Original Article

Identification of class 1 and 2 integrons from clinical and environmental *Salmonella* isolates

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Abstract

Introduction: The indiscriminate use of antimicrobials has selected for the emergence of resistant strains. Many mechanisms contribute to the spread of antimicrobial-resistant genes, and integrons play a key role in this process. The aim of this study was to describe the serotypes and resistance profiles, and to characterize the presence of integrons in *Salmonella* strains isolated from Dourados, Mato Grosso do Sul, Brazil.
Methodology: Thirty-six isolates from different sources were used. To evaluate the resistance profiles, the determination of minimum inhibitory concentrations together with polymerase chain reaction were used to screen for the presence of class 1 and class 2 integrons.
Results: The Infantis serotype of *Salmonella* was the most frequently isolated serotype. Minimum inhibitory concentrations showed that out of the 36 isolates, 11 (30.5%) were resistant to all the antimicrobials tested. These resistant isolates were separated into three groups: 4 clinical isolates (36.4%), 3 food isolates (36.4%), and 4 water isolates (27.2%). Class 1 integrons occurred in 31 (86.1%) isolates and were found in all 11 resistant isolates (35.5 %) and in 20 (64.5%) of the non-resistant isolates. Class 2 integrons were found in 3 (8.3%) isolates, which were all non-resistant.
Conclusion: The presence of an integron did not necessarily confer resistance. Future studies will seek to identify the mechanism behind integron-mediated antimicrobial resistance.

Key words: *Salmonella*; antibiotic resistance; integrons; resistance mechanisms.

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Introduction

Salmonellosis is one of the most common causes of foodborne illnesses (FBIs) worldwide due to the widespread occurrence of infected people. The economic impact of *Salmonella* infections includes the costs of medical treatment and time lost from work, as well as significant costs to the food industry (including veterinary medicine) *Salmonella* recalls, and the culling of infected livestock [1]. *Salmonella* infections result in gastroenteritis, which may even result in death in some cases [2].

In Brazil, between 2001 and 2010, *Salmonella* was the main etiologic agent of FBI in 19.16% [3] of confirmed cases. In the state of Mato Grosso do Sul, between 1999 and 2006, *Salmonella* was detected in 30.3% of the confirmed cases of FBI [4]. The transmission of salmonellosis among humans is possible, though not common. *Salmonella* species have been isolated from a variety of environmental sources, including animals destined for human consumption and drinking water, which are the most common routes of infection for people [5,6].

For the treatment of *Salmonella* infections, the Brazilian Ministry of Health recommends chloramphenicol, ampicillin, sulfatomexazol/trimethoprim, amoxicillin, quinolones, fluoroquinolones, and cetriaxona, with antipyretics for oral hydration [3]. However, the World Health Organization recommends quinolone antibiotics for adults and third-generation cephalosporins for children with serious *Salmonella* infections. In addition, drugs such as chloramphenicol, ampicillin, amoxicillin, and sulfamethoxazole/trimethoprim are suggested for occasional use [7].

Large hospital facilities initially use routine antibiotics. Following susceptibility tests, the most appropriate treatment is then selected in the treatment of *Salmonella* infection; the use of antimicrobials is only common for high-risk groups [8].

The misuse of antimicrobials by the medical and veterinary industries has led to an increase in multi-drug resistant *Salmonella* strains [9,10]. There are several antimicrobial resistance mechanisms, which include changes to outer membrane porins reducing antimicrobial uptake and the horizontal transfer of antibiotic resistance genes [2,11,12].

The main mechanism of the spread of antimicrobial resistance is gene transfer [13], with resistance being mediated through mobile genetic elements such as plasmids, transposons, and integrons [14]. Integrons are genetic elements that acquire mobility once they are inserted into plasmids or associated with transposons [15]. Gene cassettes are expressed through site-specific recombination within a variable region in the microbial genome [15]. The majority of mobile integrons have been found in Gram-negative bacteria [16,17].

Integrons are divided into class 1, class 2, class 3, class 4, and class 5, with class 1, 2, and 3 integrons being related to resistance genes [18]. Over 130 resistance gene cassettes have been identified in class 1 integrons; however, only 6 cassettes have been identified in class 2 integrons [15,18].

