

Original Article

Antimicrobial profile of non-typhoidal *Salmonella* isolated from raw sewage in the Metropolitan Region of São Paulo, Brazil

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Abstract

Introduction: Non-typhoidal *Salmonella* (NTS), are frequently found in sewage and are one of the main causes of diarrhea in developed and developing countries due to poor sanitation conditions. In addition, NTS can potentially act as reservoirs and vehicles for the transmission of antimicrobial resistance (AMR), which can be facilitated by the discharge of sewage effluents into environmental matrices. This study aimed to analyze a NTS Brazilian collection, focusing on their antimicrobial susceptibility profile and the presence of clinically relevant AMR-encoding genes.

Methodology: Forty-five non-clonal NTS strains from serotypes *Salmonella enteritidis* (n = 6), *Salmonella enterica* serovar 1,4,[5],12:i:- (S. 1,4,[5],12:i:-) (n = 25), *Salmonella cerro* (n = 7), *Salmonella typhimurium* (n = 3) and *Salmonella braenderup* (n = 4) were studied. Antimicrobial susceptibility testing was done using the Clinical and Laboratory Standards Institute guidelines (2017) and genes encoding resistance to beta-lactams, fluoroquinolones and aminoglycosides were identified by polymerase chain reaction and sequencing.

Results: Resistance to β -lactams, fluoroquinolones, tetracyclines and aminoglycosides was frequent. The highest rates were observed for nalidixic acid (89.0%), followed by tetracycline (67.0%), ampicillin (67.0%), amoxicillin + clavulanic acid (64.0%); ciprofloxacin (47.0%) and streptomycin (42.0%). The AMR-encoding genes detected were *qnrB*, *oqxAB*, *bla_{CTX-M}* and *rmtA*.

Conclusions: Raw sewage has been considered a valuable tool to evaluate epidemiological population patterns and this study supports the view that NTS with pathogenic potential and resistance to antimicrobials are circulating in the studied region. This is worrisome due to the dissemination of these microorganisms throughout the environment.

Key words: AMR; non-typhoidal *Salmonella*; NTS; sewage.

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Introduction

In the 20th century, humanity witnessed milestones in measures to prevent infectious diseases such as improved water, sanitation, and hygiene (WASH), the discovery of antibiotics, as well as other advances in medicine, science, and technology, which were significant in reducing health risks due to the breakage of the diseases' transmission chain. The improvements in WASH led to a massive drop in the rates of infectious diseases including waterborne diseases, constituting an effective barrier against fecal contamination of the environment [1,2]. The discovery of antibiotics was a great gain for public health and has saved many lives. These compounds continue to be a basis for human and animal health, as well as for the food production chain. According to Padiyara *et al.* [3], an antibiotic-free world would lead to negative impacts not only to public health but also for global development and economy

[3]. However, Bairán *et al.* [2] report that the interaction among multiple biological, sociological and cultural determinants has resulted in the development of microbial resistance [2].

In the 21st century, the population has witnessed old questions alongside new challenges that need to be faced. The rapid and uneven urbanization characterized by the lack of sanitation structure [4–6], especially in developing countries where there is low coverage of sewage collection and treatment [7], results in most residues being released directly into water bodies, leading to contamination by enteric pathogens [8], including *Salmonella*.

Another issue is the threat of antimicrobial resistance (AMR) which is considered a global challenge by the World Health Organization (WHO), both for human and animal health. Inadequate sanitation, pollution and other factors related to the

natural environment are as relevant in the spread of AMR as is the overuse of antibiotics. Therefore, prevention policies must focus on the One Health concept which is a multidisciplinary view to tackle the issue of antimicrobial resistance. AMR frequently evolves from environmental bacteria which improve and mobilize resistance mechanisms according to the selective pressure to which they are exposed [9]. Selective pressure can be exerted by antibiotics, pesticides, heavy metals, or any other molecule that represent a risk for the survival of the bacteria at specific periods and/or environments [9,10]. Sewage is considered a hotspot for the evolution and mobilization of AMR due to the presence of bacteria from several sources (homes, hospitals, and industries) and plenty of chemical molecules that can induce or activate mutations in bacteria. If there is no sewage collection or treatment, or if treatment is not effective, AMR bacteria and their genes are eventually discharged in the natural environment, interacting with environmental bacteria and improving their resistance mechanisms [11].

