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Characterization of Antimicrobial Resistance in Salmonella enterica Serovars Typhi and Paratyphi B in Zambia

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Abstract

Typhoidal *Salmonellae* are invasive and life-threatening human pathogens that cause typhoid and paratyphoid fever in many low income countries globally. People consuming contaminated food, water or working with infected livestock have the potential to become infected with *Salmonella* and may require antimicrobial therapy. Antimicrobial therapy in salmonellosis has become a global public health problem due in part to the inappropriate use of antimicrobial agents. The objective of this study was to characterize antimicrobial drug resistance in *Salmonella serovar* Typhi and *Salmonella serovar* Paratyphi isolates obtained from January 2010 to December 2012 during routine patient care at the University Teaching Hospital in Lusaka, Zambia. Seventy-seven Salmonellae from diagnostic faecal and blood samples of patients were identified by biochemical, serological and PCR testing. The isolates were analysed for drug susceptibility by the minimum inhibition concentration method, PCR drug resistance gene detection and DNA sequencing of integron class I. All the fifty *Salmonella* Typhi were resistant to sulphamethoxazole, ampicillin, trimethoprim and, cotrimoxazole, while 84% were resistant to chloramphenicol, and 4% to both ciprofloxacin and amoxicillin + clavulanic acid. Similarly, all the 27 *Salmonella* Paratyphi B isolates were resistant to ampicillin, cotrimoxazole, chloramphenicol, sulfamethoxazole trimethoprim and streptomycin, while 11.1% were resistant to amoxicillin + clavulanic acid and 7.4% to both ciprofloxacin and tetracycline. Multidrug resistance was observed in 84 % of *Salmonella* Typhi and 100% of *Salmonella* Paratyphi B isolates. Class 1 integron containing the *dfrA7* gene was detected in 66% of *Salmonella* Typhi (66%) and 81.5 of *Salmonella* Paratyphi B. In summary, multidrug resistant *Salmonella* Typhi and Paratyphi, harbouring class I integron cassettes, are emerging in Lusaka, leaving little treatment options. Therefore, regular monitoring of antibiotic susceptibility patterns is vital in guiding appropriate therapy and prevention of further emergence of drug resistance strains.

Key Words: Integron, Multidrug Resistance, *Salmonella* serovar**,** *S.* Paratyphi, *S.* Typhi

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Introduction

Enteric fever remains a serious public health problem, especially in low income countries. It is caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), and to a lesser extent, strains of *S. enterica* serovar Paratyphi (*S.* Paratyphi) A, B and C [1-3]. Enteric fever is transmitted through consumption of

contaminated food and water, and it is sometimes associated with chronic carriers and food handlers [4-6]. It is estimated that there are approximately 27 million cases and over 200 000 deaths worldwide (4). However, the data on which these estimates are based are limited, and come from isolated studies in countries with healthcare structures capable of assessing the burden of salmonellosis, and accurate figures are usually compounded by limitations in diagnostic capacity [4, 6-10].

Cases of enteric fever are often severe and require effective antibiotic therapy for treatment [4, 11-13]. However, a number of *Salmonella* isolates from human patients and food animals are resistant to multiple antimicrobial drugs. Over the years, chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole have been the drugs of choice for the treatment of enteric fever, but have now become ineffective, leading to a rapid increase in the number of multidrug-resistant (MDR) strains of *Salmonella* [14-19]. Fluoroquinolones and extended spectrum cephalosporins are now drugs of choice for typhoid fever, while azithromycin is an alternative treatment option for uncomplicated typhoid cases [20-27]. This resistance is mainly attributed to the inappropriate use of antimicrobial drugs in human and veterinary medicine that creates selective pressure for the proliferation of resistant bacteria [4,9,16,28]. Potential routes of human exposure to resistant strains of *Salmonella* include consumption of unpasteurized dairy products, undercooked meat, manure-contaminated produce or through direct contact with animals and their products [29].

Resistance to quinolones in *Salmonella* species is mostly attributed to point mutations in the quinolone resistance-determining regions (QRDRs) of the target genes *gyrA*, *gyrB*, *parC*, and *parE* [30-32] or because of decreased permeability to the agents or over-expression of efflux pumps [33-36]. More recently *qnr* genes, the products which inhibit quinolone action by binding to *gyrA* and *gyrB* subunits, have been reported [21,32]. Nucleotide changes in the QRDR of *gyrA* in *Salmonella* are more common than mutations in *gyrB* or *topoisomerase* genes [19,37,38]. In *S.* Typhi, nucleotide substitutions at Ser-83, Asp-87, Glu-133, Asp-76, Phe-72, Leu-55, and Gln-106 of *gyrA* gene have been previously reported, with mutation at codon 83 being the most common [19,38].

