.2
Hematology			
	Anemia (Hemoglobin level=70-109g/L) [c]	148	32.9
	Neutrophil count (10^3/μL), *median* [IQR]	4.9	3.2-7.2
	C-reactive protein (mg/L), *median* [IQR]	29	11.0-50.0
Treatment			
	Low-osmolarity oral rehydration solution	418	92.9
	IV Rehydration	31	6.9
	Antimicrobials	423	94.0
	Fluoroquinolones (initial treatment), *n* (%)	299	70.7
	Zinc	412	91.6
	Probiotics	300	66.7
Outcome			
	Hospital stay in days, *median* [IQR]	5	3-7
	Recovered / Improved after 3 days [d]	393	87.3
	Not improved / worse after 3 days	57	12.7

[a] Obese: weight for length z score >3SD in children age < 24months; BMI for age z score >3SD in children age ≥24months, Overweight: weight for length z score >2SD in children age < 24months; BMI for age z score >2SD in children age ≥24months, Wasted: weight for length z score <-2SD in children age < 24months; BMI for age z score <-2SD in children age≥24months, Severely wasted: weight for length z score <-3SD in children age < 24months); BMI for age z score <-3SD in children age ≥24months [31]

[b] All of the dehydrated cases classified as some dehydration according to Integrated Management of Childhood Illness [32]

[c] Hemoglobin level cut-off according to World Health Organization guidelines [19]

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

Table 2. Non-typhoidal *Salmonella* predicted serovars isolated from children with diarrheal diseases in three tertiary hospitals in Ho Chi Minh City (n=450). Antigenic formula are given in parentheses.

O-antigen group	Serovar	ST	N	Serovar	ST	N
O:4 (B)	Typhimurium (4,[5],12:i:-)*	34	120	Agona (4:f,g,s:-)	13	4
	Typhimurium (4:i:1,2)	36	30	Indiana (4:z:1,7)	17	2
		19	25	Chester (4:e,h:e,n,x)	343	1
		34	13		2063	1
	Stanley (4:d:1,2)	29	62	Schleissheim (4:b:-)	1578	1
		2615	1		2397	1
	Paratyphi B var. Java (4:b:-)	423	7	Saintpaul (4:e,h:1,2)	50	6
		135	1		27	2
	Sandiego (4:e,h:e,n,z15)	20	1			
O:7 (C_1)	Rissen (7:f,g:-)	469	9	Thompson (7:k:1,5)	26	1
	Virchow (7:r:1,2)	359	5		2417	1
		197	1	Ohio (7:b:l,w)	329	1
	Bareilly (7:y:1,5)	203	4	Mbandaka (7:z10:e,n,z15)	1602	1
		909	1			
O:8 (C_2-C_3)	Newport (8:e,h:1,2)	46	22	Albany (8:z4,z24:-)	292	2
		31	5	Hadar (8:z10:e,n,x)	33	1
		2366	1	Emek (8:g,m,s:-)	76	1
		2855	1	Litchfield (8:l,v:1,2)	214	1
	Kentucky (8:i:z6)	198	10	Muenchen (8:d:1,2)	2424	1
	Bovismorbificans (8:r:1,5)	1499	9	Brunei (8:y:1,5)	2809	1
	Corvallis (8:z4,z23:-)	1541	4			
O:9 (D_1)	Enteritidis (9:g,m:-)	11	13	Javiana (9:l,z28:1,5)	2494	3
		74	4		1547	4
	Panama (9:l,v:1,5)	48	1	Dublin (9:g,p:-)	74	1
O:3,10 (E_1)	Weltevreden (3,10:r:z6)	365	34	Anatum (3,10:e,h:1,6)	64	2
	London (3,10:l,v:1,6)	155	5	Lexington (3,10:z10:1,5)	1542	1
	Give (3,10:l,v:1,7)	516	5	Meleagridis (3,10:e,h:l,w)	3248	1
Other (G, I, K, N, O, Q)	Kedougou (13:i:l,w)	1543	3	Johannesburg (40:b:e,n,x)	512	1
	Agbeni (13:g,m:-)	2606	1	Alachua (35:z4,z23:-)	1298	1
	Hvittingfoss (16:b:e,n,x)	446	2	Wandsworth (39:b:1,2)	1498	3
	Orientalis (16:k:e,n,z15)	558	1	subsp. houtenae (43:z4,z23:-)	958	1
	Cerro (18:z4,z23:-)	367	1	subsp. salamae (48:d:z6)	3200	1

(*): monophasic

Table 3. The performance of whole genome sequencing in determine the antimicrobial susceptibility of non-typhoidal *Salmonella*

Antimicrobials	Antimicrobial resistance genes		Phenotype		Sensitivity (%[95%CI])	Specificity (%[95%CI])	PPV	NPV
			Non-Susceptible *	Susceptible				
ampicillin	*bla*$_{TEM-95}$ / *bla*$_{OXA-1}$ / *bla* $_{CARB-3}$ / *bla*$_{SHV-66}$ / *bla*$_{CMY}$ / *bla*$_{CTX-M-55}$ / *bla*$_{CTX-M-14}$ / *bla*$_{CTX-M-15}$	yes	279	1	97.2 (94.6-98.8)	99.4 (96.6-100)	99.6	95.3
		no	8	162				
ceftriaxone/ ceftazidime	*bla*$_{CTX-M-55}$ / *bla*$_{CTX-M-14}$/ *bla*$_{CTX-M-15}$	yes	52	3	91.2 (80.7-97.1)	99.2 (97.8-99.8)	94.5	98.7
		no	5	390				
imipenem	*bla*$_{NDM-1}$	yes	0	1	0.0 (0.0-52.2)	99.8 (98.8-100)	0.0	98.9
		no	5	444				
gentamicin	*aa(3)-IIa* / *aac(3)-IV* / *aac(6)-IIa*	yes	89	2	100 (95.9-100)	99.4 (98.0-99.9)	97.8	100
		no	0	358				
azithromycin	*mphA* / *mefB* / *ermT* / *ermF* / *ermB*	yes	59	7	72.8 (61.8-82.1)	98.1 (96.1-99.2)	89.4	94.3
		no	22	362				
ciprofloxacin	QRDR[$] mutation and/or PMQR[#] genes	yes	236	13	90.8 (86.8-94.0)	93.2 (88.6-96.3)	94.8	88.1
		no	24	177				
nalidixic acid	QRDR mutation and/or PMQR genes	yes	179	70	96.2 (92.4-98.5)	73.4 (67.6-78.6)	71.9	96.5
		no	7	193				
trimethoprim-sulfamethoxazole	*dfr* + *sul*	yes	163	4	94.2 (89.6-97.2)	98.6 (96.3-99.6)	97.6	96.5
		no	10	273				
chloramphenicol	*catA1* / *catB3* / *floR*	yes	224	2	93.3 (89.4-96.1)	99.0 (96.6-99.9)	99.1	92.9
		no	16	208				

(*): Fully resistance and intermediate resistance are included as non-susceptible.
($): Quinolone resistance determining region (QRDR): *gyrA*-83 + *gyrA*-87 + *parC*-80
(#): Plasmid-mediated Quinolone Resistance genes (PMQR): *qnrB6/ qnrD1/ qnrS1/ qnrS2/ oqx_AB/ patA/ aac(6)-Ib-cr*