Non-typhoidal *Salmonella* (NTS), which are frequently found in sewage, are one of the main causes of diarrhea in developed and developing countries [12]. An estimated 93.8 million cases of gastroenteritis are caused by nontyphoidal *Salmonella* worldwide, leading to 155,000 deaths per year [13]. The serovars *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* ser. Typhimurium (including monophasic variation S. 1,4,[5],12:i:-) were responsible for 31.3% of the salmonellosis cases in the USA in 2016, when almost 32,000 cases of non-typhoidal *Salmonella* infections were reported [14]. In Europe, salmonellosis is the second most reported infection with a rate of 20 cases per 100,000 population [15].

Salmonellosis has also been the cause of bacteremia in 33 out of 54 African countries [16], and is considered a threat in sub-Saharan Africa, with lethality rates greater than typhoid fever [17]. In a systematic review conducted in Latin American countries for NTS isolated from food and animal products, Quesada *et al.* (2016) [18] found elevated frequency of antimicrobial resistance, including resistance to antibiotics used to treat salmonellosis. NTS have the potential to act as reservoirs and vehicles for the transmission of antimicrobial resistance in different settings. Resistant bacteria are transferred from animals to humans via the food chain, promoting the exchange of resistance mechanisms between commensal and pathogenic bacteria. As both animal and human wastes are eventually discharged into environmental matrices, the

soil and water also represent vehicles for AMR improvement and spread [9,19,20]. Severe NTS *Salmonella* infections can be invasive and are generally treated with beta-lactams (mostly cephalosporins) or fluoroquinolones, which are also used in livestock in several countries. Therefore, resistance developed in these microorganisms is most related to these two antibiotic classes, especially through the production of extended-spectrum and AmpC beta-lactamases, as well as Qnr proteins and mutations in constitutive genes [12,21–24].

In a recent study in Brazil, our group isolated several non-typhoidal *Salmonella* serovars in sewage sludge [25] and raw sewage samples [26] from the Metropolitan Region of São Paulo. In this study, we investigated the antimicrobial susceptibility profile and the primary AMR genes carried by those strains.

Methodology

Bacterial isolates

One-hundred and four (104) non-typhoidal *Salmonella enterica* subsp. *enterica* isolates were previously recovered from raw sewage samples in 2013, according to the USEPA 2012 guidelines [26]. For the present study, enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) method [27] was used to assess the genetic similarity amongst the isolates (data not shown) and select 45 non-clonal strains for this study: 25 S.1,4,[5],12:i:-, seven *Salmonella* Cerro, six *Salmonella* Enteritidis, four *Salmonella* Braenderup and three *Salmonella* Typhimurium.

Antimicrobial susceptibility testing

AMR screening was carried out by the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) (2017) guidelines [28]. CLSI determines that, when fecal isolates of *Salmonella* are tested for clinical purposes, only ampicillin, a fluoroquinolone, and trimethoprim-sulfamethoxazole should be reported, and that routine susceptibility testing is not indicated for fecal NTS. This is because aminoglycosides, first- and second-generation cephalosporins, and cephamycins may appear active in vitro, but are not effective clinically. However, we analyzed environmental strains, which can carry and transfer important AMR genes to other Gram-negative species; therefore, we considered the Enterobacteriaceae cut-offs for all antimicrobials tested.