Antibiotic resistance genes can be propagated by mobile genomic cassettes, including integrons and transposons that can reside on bacterial chromosomes or plasmids [21, 39, 40, 41]. Plasmid-associated integrons are frequently implicated in MDR *Salmonella* [42, 43]. There are five different classes of integrons habouring different integrated gene cassettes, of which Class 1 is the most commonly associated with enteropathogens [44-46]. Integrons are made up of sequences of conserved DNA that contain an integrase gene, *IntI, which* encodes the integrase enzyme responsible for causing the incorporation of gene cassettes via site-specific gene recombination mechanisms [46]. All the integrons comprise two conserved segments (5'CS) and (3'CS), an integrase gene, a variable region and the cassette integration site, attI. The 3'CS region contains three open reading frames (ORFs): *qaEΔ1* gene which confers resistance to quaternary ammonium compounds and *sul1* gene which confers resistance to sulphonamides [46,47].

The continuous monitoring of *Salmonella* isolates resistant to commonly used antibiotics is necessary because of public health implications of a potential spread of resistant organisms. However, little research has been conducted on the characterisation of antibiotic resistant *Salmonella* in low income countries, such as Zambia, because of limited diagnostic capacity. Therefore, the objective of this study was to characterise antimicrobial drug resistance patterns of *Salmonella enterica* serovars obtained from clinical specimens from 2010-2012 at the University Teaching Hospital, in Lusaka, Zambia

Materials and Methods

Bacterial Isolates

Seventy-seven single clinical isolates of S. Typhi and S. Paratyphi B strains obtained from faecal and blood specimens from January 2010 to December 2012 at the University Teaching Hospital in Lusaka, Zambia as part of the routine hospital care were analysed in this study. Bacterial isolates were stored in Brain Heart Infusion Broth (Oxoid Diagnostics, UK) containing 20% glycerol at -80^{o}C for the duration of the study. For experimental purposes, isolates were cultured on MacConkey agar and Xylose Lysine Deoxycholate agar (Merck, Hamburg, Germany) for 18-24 hours at 37˚C. All presumptive positive suspected *Salmonella* colonies were identified using biochemical tests, followed by serogrouping using slide agglutination with hyperimmune polyvalent O antisera (Remel Europe

Ltd, Kent, United Kingdom). Isolates belonging to serogroup O:9 were then subsequently subjected to a *Salmonella* specific PCR assay for confirmation and was performed as previously described [49].

Antimicrobial Susceptibility Testing

Antibiotic resistance profiles were determined using the Sensititre System TREK Diagnostic Systems Ltd., East Grinstead, England, which is based on the classical broth microdilution method, according to manufacturer's protocol. Briefly, one to two isolated colonies from freshly streaked plates were suspended in 5 ml of demineralized water to obtain a 0.5 McFarland density. Ten microliters of the bacterial water suspension was added to 11 ml of Cation Adjusted Mueller-Hinton broth (CAMHB). A 50µl aliquot of the CAMHB cell suspension was dispensed into each well of a 96-well and were tested against a panel consisting of 17 antibiotics: amoxicillin/clavulanic Acid (AMC) ampicillin (AMP), Apramycin (APR), ceftaxime (CTX), ceftiofur (FOT), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), flofernicol (FFN), gentamicin (GEN), nalidixic acid (NAL), neomycin (NEO), spectomycin (SPE) streptomycin (STR), sulfamethoxazole (SMX), tetracycline (TET), and trimethoprim (TMP). In addition susceptibility to trimethoprim-sulphamethoxazole (cotrimoxazole) (TS) was determined using the Kirby-Bauer disk diffusion test on Mueller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines [50], and clinical breakpoints interpretative criteria for resistance (R) were used, except for ceftiofur, colistin, florfenicol, neomycin, spectinomycin, and streptomycin, respectively where epidemiological cut-off values according to EUCAST recommendations (http://www.eucast.org). *Escherichia coli* ATCC 25922 was used as the quality control strain for antimicrobial susceptibility testing and was performed.

