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A Comparative Study on *Salmonella Enteritidis*, *S. Heidelberg* and *S. Typhimurium* of Poultry Origin from Southern Brazil

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Abstract

Background: *Salmonella* spp. can cause gastrointestinal disease in animals and men, representing a significant public health issue worldwide. Poultry products are considered important sources of this bacterium. *S. Heidelberg* became lately one of the most prevalent isolate found in several countries. Hardly any information is available about the epidemiology and the resistance profile of this serovars. Therefore, the objective of this study was to compare the resistance profile of *S. Heidelberg* with *S. Enteritidis* and *S. Typhimurium* isolated from poultry.

Materials, Methods & Results: Isolates of *S. Heidelberg* (54), *S. Enteritidis* (54), and *S. Typhimurium* (54) were submitted to disk diffusion test to antibiotics routinely used in veterinary and human medicine such as: Enrofloxacin, ciprofloxacin, norfloxacin, gentamicin, ceftiofur, ceftriaxone, nalidixic acid, tetracycline, trimethoprim sulfamethoxazole, chloramphenicol. In addition, the Multi-Drug Resistance Pattern (MDRP) and the Multiple-Drug Resistance Index (MDRI) were determined. The Chi-square (X^2) test with 1% significance level was used to statistically evaluate the results by SAS. *S. Heidelberg*, *S. Typhimurium* and *S. Enteritidis* showed 18, 16.9 and 9.6% of resistance, respectively, indicating a higher degree of resistance for *S. Heidelberg*. The highest levels of resistance were observed for nalidixic acid in isolates of *S. Heidelberg* (67%), *S. Typhimurium* (65%) and *S. Enteritidis* (68.5%), as well as for tetracycline in isolates of *S. Heidelberg* (69%) and *S. Typhimuirum* (635). The gentamicin resistance was also high for *S. Typhimurium* (31.5%) followed by *S. Heidelberg* (7%). Of great importance to the poultry industry, our results show significant resistance to ceftiofur among *S. Heidelberg* isolates (32%), unlike *S. Enteritidis* (3.7%) and S. Typhimurium (1.9%). Only isolates of *S. Heidelberg* (9%) and *S. Enteritidis* (3.7%) were resistant to ceftriaxone, a common antibiotic used to treat children with salmonellosis. It is also important to point out a high resistance to the quinolone class, penicillin and cephalosporin's, reaching levels of 68, 47 and 16%, respectively. MDRP indicated that the majority of the isolates showed resistance pattern C (10%) (gentamycin-nalidixic acid-tetracycline). MDRI analyses indicated that 22.8% of the isolates were multi-drug resistant's. Among the three isolates, *S. Heidelberg* had a greater number of isolates multi-drug resistant (11.72%). The MDRI for the three isolates was 0.2. Considering each individual isolates, *S. Heidelberg* showed the greatest MDRI (0.25) ranging from 0.2 to 0.5.

Discussion: The isolates of *S. Heidelberg* were resistant to almost all antibiotics tested and also showed multi-drug resistant profile, presenting the potential for horizontal transfer of resistance genes. Additionally, ceftiofur is an important antibiotic used in poultry and it can lead to cross-resistance with ceftriaxone, used to treat salmonellosis in children. This scenario leads to the need for rational and judicious use of antimicrobials in poultry, especially ceftiofur as the only cephalosporin approved for use in food animals and special care for *S. Heidelberg*.

