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Salmonella Heidelberg: Genetic profile of its antimicrobial resistance related to extended spectrum β-lactamases (ESBLs)

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ABSTRACT

The objective of this study was to evaluate the phenotypic and genotypic profile of antimicrobial susceptibility and the possible involvement of extended spectrum beta-lactamases (ESBLs) in the resistance profile of *Salmonella* Heidelberg (SH) isolated from chicken meat. We used 18 SH isolates from chicken meat produced in 2013 in the state of Paraná, Southern Brazil. The isolates were submitted to disk-diffusion tests and from these results it was possible to determine the number of isolates considered multiresistant and the index of multiple antimicrobial resistance (IRMA) against ten antimicrobials routinely used in human and veterinary medicine. It was considered multidrug resistant the isolate that showed resistance to three or more classes of antibiotics. Another test performed was the disc-approximation in order to investigate interposed zones of inhibition, indicative of ESBLs production. In the isolates that presented multidrug resistance (18/18), a search of resistance genes involved in the production of ESBLs was performed using PCR: *bla*CMY-2, *bla*SHV-1, *bla*TEM-1, *bla*CTX-M2, *bla*OXA-1, *bla*PSE-1 and *Amp*C. The overall antimicrobial resistance was 80.55%. The highest levels of resistance were observed for nalidixic acid and ceftiofur (100%). The most commonly resistance pattern found (42.1%) was A (penicillin-cephalosporin-quinolone-tetracycline). The results were negative for ghost zone formation, indicative of ESBLs. However, PCR technique was able to detect resistance genes via ESBLs where the *bla*TEM-1 gene showed the highest amplification (83.33%), and the second most prevalent genes were *bla*CMY-2 (38.88%) and *Amp*C gene (38.88%). The *bla*OXA-1 and *bla*PSE-1 genes were not detected. These results are certainly of concern since SH is becoming more prevalent in the South of Brazil and able to cause severe disease in immune compromised individuals, showing high antimicrobial resistance to those drugs routinely used in the treatment and control of human and animal salmonellosis.

1. Introduction

Salmonellosis is a global disease of concern to the health authorities and constitutes an important barrier to international trade of food, mainly those of animal origin [1]. According to Sousa; Conceição; Ferreira [2], there is an increasing number of Salmonella strains multi-resistants to many antimicrobials used in poultry and humans, and their early detection is important for proper treatment and to improve control and prevention of the disease in animals and humans.

The dissemination of ESBLs-producing strains in hospitals, environment, food chain, animals and humans is becoming more prevalent, increasing the need to better understand this mechanism of antimicrobial resistance in order to develop new therapy and prevention policies in human and veterinary medicine with the idea of One Health Concept in mind. ESBLs are most commonly found in *Klebsiella pneumoniae*, but they have been found with increasing frequency in *Escherichia coli*, *Proteus mirabilis* and other Gram negative bacilli such as *Salmonella* spp [2].

Salmonellosis can be a serious illness mainly in immunosuppressed individuals and chicken meat may transmit *Salmonella* spp.

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the causative agent of the disease. Beta-lactam antimicrobials, such as third generation cephalosporins, are the most commonly used antimicrobial to combat this disease in humans, specially ceftriaxone which is recommended for children with salmonellosis. Mechanisms of bacterial resistance, such as the production of ESBLs, may inhibit the action of this type of antimicrobial. Thus, the evaluation of ESBL indices in strains isolated from chicken meat samples is necessary in order to determine the degree and type of multiresistance present in these isolates.

Given the lack of studies regarding the presence of ESBLs in *Salmonella* spp. isolates from chicken meat in Brazil, the objective of this study was to evaluate the susceptibility and genetic profile of *Salmonella* Heidelberg regarding the ESBLs mechanism of antimicrobial resistance.

2. Material and methods

2.1. Sampling

Eighteen isolates of *Salmonella* Heidelberg were kindly provided by a private laboratory accredited for Salmonella isolation. These isolates were obtained in 2013 from a slaughterhouse in Paraná State, Southern Brazil. They were maintained in eppendorf tubes containing PCA agar and glycerin at −20 °C up to use in the Laboratory of Molecular Biology, Immunology and Microbiology (LABMIM) at the Universidade do Estado de Santa Catarina (UDESC), Chapecó, Brazil.