The 17 antimicrobials and their respective dosages were amoxicillin (20 µg); ampicillin (10 µg); aztreonam

(30 µg); cefazolin (30 µg); ceftazidime (30 µg); ceftriaxone (30 µg); cefepime (30 µg); meropenem (10 µg); ciprofloxacin (5 µg); chloramphenicol (30 µg); streptomycin (10 µg); fosfomicin (200 µg); gentamicin (10 µg); nalidixic acid (30 µg); sulfamethoxazole/trimethoprim (23.75 µg); tetracycline (30 µg). Quality control was performed using the standard strain *Escherichia coli* ATCC 25922.

Polymerase chain reaction (PCR) amplification of resistance genes

PCR amplifications of resistance-encoding genes were performed for the most clinically relevant antimicrobials for Gram-negative infections: beta-lactams, (fluoro)quinolones and aminoglycosides, totaling 16 genes [29–34]. Table 1 shows the primers used in each reaction. PCR mixtures were submitted to a denaturation step of 5 minutes at 94 °C, followed by 30 cycles of amplification (45 seconds of denaturation at 94 °C, 1 minute of annealing at 55 °C, 1 minute of elongation at 72 °C), and 10 minutes at 72 °C for final elongation. PCR products were visualized in agarose

gels after electrophoresis and sequenced to confirm the results.

Results

Antimicrobial susceptibility results for the seventeen evaluated compounds are shown in Table 2. Almost 70% of the 45 isolates expressed a multidrug resistant phenotype, and resistance was frequently to β-lactams, fluoroquinolones, tetracyclines and aminoglycosides. Considering all serotypes, the highest resistance rates were observed for nalidixic acid (89.0%), followed by ampicillin (67.0%), tetracycline (67.0%), amoxicillin-clavulanate (64.0%), ciprofloxacin (47.0%) and streptomycin (42.0%). One *Salmonella* Braenderup isolate produced an extended-spectrum beta-lactamase (ESBL). *Salmonella* Typhimurium and S. 1,4,[5],12:i:- showed , 66.7% and 60% of ciprofloxacin resistance respectively, and S. 1,4,[5],12:i:- also showed 96% resistance to ampicillin.

The antimicrobial resistance genes identified are presented in Table 3. The plasmidial-mediated quinolone resistance (PMQR) gene *qnrB* was detected

Table 1. Oligonucleotide sequences of primers used for the detection of antimicrobial resistance genes.

Assay	Primers	5'-3' Sequences	Target gene	Product (bp)	Reference
Extended-spectrum beta-lactamases (CTX-M group)	CTX-M-geral F CTX-M-geral R	SCSATGTGCAGYACCAGTAA CCGCRATATGRTTGGTGGTG	<i>bla</i> _{CTX-M}	543	[28]
AmpC β-lactamases (CMY group)	CMY-F CMY-R	ATGATGAAAAAATCGTTATGC GCTTTTCAAGAATGCGCCA	<i>bla</i> _{CMY}	1166	This study
Qnr proteins	qnrA-F	AGAGGATTTCTCACGCCAGG	<i>qnrA</i>	580	[29]
	qnrA-R	TGCCAGGCACAGATCTTGAC			
	qnrB-F	GGMATHGAAATTCGCCACTG	<i>qnrB</i>	264	
	qnrB-R	TTTGCYGYCGCCAGTCGAA			
	qnrC-F	ATGGGTTGTACATTTATTGAATC	<i>qnrC</i>	537	
	qnrC-R	TCAAAACACTTTGTCTGGAAT			
	qnrD-F	ATGGAAAAGCACTTTATCAATG	<i>qnrD</i>	645	
	qnrD-R	TTATCGGTGAACAATAACACC			
qnrS-F	GCAAGTTCATTGAACAGGGT	<i>qnrS</i>	428		
qnrS-R	ATTTTGATACCTGATGTATCGACT				
Efflux Pumps	qepA-F	ATGTCCGCCACGCTCCAC	<i>qepA</i>	1536	This study
	qepA-R	TCAACCAGATGCGAGCGCT			
	oqxA-F	CTCGGCGCGATGATGCT	<i>oqxA</i>	392	
	oqxA-R	CCACTCTTACGGGAGACGA			
	oqxB-F	TTCTCCCCGGCGGAAGTAC	<i>oqxB</i>	512	
oqxB-R	CTCGGCCATTTGGCGCGTA				
6'-N-acetyl-transferase	Faa(+) Faa(-)	GCAACGCAAAAACAAAGTTAGG GTGTTTGAACCATGTACA	<i>aac(6')-Ib-cr</i>	561	[31]
<i>16S rRNA</i> methyltransferases	RmtA F	CTAGCGTCCATCCTTTCCTC	<i>rmtA</i>	635	[32]
	RmtA R	TTGCTTCCATGCCCTTGCC			
	RmtB F	GCTTTCTGCGGGCGATGTAA	<i>rmtB</i>	173	
	RmtB R	ATGCAATGCCGCGCTCGTAT			
	RmtD F	CGGCACGCGATTGGGAAGC	<i>rmtD</i>	401	
	RmtD R	CGGAAACGATGCGACGAT			
	RmtG F	AAATACCGCGATGTGTGTCC	<i>rmtG</i>	300	
	RmtG R	ACACGGCATCTGTTT CTTC			
	ArmA F	ATTCTGCCTATCCTAATTGG	<i>armA</i>	315	
	ArmA R	ACCTATACTTTATCGTTCGTC			