Given the increasing prevalence of *Salmonella* isolates resistant to antibiotics, it is important to determine the mechanisms responsible for resistance to assist practitioners. The aim of our research was to determine the antimicrobial resistance profile in *Salmonella* and to associate this with the presence of class 1 and class 2 integrons in isolates from Dourados, Mato Grosso do Sul.

Methodology

Bacterial isolates

This study included 36 isolates of *Salmonella*, isolated between 2010 and 2011 from the Laboratory of Applied Microbiology, School of Environmental and Biological Sciences, Federal University of Grande Dourados. Seventeen strains were isolated from chicken and fish (food), nine strains were isolated from fish farming lakes and ponds (water), and ten strains were isolated from patients with clinical infections at the University Hospital of Dourados.

Identification of Salmonella

Isolates from food, water, and clinical infections were identified using specific polyvalent and monovalent antisera to the somatic and flagellar antigens [2]. In this study, isolates were sent to Instituto Oswaldo Cruz Foundation (FIOCRUZ/RJ) for serotypic characterization.

Minimum inhibitory concentrations

The resistance profile of each isolate was determined using the minimum inhibitory concentration (MIC), with the measurements performed in triplicate. This was carried out according to the Clinical and Laboratory Standards Institute document M11-A8 [19]. Six antibiotics were tested: ampicillin (AMP, 10 μg/mL), ciprofloxacin (CIP, 5 μg/mL), gentamicin (GEN, 10 μg/mL), norfloxacin (NOR, 10 μg/mL), tetracycline (TET, 30 μg/mL), and sulfamethoxazole/trimethoprim (SUT, 23.75/1.25 μg/mL).

The antibiotics were prepared according to the recommendations of the CLSI document M100-S22 [20]. *Salmonella* ATCC 1406 was used as the control.

The results of the resistance profiles were determined according to the CLSI document M100-S23 [21]. Microorganisms were classified as either resistant (resistance profile to one or more antimicrobials) or non-resistant (sensitive or intermediate to all agents tested).

Identification of class 1 and 2 integrons

DNA was extracted according to Chagas *et al*. [22], and the polymerase chain reaction (PCR), adapted from Santos *et al*. [23], was performed using three pairs of primers. To detect the integrase genes *int1* and *int2* [24], PCR was performed using the oligonucleotide primers described in Table 1. Each reaction was prepared separately with individual primer pairs, with a final volume of 25 μL, including 12.5 μL of PCR Master Mix (Thermo Scientific, San Jose, USA), 1.5 μL (10 pM/μL) of each primer (IDT, Coralville, USA), 10–50 ng of genomic DNA, and ultrapure water up to 25 μL. PCR was performed in a thermocycler (Biorad, Hercules, USA) with initial denaturation at 94°C for 5 minutes followed by 35

Table 1. Primers used for amplification of genes of *Salmonella* isolates

Class integron	Name/ designation	Target region	Nucleotide sequence (5 '– 3')	Size in base pairs (bp)
Class 1	INT1F	*Intl1*	F – CAGTGGACATAAGCCTGTTC	210–230
	INT1R	*Intl1*	R – CCCGACGCATAGACTGTA	
Class 2	INT2F	*Int2*	F – TTGCGAGTATCCATAACCTG	400
	INT2R		R – TTACCTGCACTGGATTAAGC	

cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. All the reactions included a negative control where the DNA was replaced with an equal volume of ultrapure water. Each isolate was tested twice to confirm reproducibility. Each PCR sample (10 μL) was added to loading buffer (Thermo Scientific) and subjected to electrophoresis on a 2.0% agarose gel at 120 volts for 30 minutes. A 50-bp DNA ladder (Thermo Scientific) was used. The DNA bands of amplified products were visualized under ultraviolet light and photographed on a PhotoDoc-It system (UVP, Upland, USA).