2.2. Disc-diffusion test

Disc-diffusion test were performed according to the methodology approved by the Clinical and Laboratory Standards Institute [3] and by the National Agency of Sanitary Surveillance (ANVISA), which is included in IN-2 A-8 Standardization of Antimicrobial Sensitivity Tests by Disk-diffusion [4]. The antimicrobials used were: amoxicillin associated with clavulanic acid 10 μg (penicillin), nalidixic acid 30 μg (quinolone), cefotazyme 30 μg, ceftazidime 30 μg, ceftiofur 30 μg, ceftriaxone 30 μg (cephalosporin), enrofloxacin 5 μg (fluoroquinolones), 10 μg streptomycin, 10 μg gentamicin (aminiglycosides), and 30 μg tetracycline. The disks were manufactured by Laborclin®. A sample of *E. coli* ATCC® 25922 was used as reference.

2.3. Disc-approximation test

For the detection of extended-spectrum beta-lactamase enzymes (ESBLs) the technique adapted according to Lezameta [5] disc-approximation was used. Briefly, the bacteria (SH) was inoculated into a tube containing 3 mL of Brain Heart Infusion (BHI), and incubated at 37 °C + 1 for 18 h. After incubation, using a sterile swab moistened in the bacterial suspension, the sample was gently seeded in all directions of a petry dish containing Muller-Hinton agar. The disks containing each antimicrobial were placed between distance of 25 mm. The discs used for this test were: amoxicillin/clavulanic acid (AMC) (20/10 μg), azetreonam (AZM) (30 μg) ceftazidime (CAZ) (30 μg), ceftriaxone (30 μg), cefepime (FEP) (30 μg), then incubated at 37 °C for 18–24 h. A sample of *E. coli* ATCC® 25922 was used as reference. ESBLs were verified by the presence of a "gost zone", represented by an enlargement of the inhibition zone between the disks.

2.4. Multiple drug resistance (MDR)

From the disk-diffusion results, it was possible to calculate the number of isolates that were multiresistant. According to Frye and Cray [6], they were considered multiresistant when resistant to three or more antimicrobials.

2.5. Multiple antibiotic resistance index (IRMA)

The antimicrobial multiple resistance index (IRMA) for each sample was calculated according to the methodology described by Krumperman [7], where IRMA revealed the relationship between the number of resistant antimicrobials and the total number of classes of antimicrobial.

2.6. Search for resistance genes related to ESBLs

The PureLink® Genomic DNA For Purification of Genomic DNA kit (Invitrogen, Life Technologies, Carlsbad) was used for the extraction of genomic DNA from multiresistant *Salmonella* Heidelberg isolates after the disk-diffusion test.

2.7. Polymerase chain reaction (PCR) for ESBL genes

Detection of resistance genes related to beta-lactam antibiotics (*bla*CTY-M2, *bla*SHV-1, *bla*TEM-1, *bla*CTX-M2, *bla*OXA-1, *bla*PSE-1 and *Amp*C) were performed by standard PCR. Reactions were performed as directed by Platinum® PCR SuperMix (Invitrogen, Life Technologies, Carlsbad, USA). Amplification conditions for *inv*A gene (used as PCR positive control) were obtained as described by Singh and Mustapha [8]: 95 °C for 10 min, 40 cycles of denaturation by 95 °C for 15 s, annealing and extension 60 °C for 45 s. The conditions for amplification of *bla*CTY-M2, *bla*SHV-1, *bla*TEM-1,*bla*CTX-M2, *bla*OXA-1, *bla*PSE-1 genes were obtained according to Chen et al. [9] and conditions for amplification of the *Amp*C gene were performed according to Alcaine et al. [10], as can be observed in Table 1. Two types of control were used for the analysis: negative reaction control and positive control. The negative control of the amplification reaction constituted in a sample containing the reagents of the reaction, but without addition of extracted DNA. This control was used to detect a possible occurrence of non-specific genetic material amplification. The positive control of the amplification reaction constituted in a sample of SH, all reagents of the PCR reaction and primers for the *inv*A gene.

PCR products were analyzed by agarose gel electrophoresis (1%), stained with ethidium bromide (Ludwig), using a 1 K molecular marker (Ludwig) at 110 V, 150 mA, 110 W for 60 min. After these procedures, each gel was revelead under UV (L-PIXEX).