Keywords: Antibiotic; Multi-drug resistance; Public health; *Salmonella*

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Introduction

Salmonellosis is a gastrointestinal disease caused by infection caused by bacteria of the genus *Salmonella* spp. belonging to the family *Enterobacteriaceae* [1,2]. For even affect humans, it represents a significant public health problem in many countries. The ingestion of contaminated food is the main form of human contamination, especially for poultry products, especially raw egg [3,4]. The *S. Heidelberg* is among the top three serovars isolated from people infected with salmonellosis in Canada. Moreover, it seems to be more invasive than other serotypes that cause gastroenteritis. *S. Heidelberg* alternates with *S. Enteritidis* as the second or third most prevalent Salmonella serotype in human infections [5-7]. Increased resistance to broad-spectrum cephalosporins among *S. Heidelberg* is of significant interest to public health because ceftriaxone is an important drug for the treatment of children with the severe form of salmonellosis. Considering that the microorganisms resistant to ceftiofur are cross-resistant to ceftriaxone, the use of this antimicrobial agent in food animals is under increased scrutiny, as a potential agent responsible for the emergence and spread of resistance to ceftriaxone in *Salmonella* spp. and other enteric pathogens [8,9]. Given the increasing concern about the impact of antimicrobial resistance in animal-derived bacteria on public health, the objective of this study was to determine the phenotypic profile of resistance and multi-drug resistance of isolates of S. Heidelberg (54), compare it with the isolates of *S. Enteritidis* (54) and *S. Typhimurium* (54) of poultry origin from refrigerator samples, and field several states of southern Brazil.

Materials and Methods

Salmonella isolates

All *Salmonella* spp. samples used in this study were from poultry materials as described in Table 1. We analyzed 162 isolates of *S. Enteritidis, S. Typhimurium* and *S. Heidelberg,* reaching a total of 54 isolates for each species from 2009 to 2013 of the three most important states exporting broiler meat (Parana, Santa Catarina and Rio Grande do Sul) to more than 140 different countries (APBA, 2015). Isolation procedures were done by a private veterinary laboratory accredited by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) and State Agricultural Foundation in the state of Rio Grande do Sul (FEPAGRO). Later, these isolates were serotypes by the Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, RJ, Brazil). At the Laboratory of Molecular Biology, Immunology and Microbiology (LABMIM) of the State University of Santa Catarina (UDESC) all samples were subcultures following the methodology of conservation and maintenance of micro-organisms described by Sola et al. [10].

Disk diffusion tests

The disk diffusion tests were performed in the LABMIM using the methodology approved by the NCCLS (National Committee for Clinical Laboratory Standards) and ANVISA (National Health Surveillance Agency) contained in the Normative Instruction number M-2, Standardization A-8 of Antimicrobial Susceptibility Testing by Disco-diffusion [11]. Antimicrobials used (Probac, Sao Paulo, Brazil) to verify the sensitivity of the 162 isolates were: Enrofloxacin (5 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), gentamicin (10 µg), ceftiofur (30 µg), ceftriaxone (30 µg), nalidixic acid (30 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg) and chloramphenicol (30 µg). *Escherichia coli* ATCC 25922 was used as reference strain (positive control). Duplicate plates were made for each isolate and were incubated for 24 h at 35ºC. The plates were analyzed for the presence of a confluent halo surrounding the antibiotic disk. With the aid of a ruler, the diameter of each halo was measured and ranked according to the tables previously described and isolates were classified as sensitive or resistant [11].

Table 1: Number of *S. Heidelberg, S. Enteritidis,* and *S. Typhimurium* according to their site of poultry origin.

Field		Total	Number of Samples (SH/SE/ST)*
Broiler house	Cupboard box		0/2/0
	Drag swab	8	28/13/37
	Meconio		0/2/0
	Feces		0/1/0
Hatchery	Pipped eggs		0/2/0
	Yolk sac		0/4/4
	Feathers		0/1/0
Feedmill	Feed paper bag		0/1/0
	Bonemeal		0/0/1
Broiler organs from necropsy	Died broiler		2/4/0
	Cecum		0/1/1
	Spleen		0/0/1
	Intestines		0/3/0
	Crop		0/0/1
	Fabricius Bursa		0/0/1
	Culture (diverse)	2	6/9/7
Field Total		32	36/43/53
Slaughter house			
Poultry products	Liver/heart		0/1/0
	Neck		0/1/0
	Broiler parts (meat)	4	18/6/0
	Wing		0/1/0
	Mechanical separated Meat		0/1/0
	Carcass		0/0/1
	Broiler thigs		0/1/0
Slaugther house total		0	18/11/1
Total		62	54/54/54

Multi-drug resistance pattern (MDRP)

According to the disk diffusion data, it was possible to determine the number of isolates that were multi-drug resistant to three or more antibiotics [9].

Multi-drug resistance index (MDRI)

MDRI was calculated according to the methodology described by Krumperman considering the ratio between the number of drugs against which each isolate was resistant and the total number of tested antimicrobials [12].