Results

From the 36 isolates, 12 different serotypes were identified. The most common serotypes were *Salmonella* Infantis (47.2%; n = 17), *Salmonella enterica* subsp. *enterica* (O:6,7:e,h:-) (16.6%; n = 6), and *Salmonella* Typhimurium (11.1%; n = 4) (Table 2). The serotypes most commonly identified in the food samples were *Salmonella* Infantis 58.8% (n = 10) and *Salmonella enterica* subsp. *enterica* (O:6.7:e,h) (11.7%; n = 2). The most common serotypes identified in the water samples were *Salmonella enterica* subsp. *enterica* (O:6.7:e,h:-) (44.4%; n = 4) and *Salmonella* Infantis 33.3% (n = 3). In the clinical infection samples, *Salmonella* Infantis (40%; n = 4) and *Salmonella* Typhimurium (40%; n = 4) were identified. The MIC showed that out of the 36 isolates, 11 (30.5%) were resistant to all the antimicrobials tested. These resistant isolates were separated into three groups: 4 clinical isolates (36.4%), 3 food isolates (36.4%), and 4 water isolates (27.2%). Class 1 integrons occurred in 31 (86.1%) isolates, and were found in all 11 resistant isolates (35.5 %) and in 20 (64.5%) of the non-resistant isolates. Class 2 integrons were found in 3 (8.3%) isolates, which were all non-resistant. Five resistance patterns were identified through this technique (Table 3).

For the MIC50 (minimum inhibitory concentration to inhibit the growth of 50% of the samples), there was no difference among the isolates. The MIC90 (minimum inhibitory concentration to inhibit the growth of 90% of the samples) was higher in isolates exhibiting a high prevalence of antibiotic resistance when compared to those that showed a lower prevalence of antibiotic resistance (Table 4). The antibiotic that showed the highest number of resistant isolates was sulfamethoxazole/trimethoprim with 19.4% (n = 7), followed by ampicillin with 13.8% (n = 5) resistant isolates.

The integrase 1 gene (*int1*) was present in 31 of 36 *Salmonella* isolates (88%), in 9.6% (n = 11) of the 12 resistant strains, and in 87.5 % (n = 20) of the 24 non-resistant isolates. The integrase 2 gene (*int2*) was found in only 8.3% (n = 3) of the non-resistant *Salmonella* isolates and was not detected in any of the resistant isolates (Table 3).

Isolates possessing the class 1 integron showed higher MIC90 values to all antibiotics except for ampicillin and sulfamethoxazole / trimethoprim compared to those isolates without the class 1 integron. There was no difference in MIC50 values between isolates with or without the class 1 integron, except for gentamicin (Table 4).

Table 2. *Salmonella* serotypes from water, food, and clinical samples

Serotype	Food n (%)	Water n (%)	CI n (%)	Total n (%)
S. Infantis	10 (58)	3 (33.3)	4 (40)	17 (47.2)
S. enterica (O:6,7:e,h:-)	2 (11.7)	4 (44.4)	0	6 (16.6)
S. Typhimurium	0	0	4 (40)	4 (11.1)
S. Agona	1 (5.8)	0	0	1 (2.7)
S. Paratyphi B	1 (5.8)	0	0	1 (2.7)
S. enterica (O:4,5:e,h:-)	1 (5.8)	0	0	1 (2.7)
S. enterica (O:6,7:r:-)	1 (5.8)	0	0	1 (2.7)
S. Saintpaul	1 (5.8)	0	0	1 (2.7)
S. enterica (O:6,7)	0	1 (11.1)	0	1 (2.7)
S. Ealing	0	1 (11.1)	0	1 (2.7)
S. Heidelberg	0	0	1 (10)	1 (2.7)
S. enterica (O:4,5:I,v:-)	0	0	1 (10)	1 (2.7)

CI: clinical infections

Table 3. Standard/Pattern in *Salmonella* isolates according to the minimum inhibitory concentration (MIC) and the presence of class 1 and class 2 integrons