Integron Screening and DNA Sequencing

Bacterial DNA was extracted on the easyMag instrument (bioMérieux Inc, Durham, NC, USA) using the "off-board lysis" protocol as recommended by the manufacturer. The isolates were screened for class 1 integrons by PCR as described previously [51] using primers *intF*: GGCATCCAAGCAGCAAG and *intR*: AAGCAGACTTGACCTGA. Briefly, amplification was performed in a final volume of 25µl. Each reaction mixture contained 5.5µl molecular grade water, 12.5µl PCR master mix 2X (0.05u/µl *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP) (Thermo Fisher Scientific Inc, SA), 2.5µl of each primer and 2µl of bacterial DNA. Amplification reaction was carried on a GeneAmp System 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation at 94^{o}C for 5 minutes, followed by 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing for 55^{o}C for 30 seconds and extension for 72^{o}C for 2 min 30 seconds, and a final extension for 10 minutes at 72^{o}C. Amplified products were then purified using Qiaquick purification columns (QIAGEN, Crawley, United Kingdom) according to the manufacturer's instructions.

Sequencing was performed using the above-named primers. Forward and reverse linear amplification was performed in 10µl using 2µl of the purified PCR product (about 20 to 200ng), 2µl BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA**)**, 1µl BigDye Sequencing Buffer (Applied Biosystems, Foster City, CA, USA**)** 1µM of each primer. Linear amplification consisted of 25 cycles of denaturation at 96^{o}C for 10s, annealing at 60^{o}C for 30s and elongation at 72^{o}C for 60s using the iCycler Thermocycler (Bio-Rad, Hercules, CA, USA). The entire extension products were transferred into 80ml of freshly prepared precipitation solution (3ml of 3M sodium acetate [pH 4.6], 62.5ml of non-denatured 95% ethanol and 14.5ml deionised water), incubated for at least 1hr at room temperature and centrifuged at 14000rpm for 20min. After carefully removing the supernatant, 250µl of 70% ethanol was added to the pellet, vortexed and the contents re-centrifuged at 14000rpm for 8min. The ethanol was carefully aspirated and the pellet air-dried for 15min at room temperature. The samples were analysed on an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). The DNA sequence reads were edited using Ridom TraceEdit software (Ridom Bioinformatics GmbH, Würzburg, Germany) and used to search the National Center for Biotechnology Information (NCBI) RefSeq database using BLASTN software (http://www.ncbi.nlm.nih.gov/BLAST).

Ethics approval: Ethics approval for this study was granted by the University of the Zambia Biomedical Research Ethics Committee.

Results

Antimicrobial Susceptibility of *S.* Typhi and *S.* Paratyphi B Isolates

Among the antimicrobial tested, *S.* Typhi and *S.* Paratyphi B were 100% resistant to ampicillin, sulfamethoxazole, trimethoprim, cotrimoxazole and streptomycin. In addition *S.* Paratyphi B was also 100% to chloramphenicol and spectinomycin, while *S.* Typhi was 84% resistant to chloramphenicol. *S.* Typhi was least resistant to ciprofloxacin (4%), spectinomycin (4%), amoxicillin and clavulanic acid (2%), tetracycline (2%), while S. Paratyphi B was least resistant to gentamycin (3.7%), ciprofloxacin (7.4%), tetracycline (7.4%), colistin (11.1%), amoxicillin and clavulanic acid" (11.1%) (Figures 1 and 2). All the isolates were at least resistant to five or more antimicrobial drugs. The majority of the *S.* Typhi isolates (72%) were resistant to five antibiotics, while 74.1% *S.* Paratyphi B isolates were resistant to seven antimicrobial drugs (Table 1). Multidrug resistance (MDR) was observed in 84% of S. Typhi and 100% of *S.* Paratyphi B isolates. Multidrug resistance to *Salmonella* was defined as being resistance to ampicillin, chloramphenicol and cotrimoxazole.