Table 2. Resistance percentage to antimicrobial agents in non-typhoid *Salmonella* (NTS) serotypes in raw sewage from the Metropolitan Region of São Paulo, Brazil.

<i>Salmonella</i> serotypes	Antimicrobial agents																
	b-Lactam antibiotics									Quinolones		Aminoglycosides		Tetracyclines	Sulfonamides	Amphenicols	
	AMP	AMC	CFZ	CFO	CAZ	CRO	CPM	ATM	MPM	NAL	CIP	EST	GEN	AMI	TET	SUT	CLO
<i>S.</i> 1,4,[5],12:i:- (n=25)	96.0	92	20.0	12.0	0.0	0.0	4.0	0.0	0.0	96.0	60.0	60.0	4.0	0.0	88.0	0.0	0.0
Cerro (n = 7)	28.6	28.6	43.0	28.6	0.0	0.0	0.0	0.0	0.0	71.0	0.0	14.0	28.6	0.0	28.6	0.0	0.0
Enteritidis (n = 6)	17.0	17.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	17.0	17.0	0.0	17.0	33.0	0.0	17.0
Braenderup (n = 4)	50.0	50.0	0.0	0.0	25.0	25.0	25.0	25.0	0.0	75.0	50.0	25.0	0.0	0.0	75.0	25.0	0.0
Typhimurium (n = 3)	33.3	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	66.7	66.7	33.3	0.0	0.0	33.3	66.7	0.0
Total NTS isolates (n = 45)	67.0	64.0	18.0	11.0	2.0	2.0	4.4	2.2	0.0	89.0	47.0	42.0	7.0	2.0	67.0	7.0	2.0

AMC: amoxicillin; AMP: ampicillin; ATM: aztreonam; CFZ: cefazolin; CPM: cefepime; CFO: cefoxitin; CAZ: ceftazidime; CRO: ceftriaxone; CIP: ciprofloxacin; CLO: chloramphenicol; EST: streptomycin; GEN: gentamicin; MPM: meropenem; NAL: nalidixic acid; SUT: sulfamethoxazole/trimethoprim; TET: tetracycline. All isolates were susceptible to fosfomicin.

in four isolates from three different serotypes, while the efflux pump-encoding genes *oqxAB*, also mediating resistance to fluoroquinolones were detected in two isolates from *S.* 1,4,[5],12:i:-. Extended-spectrum beta-lactamase genes *bla_{CTX-M}* were detected in seven isolates, from serotypes *Salmonella* *S.* 1,4,12:i:-, *S.* Cerro and *S.* Braenderup. Finally, two *S.* 1,4,[5],12:i:- carried the 16S rRNA methyltransferase gene *rmtA*.