Source	Food	Species	MIC standard	IF1	IF2
Food	F1	*S.* Infantis	Not resistant	+	-
	F2	*S.* Agona	Not resistant	+	-
	F3	*S.* Paratyphi B	Not resistant	-	-
	F4	*S. enterica* (O:6,7:e,h:-)	Not resistant	+	+
	F5	*S.* Infantis	Not resistant	+	-
	F6	*S.* Infantis	Not resistant	+	-
	F7	*S.* Infantis	Not resistant	+	+
	F8	*S.* Infantis	Not resistant	+	-
	F9	*S.* Infantis	Not resistant	+	-
	F10	*S. enterica* (O:6,7:e,h:-)	Not resistant	+	-
	F11	*S.* Infantis	SUT	+	-
	F13	*S.* Infantis	GEN	+	-
	F14	*S.* Infantis	SUT	+	-
	F15	*S. enterica* (O:4,5:e,h:-)	Not resistant	+	+
	F16	*S. enterica* (O:6,7:r:-)	Not resistant	+	-
	F17	*S.* Saintpaul	Not resistant	-	-
	P1	*S.* Infantis	AMP - SUT	+	-
Water	A1	*S. enterica* (O:6,7:e,h:-)	Not resistant	+	-
	A2	*S. enterica* (O:6,7:e,h:-)	SUT	+	-
	A3	*S. enterica* (O:6,7:e,h:-)	Not resistant	+	-
	A4	*S.* Infantis	Not resistant	+	-
	A5	*S. enterica* (O:6,7)	SUT	+	-
	A6	*S.* Infantis	SUT	+	-
	A7	*S. enterica* (O:6,7:e,h:-)	Not resistant	+	-
	A8	*S.* Ealing	Not resistant	+	-
	A12	*S.* Infantis	Not resistant	-	-
CI	H1	*S.* Infantis	Not resistant	-	-
	H2	*S.* Infantis	Not resistant	+	-
	H3	*S.* Typhimurium	AMP - SUT	+	-
	H4	*S.* Typhimurium	AMP - GEN - TET	+	-
	H5	*S.* Heidelberg	AMP	+	-
	H6	*S.* Infantis	Not resistant	+	-
	H7	*S.* Typhimurium	AMP - GEN - TET	+	-
	H8	*S.* Typhimurium	Not resistant	-	-
	H9	*S.* Infantis	Not resistant	+	-
	H10	*S. enterica* (O:4,5:I,v:-)	Not resistant	+	-

AMP: ampicillin; GEN: gentamicin; SUT: sulfamethoxazole/trimethoprim; TET: tetracycline. +: presence of the gene; -: absence of gene; CI: clinical infection

Table 4. MIC50, MIC90 and prevalence of resistance to all strains of *Salmonella* isolates according to their source of isolation (food, water, clinical infections) and according to the presence/absence of class 1 integrons

Antibiotic	MIC_{50} (µg/mL) for all isolates MIC_{50} (µg/mL) for food/water/CI	MIC_{90} (µg/mL) for all isolates MIC_{90} (µg/mL) for food/water/CI	MIC_{50} (µg/mL) for all isolates MIC_{50} (µg/mL) for isolates with int1/without int1	MIC_{90} (µg/mL) for all isolates MIC_{90} (µg/mL) for isolates with int1/without int1
AMP	1 1 / 1 / 1	≥512 1 / 4 / ≥512	1 1 / 1	≥512 ≥512 / ≥512
CIP	≤0.03 ≤0.03 / 0.06 / ≤0.03	0.125 0.125 / 0.125 / ≤0.03	≤0.03 ≤0.03 / ≤0.03	0.125 0.125 / ≤0.03
GEN	4 8 / 4 / 8	8 8 / 8 / ≥256	4 8 / 4	8 16 / 8
NOR	≤0.5 ≤0.5 / 1 / ≤0.5	1 1 / 1 / ≤0.5	≤0.5 ≤0.5 / ≤0.5	1 1 / ≤0.5
SUT	0.25–4.75 0.25–4.75 / 0.5–0.95 /0.5-9.5	4–76 8–152 / 16–304 / 4–76	0.25–4.75 0.25–4.75 / 0.25–4.75	4–76 8–152 / 16–304
TET	0.5 0.5 / 0.5 / 0.5	4 1 / 4 / 128	0.5 0.5 / 0.5	4 4 / 1

MIC_{50}: concentration (mg/uL) minimal to inhibit the growth of 50% of isolates; MIC_{90}: concentration (mg/uL) minimal to inhibit the growth of 90% of isolates; ; CI: clinical infection; AMP: ampicillin; CIP: ciprofloxacin; GEN: gentamicin; NOR: norfloxacin; SUT: sulfamethoxazole/trimethoprim; TET: tetracycline;; Int1: class 1 integron.