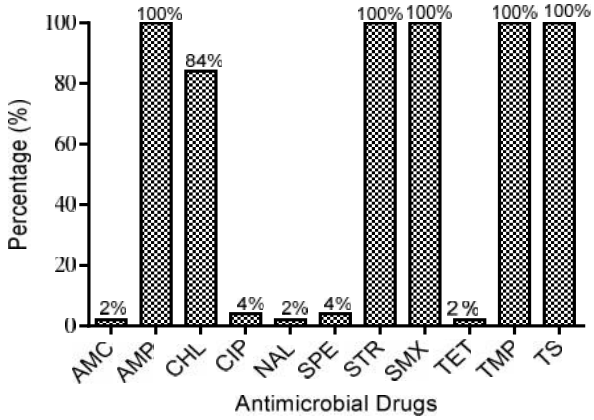

Figure 1: Antibiotic resistance profile of *S.* Typhi. AMC-amoxicillin/clavulanic acid, AMP-ampicillin, CHL-chloramphenicol, CIP-ciprofloxacin, NAL-nalidixic acid, SPE-spectinomycin, STR-streptomycin, SMX-sulfamethoxazole, TET-tetracycline, TMP-trimethoprim and TS-cotrimoxazole. S. Typhi was completely susceptible to apramycin, cefotaxime, colistin, florfenicol, gentamycin and ceftiour.

Integron Screening and DNA Sequencing

PCR-based detection of class 1 integrons was performed on all the 77 *Salmonella* isolates analysed. Of the fifty *S.* Typhi isolates, 33(66%) carried class I integrons, while 22 (81.5%) of the 27 *S.* Paratyphi B isolates analysed were also positive for the class I integrons (Figure 3). The integrons showed variable fragment sizes, and the most common size among the two serovars was 800bp. Sequence analysis of the integrons revealed that in the 29/50 (77.8%) integron positive *S.* Typhi had a common resistance pattern of ampicillin-chloramphenicol-sulfamethoxazole-trimethoprim-cotrimoxazole, while those of *S.* Paratyphi B (22/27, 81.5%) had the ampicillin-chloramphenicol-spectinomycin-sulfamethoxazole-trimethoprim-cotrimoxazole. The most common gene cassette detected in both serovars was that of *dfrA7*.

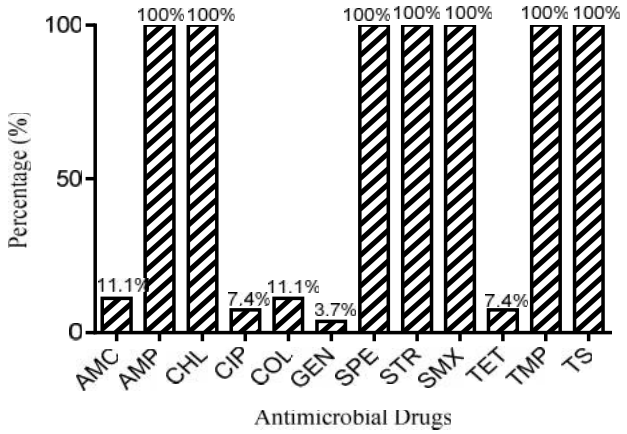

Figure 2: Antibiotic resistance profile of *S.* Paratyphi B. AMC-amoxicillin/clavulanic acid, AMP-ampicillin, CHL-chloramphenicol, CIP-ciprofloxacin, COL-colistin, GEN-gentamycin, SPE-spectinomycin, STR-streptomycin, SMX-sulfamethoxazole, TET-tetracycline, TMP-trimethoprim and TS-cotrimoxazole. *S.* Paratyphi B was completely susceptible to apramycin, cefotaxime, nalidixic acid, florfenicol and ceftiour

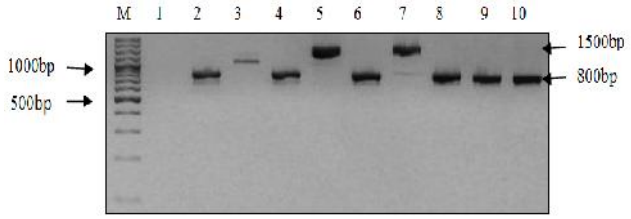

Figure 3: PCR of class 1 integron among clinical isolates of *S.* Typhi and *S.* Paratyphi. M: 100bp Marker, Lane 1: Negative control, Lane 2: Positive control, Lane 3-10, Positive clinical isolates.

When the association of antimicrobial drug resistance and the presence of integron class I was investigated, 33 (66%) of the integron-positive isolates were found to be statistically more resistant to chloramphenicol than the 17(34%) integron-negative isolates (p=0.03) (Table 2). This was only

observed in *S.* Typhi isolates. There was no statistically significant difference with the other antibiotics.