2.8. Statistical analysis

The concordance ratio is an index that measures the degree of agreement between two or more methods. The total agreement coefficient used to measure accuracy uses a scale of 0–1, where 0 means no agreement and 1 means perfect agreement [11]. For the calculation of the concordance ratio, the model of diagonal was used [12,13].

3. Results

3.1. Disk-diffusion test

This test showed different susceptibility profiles for *S.* Heidelberg isolates as shown in Table 2. The overall resistance found was 80.55% for SH. The highest level (100%) of resistance were observed for nalidixic acid and ceftiofur. The most prevalent resistance pattern was A (penicillin-cephalosporin-quinolone-tetracycline), present in 42.1% of the isolates.

Table 1
Resistance genes related to ESBLs, primer sequences, conditions for amplification, product size (Pb) and references.

Gene	Primers (5′–3′)	PCR settings	Cicles	Pb	References
*bla*CMY-2	(F) TGG CCG TTG CCG TTA TCT AC (R) CCC GTT TTA TGC ACC CAT GA		30	870	[9]
*bla*SHV-1	(F) GGC CGC GTA GGC ATG ATA GA (R) CCC GGC GAT TTG CTG ATT TC		30	714	[9]
*bla*TEM-1	(F) CAG CGG TAA GAT CCT TGA GA (R) ACT CCC CGT CGT GTA GAT AA	95 °C-10 min; 95 °C-30 s; 55 °C-1 min; 72 °C-1 min; 72 °C-7 min (for all bla genes)	30	643	[9]
*bla*CTX-M2	(F) GGC GTT GCG CTG ATT AAC AC (R) TTG CCC TTA AGC CAC GTC AC		30	486	[9]
*bla*OXA-1	(F) AAT GGC ACC AGA TTC AAC TT (R) CTT GGC TTT TAT GCT TGA TG		30	595	[9]
*bla*PSE-1	(F) TGC TTC GCA ACT ATG ACT AC (R) AGC CTG TGT TTG AGC TAG AT		30	438	[9]
AmpC	(F) AAC ACA CTG ATT GCG TCT GAC (R) CTG GGC CTC ATC GTC AGT TA	95 °C-9.5 min; 95 °C-45 s; 59 °C-45 s; 72 °C-1 min; 72 °C-7 min	40	1226	[10,22]

3.2. Disc-approach test

We were unable to visualize “ghost zones” that could indicate the presence ESBLs producers.

3.3. Multiple antimicrobial resistance index (IRMA) and Multiple Drug Resistance (MDR)

Through the results obtained by the disc-diffusion test it was possible to define the Multiple Resistance Index to the Antimicrobials (IRMA) and the Multiple Drug Resistance (MDR), according to Table 3. It was considered multidrug resistant the isolate that showed resistance to three or more different classes of antimicrobials. The Multiple Antimicrobial Resistance Index (IRMA) according to the pre-established resistance standard for isolates from poultry products obtained an average of 0.77 (Table 3).

3.4. PCR results for resistance genes ESBL-related

Table 3 shows the detection of seven resistance genes related to ESBLs multiresistant SH strains, where the highest prevalence rate was observed for *bla*TEM-1 gene (15/18), totaling 83.33% of all amplifications. The second most prevalent genes were *bla*CMY-2 (7/18) and *AmpC* gene (7/18), with 38.88% each. However, *bla*OXA-1 and *bla*PSE-1 genes were not detected. In the strains of number 78 and 79, four resistance genes related to ESBLs were detected: *bla*CMY-2, *bla*SHV-1, *bla*TEM-1 and *AmpC*. Fig. 1 shows the amplification of only the most prevalent gene (*bla*TEM-1).