Discussion

According to the Country Databank [25] of the World Health Organization, a network that gathers information on the 15 *Salmonella* serotypes prevalent worldwide, the most common serotypes in South America are *S.* Enteritidis and *S.* Typhimurium, in both human and non-human sources. In Brazil, *S.* Typhimurium, *S.* Infantis, and *S.* Enteritidis are the most commonly found serotypes in food, in the environment, and in humans, respectively. *S.* Typhimurium is one of the most common serotypes found in humans and in food in Brazil and South America. In our study, this serotype was found exclusively in humans. In addition, we failed to isolate *S.* Enteritidis, despite its high global distribution and frequency. A previous study that analyzed poultry and chiller water samples from slaughterhouses in the state of Mato Grosso do Sul identified the serotype *S.* Schwarzengrund as the most prevalent (37.6%), followed by *S.* Typhimurium (17.2%), *S.* Corvallis (13.8 %), *S.* Enterica (O:4.5:-:1.2) (10.34%), and *S.* Enteritidis (10.34%) [26].

The emergence of multidrug-resistant species is related to the non-therapeutic use of antimicrobials, which has become an established part of intensive farming practice [27]. The presence of multidrug-resistant isolates in food animal production threatens the effectiveness of antimicrobials in the treatment of human diseases due to the horizontal gene transfer of multidrug resistance [10,27]. This is exemplified by our MIC results, where 30.5% of the isolates demonstrated a resistance profile. Several studies have identified antimicrobial resistance in *Salmonella* [8,28-30]. In countries such as South Korea, the quinolone group of antibiotics is no longer considered effective in combating salmonellosis due to the spread of resistance genes. This was shown by Lee *et al*. [9], who reported the ineffectiveness of quinolone antibiotics in treating *S. gallinarum*, the causative agent of typhoid fever in chickens. In this study, the prevalence of resistant isolates was lower than reported previously [9,30-33]. Although no quinolone resistance was observed, many isolates showed intermediate resistance. A study conducted by Kiffer *et al*. [34] revealed that the consumption of antibiotics was proportional to the rate of resistance in the regions around São Paulo's municipalities, where antimicrobial consumption is higher; thus, there was an increase in resistance. Based on the above results, a cautious approach in the administration of antimicrobials must be adopted in the studied region in order to prevent the increase of multidrug-resistant strains. Beier *et al.* [30] determined the MIC50 for *Salmonella* strains isolated from chickens, and reported the following results: ciprofloxacin (0.03 μg/mL), ampicillin (≤ 1 μg/mL), gentamicin (≤ 0.25 μg/mL), and tetracycline (≤ 4 μg/L). The MIC90 for these antibiotics was 0.03 μg/mL, 2 μg/mL, 16 μg/mL, and > 32 μg/mL, respectively. Our study showed similar results of the MIC50 for ampicillin; however, our isolates were more sensitive to tetracycline and less sensitive to gentamicin. A Danish study revealed that people who were susceptible to *Salmonella* infections showed a higher mortality rate compared to the rest of the population. The mortality rate for people with infections caused by multidrug-resistant *Salmonella* is estimated to be 10 times higher compared to the population in general [5]. The resistance of microorganisms to antibiotics may be associated with alterations in the porins present in the outer membrane, mutations, and genes carried by plasmids, among others [2,13,16]. The role of integrons in antimicrobial resistance has attracted the attention of researchers over the last decade, and several studies have identified a connection between the presence of integrons and multidrug resistance in *Salmonella* strains. Firoozeh *et al*. [35] isolated 43 multidrug-resistant *Salmonella* serovars and identified the presence of a class 1 integron in 88.3% of the isolates. Ahmed and Shimamoto [36] isolated 17 multidrug-resistant *Salmonella* strains, of which 42.9% contained a class 1 integron and 14.3% contained a class 2 integron. However, the present study showed a high prevalence of class 1 integrons in both resistant and non-resistant samples. Genes contained in integrons are often associated with multidrug resistance [35,37]. Integrons are most frequently found in clinical isolates, although their presence has also been reported in the environment and foods, which was confirmed by our results [37,38]. In addition to multidrug-resistant genes, integrons can also encode genes related to adaptation to different environments [39]. Several factors can influence the expression of resistant genes contained in integrons; a major factor is that genes found near the promoter tend to be expressed more effectively than those that are located further away. Therefore, effective multidrug resistance selects for the placement of resistance genes closer to the promoter [40]. Our research shows that there is no connection between resistance profiles and the presence or absence of the class 1 and 2 integrons. Our study highlights the need for more research on understanding how integrin-encoded genes contribute to antimicrobial resistance.

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