Pattern	**No. of Isolates (%)**	**Resistance to antimicrobial drugs (n)**
S. Typhi		
AMP-CHL-CIP-NAL-STR-SMX-TMP-TS	4	8
AMC-AMP-CHL-SMX-STR-TMP-TS	2	7
AMP-CHL-SMX-STR-TET-TMP-TS	2	7
AMP-CHL-SPE-STR-SMX-TMP-TS	4	7
AMP-CHL-SMX-STR-TMP-TS	72	6
AMP-SMX-STR-TMP-TS	16	5
S. Paratyphi B		
AMP-CHL-COL-SPE-SMX-STR-TMP-TET-TS	7.4	9
AMP-CHL-COL-SPE-SMX-STR-TMP-TS	3.7	8
AMP-CHL-GEN-SPE-SMX-STR-TMP-TS	3.7	8
AMC-AMP-CHL-SPE-SMX-STR-TMP-TS	3.7	8
AMP-CHL-CIP-SPE-SMX-STR-TMP-TS	7.4	8
AMP-CHL-SPE-SMX-STR-TMP-TS	74.1	7

Table 1: Antimicrobial Resistance Patterns of S. Typhi and S. Paratyphi B; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; FFN, florfenicol; GEN, gentamicin; NAL, nalidixic acid; SPE, spectinomycin; STR, streptomycin; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; SXT, and trimethoprim-sulfamethoxazole.

Antibiotic	Integron-Positive (n=33)		Integron-negative (n=17)		*p*-Value
	%S	%R	%S	%R	
AMC	97	3	100	0	1.000
AMP	0	100	0	100	1.000
APR	100	0	100	0	1.000
CTX	100	0	100	0	1.000
CHL	6.1	93.9	35.3	64.7	**0.013**
CIP	93.9	6.1	100	0	0.542
COL	100	0	100	0	1.000
FFN	100	0	100	0	1.000
GEN	100	0	100	0	1.000
NAL	97	3	100	0	1.000
NEO	100	0	100	0	1.000
SPE	93.9	6.1	100	0	0.542
STR	0	100	0	100	1.000
SMX	0	100	0	100	1.000
TET	97	3	100	0	1.000
TMP	0	100	0	100	1.000
TS	0	100	0	100	1.000
XNL	100	0	0	100	1.000

Table 2. Antibiotic susceptibility of integron-positive and integron-negative strains of *S.* Typhi; %S, percentage susceptible; %R, percentage resistant; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; APR, apramycin, CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; CTX, cefotaxime; FFN, florfenicol; GEN, gentamicin; NAL, nalidixic acid; NEO, neomycin; SPE, spectinomycin; STR, streptomycin; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole and XNL, ceftiour. Statistical significance (*p*-Value) was calculated using the Fishers exact test in terms of the number of resistant strains and susceptible strains in the integron-positive and integron-negative groups.

Discussion

In this study nearly all the *S.* Typhi and *S.* Paratyphi B isolates were resistant to ampicillin, cotrimoxazole, streptomycin, sulfamethoxazole and trimethoprim, while 84% of *S.* Typhi and 100% *S.* Paratyphi B were resistant to chloramphenicol. These findings are consistent with other studies in other regions of the world where it was observed that there

was a huge number of *Salmonella* isolates that were resistant to ampicillin, chloramphenicol and cotrimoxazole [27, 43, 44, 52, 53]. Similar results were reported on the Malawi-Mozambique border in which 100% of *S.* Typhi isolates were found to be resistant to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim [23]. Another study conducted in Uganda showed that 76% of *S.* Typhi were resistant to ampicillin, streptomycin, sulphisoxazole, tetracycline, and cotrimoxazole, but were susceptible to chloramphenicol [54]compared to 100% ampicillin and cotrimoxazole and 84% to chloramphenicol resistance reported in our study. *S.* Typhi was only completely susceptible to cefotaxime, gentamicin, and spectinomycin. These drugs could be used for treating affected patients.

The high susceptibility rates to flouroquinolones are important as these antibiotics are alternatives in the treatment of resistant cases [55,56]. In this study flouroquinolone resistance was found to be low. Reduced resistance to ciprofloxacin was found to be comparatively lower as compared to 15.4% in the Democratic Republic of Congo [30], 100% in Turkey [56], and 81% in the USA [57]. Nalidixic acid resistance was also found to be low in this study compared to other studies [30,58,59]. The sensitivity of the isolates to quinolones and cephalosporins suggests that this group of antibiotics could be used for the treatment of enteric fever cases. However, the use of antimicrobial drugs for the treatment of enteric fever should be carefully monitored in order to avoid the development of drug resistant strains.