According to the results, the concordance test proposed by Goodman [12,13] was used. This test measures the degree of agreement of two or more observers in the interpretation of a given result. When comparing the techniques of disk approximation and disk diffusion (AMP, CAZ, CTX, CTF, NAL, TET and CRO) there is no agreement among the samples identified as resistant. The disk approximation test using ENO, GEN, EST, has moderate agreement (c = 0.61, 0.61 and 0.66, respectively). When we compared the disc-approximation with the PCR technique, we found moderate to high agreement (*AmpC* and *bla*CMY-2, c = 0.61, *bla*SHV-1, c = 0.88, *bla*CTX-M2, c = 0.94, *bla*OXA-1 and *bla*PSE-1, c = 1.0), with the exception of *bla*TEM-1, which showed very low concordance (c = 0.16). When we compared the various techniques of disk diffusion and PCR, we did not find perfect agreement between them, but we found high agreement between AMP, CAZ, CTX, CTF, NAL, TET and *bla*TEM-1 (c = 0.83); ENO and *AmpC*, *bla*CMY-2 (c = 0.78), CRO and *bla*TEM-1 (c = 0.77); GEN and *bla*CMY-2, *bla*CTX-M2, *bla*SHV-1 (c = 0.72), moderate between GEN and *bla*OXA-1, *bla*PSE-1 (c = 0.67); EST and *bla*CMY-2, *bla*CTX-M2, *bla*SHV-1 (c = 0.67) and *bla*OXA-1, *bla*PSE-1 (c = 0.61); ENO and *bla*PSE-1 (c = 0.61), *bla*SHV-1 (c = 0.56). The other comparisons showed minimal agreement or did not agree.

4. Discussion

As already reported by Neves et al. [14] and Krumperman [7], the present study shows that SH is a real challenge regarding antimicrobial resistance when tested against antibiotics routinely used in the treatment of samonelosis in veterinary and human medicine. These authors obtained similar results for the different classes of antimicrobials tested, a fact that may also be related to horizontal transmission of resistance genes.

The expression of sensitivity or resistance, *in vitro*, depends on the enzymatic concentration [15], which can be correlated to the results obtained in the present study, where in the tested strains we did not find ESBL production in the disc-approximation test, but some genes related to ESBLs were detected by PCR. Future studies evaluating the gene expression of these detected genes would be helpful to correlate the phenotype with the respective bacterial genotype.

In the tests for beta-lactam antimicrobial resistance genes (*bla*CMY-2, *bla*SHV-1, *bla*TEM-1, *bla*CTX-M2, *bla*OXA-1, *bla*PSE-1 and *AmpC*), we found that the highest amplification index was for the gene *bla*TEM-1 (83.33%) and these results are similar to those reported by Eguale et al. [16] that reported *bla*TEM-1 as the main gene detected and involved in the mechanism of resistance to beta-lactamase. In the mechanism specifically related to the resistance to cephalosporins, directly linked to ESBLs, the gene *bla*CMY-2 is the most commonly detected [17,18].

Resistance to beta-lactam antibiotics, such as ceftriaxone, is linearly correlated to an increase in the expression level of EBLs enzymes [19]. Third generation cephalosporins are used to treat infections caused by *Salmonella* spp in humans and animals. There is a growing global concern regarding the appearance of strains multiresistants to cephalosporins [6], a fact that can be confirmed according to the results of the present study, where 100% resistance to cephalosporins was obtained.

According to Krumperman [7], multiresistant *E. coli* are considered when presenting IRMA above 0.2. In this study, the majority of the isolates presented indexes above 0.60 which indicate potential ability to transfer resistance genes.

The presence and the concern with multi-resistance to antimicrobials was also reported by Ribeiro et al. [20] who, when studying Salmonella isolates from samples collected from foods derived poultry and swine, detected the presence of the AadA-Sul1-Sul2 and *bla*TEM-1 genes conferring resistance to aminoglycosides,

Table 2
Results of the disc-diffusion, disc-approximation and PCR performed on *S.* Heidelberg isolates from chicken meat.

ID	Disk-diffusion test										Ghost zone	PCR on resistance genes						
	Penicillin	Cephalosporin			Quinolone	Fluorquinolone	Aminoglycosides		Tetracycline			*bla*	*bla*	*bla*	*bla*	*bla*	*AmpC*
	AMC	CAZ	CTX	CRO	CTF	NAL	ENO	EST	GEN	TET		CMY-2	SHV-1	TEM-1	CTX-M2	PSE-1	OXA-1	
52	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.			X				
53	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.			X				
54	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	Neg.	X	X		X			
62	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.							
63	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.							
64	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.			X				X
65	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	Neg.			X				X
66	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	Neg.			X				X
67	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	S	**R**	Neg.			X				
68	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	Neg.			X				X
69	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.			X				X
70	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	Neg.	X		X				X
71	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	Neg.			X				X
73	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	Neg.	X		X				
74	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.	X		X				X
78	**R**	**R**	**R**	S	**R**	**R**	S	**R**	**R**	**R**	Neg.	X	X	X				X
79	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	**R**	Neg.	X	X	X				X
93	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	Neg.	X		X				
R %	100	100	100	94.4	100	100	38.9	38.9	33.3	100		38.88	16.66	83.33	5.55	0	0	38.88
Overall Resistance (%) 80.55												**ESBL Genes Prevalence mean (%) 26.18**							