Discussion

Salmonellosis is among the leading causes of morbidity and mortality in developing countries due to poor sanitation conditions. *Salmonella* is often found in environmental samples and is usually present in high concentrations in raw or poorly treated sewage, and even in treated effluents, which may be eventually discharged into natural water bodies. The bacterial load released into the environment will depend on the sanitation conditions of each geographical region. The circulation of *Salmonella* through environmental matrices is a factor of great concern for public health because it may facilitate its route through water supply and soil [35,36].

AMR is a serious global concern that also involves interrelationships among people, animals, and the environment as well as the challenge of treating infections caused by multidrug resistant strains. Resistant bacteria and their resistance-encoding genetic elements can reach environmental matrices through several routes, with great potential for resistance genetic interchange between clinical and native bacteria, as well as dissemination through water and soil [10,37]. Resistance to clinically important drugs including ciprofloxacin (quinolone) has increased in

non-typhoidal *Salmonella* since 1996. In 2011, about 5% of *Salmonella* tested by the Centers for Disease Control and Prevention (CDC) were resistant to five or more types of drugs [12,24]

We looked at the antimicrobial profile of 45 non-clonal NTS from 5 serotypes: *S.* 1,4,[5], 12:i:-, Cerro, Enteritidis, Braenderup and Typhimurium, that are generally associated with cases of human and animal infections [38–40]. The strains were isolated from raw sewage, which was treated by activated sludge and then released into natural rivers. Therefore, it is possible that these pathogens have reached the environment [26,35,41]. While most human *Salmonella* infections appear to be foodborne, salmonellosis can also be acquired through contaminated drinking water [42,43]. It is also relevant to mention that if *Salmonella* reaches the soil, it can also contaminate groundwater and/or internalize several agricultural products, which leads to additional possibilities of its dissemination through the food chain [42,44].

Regarding the serotypes isolated in this study, *S.* 1,4,[5],12:i:- represented 55.6% (n = 25) of isolates. This monophasic variant of *Salmonella* Typhimurium has been rarely identified before the mid-1990s, but now it is among the five most important serotypes that have been isolated from human and non-human sources associated with foodborne outbreaks in intestinal and extra-intestinal infections [14,45]. It has already caused substantial outbreaks in several countries, with reports of severe infections and deaths [46,47], including cases of septicemia in Thailand and Brazil [45,46]. Mandilara et al. [47] investigated 403 cases of salmonellosis in Greece that were caused by monophasic *S.* Typhimurium, of which 18.4% were related to two

Table 3. Number of positive isolates for the presence of antimicrobial resistance genes in non-typhoidal *Salmonella* (NTS) serotypes in raw sewage from the Metropolitan Region of São Paulo.

Serovar (number of isolates)	<i>qnrB</i>	<i>oqxA</i>	<i>oqxB</i>	<i>bla_{CTX-M}</i>	<i>rmtA</i>
<i>S.</i> 1,4,[5],12:i:- (n = 25)	1	2	2	4	2
Cerro (n = 7)	0	0	0	2	0
Enteritidis (n = 6)	0	0	0	0	0
Braenderup (n = 4)	1	0	0	1	0
Typhimurium (n = 3)	2	0	0	0	0
Total (n = 45)	4 (8.9%)	2 (4.4%)	2 (4.4%)	7 (15.6%)	2 (4.4%)