This study also demonstrated the occurrence of MDR strains of *S.* Typhi and *S.* Paratyphi B. The commonest resistance pattern was observed with ampicillin, chloramphenicol, sulfamethoxazole, streptomycin, trimethoprim and cotrimoxazole for *S.* Typhi and ampicillin, chloramphenicol, spectinomycin, sulfamethoxazole, streptomycin, trimethoprim and cotrimoxazole for *S.* Paratyphi B. Demczuk and colleagues [5] observed 26 resistance patterns with the commonest patterns being nalidixic acid-resistant (NAR) and ampicillin-chloramphenicol-nalidixic acid-streptomycin-sulphisoxazole-cotrimoxazole in *S.* Typhi. This study demonstrated that most of the isolates were resistant to five or more antibiotics. The MDR detection rate was 84% for *S.* Typhi and 100% for *S.* Paratyphi. Similar findings of MDR strains of *S.* Typhi were reported in Pakistan (58.7%), Vietnam (89.9%) and Turkey (100%) [56, 60, 61]. However, in Tajikistan and the Democratic Republic of Congo resistance was considerably found to be low, 27% and 30.3% [30, 62].

In this study antimicrobial drug resistance could be attributed to the inappropriate use of these drugs and also the use of cotrimoxazole in HIV/AIDS patients as routine prophylaxis for opportunistic infections prevention [63, 64]. The other reason could be that the laws and regulations are not strict enough to control the abuse of antimicrobial drugs in Zambia [65]. A similar observation was made in India and this was attributed to the widespread availability and uncontrolled use of antibiotics, leading to selective pressure on a large bacterial population of endemic *Salmonella* species [58]. These practices, together with horizontal gene transfer, have contributed to the emergence of *Salmonella* isolates that are resistant to commonly used antimicrobial drugs used for therapy.

MDR is known to be associated with integrons, especially those in class I, which are widely distributed among Gram-negative bacteria, and are implicated in the horizontal transfer of drug resistance genes [45,60,66-68]. In our study 66% of *S.* Typhi isolates and 81.5% of *S.* Paratyphi B harboured class I integrons. In contrast an Iranian study found that 23% of *S.* Typhi and 45% of *S.* Paratyphi contained class I integrons [69]. Our findings also support the notion that class I integrons are widespread, especially in Gram negative bacteria, including *Salmonella* species.

Integron-carrying bacterial isolates are usually MDR in nature, whereas integron-lacking isolates are more susceptible to integron-associated antibiotics, and in many cases, they have been associated with the presence of other drug resistance genes, including *aadA, aadB, strA and strB* [48,70]. In this study, DNA sequencing of class I integron elements revealed a *dfrA7* gene cassette, which encodes resistance to trimethoprim. All the isolates were resistant to trimethoprim-sulfamethoxazole. Similar studies conducted in Canada and Pakistan showed similar patterns [42,60].

When drug resistance and the presence of integrons were compared, it was revealed that resistance to chloramphenicol was associated with the presence of the integrons, indicating that isolates containing class I integrons were more likely to be resistant to chloramphenicol than those lacking the integrons. This was surprising because no drug resistance gene cassette conferring resistance to chloramphenicol were detected in the integrons.

There was no association between drug resistance to the other drugs tested and the presence of integrons. This finding suggests that resistance to these drugs was due to other drug resistance mechanisms other than those associated with the presence of class I integrons.

Conclusion

This study revealed a high prevalence of MDR *S.* Typhi and *S.* Paratyphi B isolates. This is alarming since resistance to first-line drugs will require more expensive drugs for effective treatment of enteric fever. Class I integrons were prevalent in the majority of the isolates, and probably play a major role in MDR mechanisms of *S.* Typhi and *S.* Paratyphi B isolates. A better understanding of the factors that potentially contribute to the development and dissemination of drug resistant strains of *Salmonella* could allow for improved measures to treat salmonellosis and minimize antimicrobial resistance.

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Conflict of Interest: The contents of this paper reflect the views of the authors who are responsible for the facts and accuracy of the data presented herein and do not necessarily reflect the views or policies of any institution or agency. This paper does not constitute a standard, specification, nor is it intended for design, construction, bidding, contracting, or permit purposes. The authors have no conflicts of interest.

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