Legend: ID: Sample identification, R%: percentage of resistance, S: Sensitive, R: Resistant, Penicillin (AMP: Amoxicillin + clavulanicacid); Cephalosporins (CAZ: Ceftazidime, CTX: Cefotaxime, CRO: Ceftriaxone, CTF: Ceftiofur); Quinolone (NAL: NalidixicAcid); Fluorquinolone (ENO: Enrofloxacin); Aminoglycosides (EST: Streptomycin, GEN: Gentamicin); Tetracycline (TET: Tetracycline).

Table 3
Antibiotic multiple resistance index (IRMA) of *S.* Heidelberg isolated from chicken meat.

MDR pattern	Standard	IRMA	N	Percentage
PEN-CEF-QUI-TET	A	0.6	8	42.1
PEN-CEF-QUI-FLU-TET	B	0.83	3	15.78
PEN-CEF-QUI-AMI-TET	C	0.83	1	5.26
PEN-CEF-QUI-FLU-TET	D	0.83	1	5.26
PEN-CEF-QUI-AMI-TET	E	0.83	1	5.26
PEN-CEF-QUI-FLU-AMI-TET	F	1	4	21.05

Legend: PEN: Penicillin (Amoxicillin + clavulanic acid); CEF: Cephalosporins (Ceftazidime, Cefotaxime, Ceftriaxone, Ceftiofur); QUI: Quinolone (Nalidixic Acid); FLU: Fluorquinolone (Enrofloxacin); AMI: Aminoglycosides (Streptomycin, Gentamicin); TET: Tetracycline (Tetracycline).

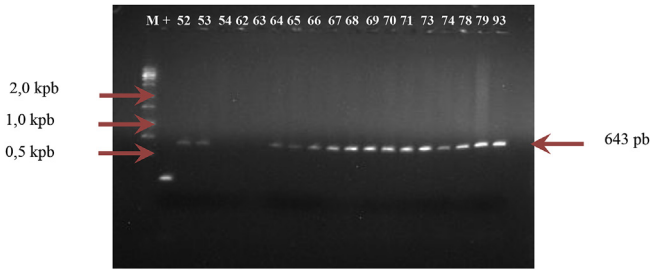

Fig. 1. Agarose gel (1%) with the amplification of *bla*TEM-1 gene (643 bp). M: Molecular marker with 1 Kb of weight; control +: consisting of an SH isolate with the amplified of the *inv*A gene. Lanes 52, 53, 64, 65, 66, 67, 68, 69, 70, 71, 73, 74, 78, 79 and 93 show the amplification of the *bla*TEM-1 gene.

sulfonamides and cephalosporins, respectively.

In addition to the *bla*CMY and *AmpC* genes, other variant genes, including *bla*TEM, *bla*SHV, *bla*CTX-M have also been implicated as responsible for the appearance of resistance to third generation cephalosporins [6,21], highlighting the importance of research and study of these genes, correlating them with the emerging strains of *Salmonella* spp, we observed that the presence of resistance and multi-resistance of the isolated strains to the antimicrobials tested are directly related to the detection of these genes involved through the PCR technique. Considering the widespread degree of resistance observed in this and other studies related to SH, caution should be exercised when using antimicrobials that are still efficient, in order to avoid or at least delay the resistance and, consequently, the appearance of new super-resistant bacterial strains.

This study was able to detect the main resistance genes involved in the production of extended-spectrum beta-lactamases enzymes present in the genomic DNA of *S.* Heidelberg isolates from chicken meat. These enzymes are involved in antimicrobial resistance to beta-lactamics, including third generation cefalosporin, that is routinely used in the treatment and control of human and animal salmonellosis. Therefore, our results indicate that new policies on the rational use of antimicrobials should be developed and implemented in veterinary, as well as in human medicine, in addition to the use of sensitivity tests to detect the presence of ESBLs before the administration of antibiotic therapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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