community outbreaks in 2017 that were caused by raw milk and pork [47]. Similar to what we found in this study, AMR was reported against ampicillin, streptomycin, sulfamethoxazole, tetracycline, trimethoprim, and chloramphenicol. *S. Cerro*, represented by seven (15.6%) isolates in this study, is generally less prevalent than other serotypes, maybe due to its low pathogenic potential [39], although recently Cohn *et al.* [48] have described its potential transcriptional mechanisms which may facilitate its survival in and adaptation to the respective hosts and impact its ability to cause disease [48]. On the other hand, several studies report that *S. Enteritidis* is endemic in the state of São Paulo, due to its high prevalence among isolates from human and non-human sources [21,45,49]. In the present study, six (13.3%) strains were identified, and the presence of this serotype in raw sewage may indicate the circulation of this pathogen in the population and a potential risk of environmental spread. Although serotype *S. Braenderup* is represented by only four isolates (8.9%) in this study, the importance of this serotype should not be ignored. The CDC (2018) reports an outbreak of this serotype where epidemiological, laboratory, and tracing evidence indicate that shell eggs produced by the Rose Acre farm are the likely source of this outbreak in many states of the USA. Laboratory testing identified the outbreak strain of *Salmonella* Braenderup in environmental samples taken in the farm [50].

AMR in *Salmonella* can have potential public health impact. In our study, the highest resistance rate among all serotypes was observed for the (fluoro) quinolones nalidixic acid and ciprofloxacin, the latter being extensively used in salmonellosis treatment [23]. In addition, Hayes *et al.* have recently reported that the presence of ciprofloxacin in wastewater poses a significant risk of AMR selection by the microbial community [51]. It is to be noted that *S. 1,4,[5],12:i:-*, that was represented by more than a half of the isolates, showed 96% and 60% resistance to nalidixic acid and ciprofloxacin, respectively (Table 2). Casas *et al.* also found that *S. 1,4,[5],12:i:-* may be highly associated with nalidixic acid and ciprofloxacin resistance, and that this serotype may be involved in human diseases and foodborne outbreaks [21]. Although represented by a smaller number of representative isolates, the serotypes *S. Typhimurium* and *S. Braenderup* also showed high rates of (fluoro) quinolone resistance. Corroborating phenotypic results, these three serotypes carried the plasmidial-encoded gene *qnrB*, while *S. 1,4,[5],12:i:-* also carried the PMQR efflux pump genes *oqxAB* which can all be easily transferred amongst

Gram-negative bacteria by conjugation. Regarding beta-lactams, the highest resistance rates were observed for ampicillin and amoxicillin-clavulanate, again with emphasis on serotype *S. 1,4,[5],12:i:-*, which had more than 90% of isolates resistant to these antimicrobials. In addition, one isolate from this serotype and two from *S. Braenderup* were resistant to third-generation cephalosporins and produced an extended-spectrum beta-lactamase, which was confirmed by PCR results that indicated the presence of *bla_{CTX-M}* genes in these strains. CTX-M-encoding genes were also detected in a *S. Cerro* strain that did not express the ESBL phenotype. Further molecular analysis will be necessary to explain these results. Aminoglycoside resistance was observed in all the serotypes. In Gram-negative bacteria, the transferable genes responsible for resistance to these compounds encode 16S rRNA methyltransferases *Rmt* [34]. In this study, we detected two *S. 1,4,[5],12:i:-* isolates carrying *rmtA* genes, while the other resistant strains may express different kinds of aminoglycoside resistance.

Undoubtedly, *S. 1,4,[5],12:i:-* stood out from the other serotypes in this study due to the number of isolates, the percentage of antimicrobial resistance characterizing most of them as multidrug-resistant, and the carriage of five plasmidial resistance genes. These findings reinforce the clinical importance of this serotype, while raising a concern regarding its role in AMR transmission through the food chain and the environment.

Conclusions

The presence of NTS with pathogenic potential and resistance to antibiotics in raw sanitary sewers in the studied region indicates that these serotypes are circulating in the Metropolitan Region of São Paulo. Considering that raw sewage may represent a valuable tool to investigate the epidemiological status of populations, our study supports the view that NTS are being excreted by infected and ill people. The dissemination and transmission of pathogens throughout the environment is worrisome, especially in developing countries with low level of sewage collection coverage. Improvement in basic sanitation could provide a better infectious diseases' scenery in Brazil, and investments in new sewage treatment technologies could reduce the risk of AMR development, acquisition, and selection.

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