Statistical analysis

The chi-square (X^2) by SAS (2007) with 1% significance level ($P<0.01$) was used to statistically evaluate the results from the disk test.

Results

Disk diffusion test

The number and the percentage of resistant isolates of *S. Heidelberg, S. Enteritidis,* and *S. Typhimurium* are shown in Table 2. In all cases it was observed significance between the resistance profile and the antibiotic tested ($P<0.01$), except ($p=0.3505$) for *S. Enteritidis*

Table 2: Resistance profile of *S. Heidelberg (SH)*, *S. Enteritidis (SE)*, and *S. Typhimurium (ST)* of poultry origin isolated from field and slaughter house according to the disk diffusion test.

Antibiotics	Field and Slaughter house			Field			Slaughter house		
	SH (%)	ST (%)	SE (%)	SH (%)	ST (%)	SE (%)	SH (%)	ST (%)	SE (%)
Ceftiofur	17/54 (31.5)	1/54 (1.9)	2/54 (3.7)	10/36 (27.8)	1/53 (1.9)	2/43 (4.7)	7/18 (38.9)	0/1	0/11
Gentamicin	4/54 (7.4)	17/54 (31.5)	2/54 (3.7)	3/36 (8.3)	17/53 (32)	2/43 (4.7)	1/18 (5.6)	0/1	0/11
enrofloxacin	0/54	0/54	0/54	0/36	0/53	0/43	0/18	0/1	0/11
Ciprofloxacin	0/54	0/54	0/54	0/36	0/53	0/43	0/18	0/1	0/11
Ceftriaxone	5/54 (9.3)	0/54	2/54 (3.7)	3/36 (8.3)	0/53	2/43 (4.7)	2/18 (11.1)	0/1	0/11
Nalidixic Acid	36/54 (66.7)	37/54 (68.5)	37/54 (68.5)	36/36 (100)	36/53 (67.9)	37/43 (86)	0/18	1/1 (100)	0/11
Chloramphenicol	0/54	0/54	1/54 (1.9)	0/36	0/53	0/43	0/18	0/1	1/11 (9.1)
Sulfa-trimethoprim	0/54	0/54	1/54 (1.9)	0/36	0/53	1/43 (2.3)	0/18	0/1	0/11
Tetracycline	35/54 (64.8)	35/54 (63)	7/54 (13)	35/36 (97.2)	33/53 (62.3)	6/43 (14)	0/18	1/1 (100)	1/11 (9.1)
Norfloxacin	0/54	0/54	0/54	0/36	0/53	0/43	0/18	0/1	0/11
Average(%)	**18**	**16.9**	**9.6**	**24.2**	**16.8**	**11.6**	**5.6**	**20**	**1.8**

Table 3: Number of *S. Heidelberg (SH), S. Enteritidis (SE) and S. Typhimurium (ST)* isolates of avian origin resistant to none, one, two, three or more antibiotics (atbs).

Multi-resistance percentage										
	Total	no atb		1 atbs		2 atbs		3 atbs		
	nº	nº	%	nº	%	nº	%	nº	%	
SE	54	5	9.3	42	77.8	3	5.6	4	7.4
ST	54	14	25.9	6	11.1	19	35.2	15	27.8
SH	54	0	0	0	0	36	66.7	14	25.9
Total	**162**	**19**	**11.7**	**48**	**29.6**	**58**	**35.8**	**33**	**20.4**

and *S. Typhimurium* isolated from chicken meat (slaughterhouse).

Multi-drug resistance pattern (MDRP)

Table 3 shows the results about the number and the percentage of isolates of *S. Enteritidis*, *S. Typhimurium* and *S. Heidelberg* that were resistant to any one, two, three or more antibiotics. Of all the isolates, only 30 were resistant to three drugs. Resistance to four drugs was low (3/162), one from each studied serovar. It was not observed isolates resistant to more than five antibiotics.

Multi-drug resistance index (MDRI)

The MDRI for isolates of *S. Enteritidis*, *S. Typhimurium* and *S. Heidelberg* was 0.2. Considering each individual isolates, *S. Heidelberg* showed the greatest MDRI (0.25) ranging from 0.2 to 0.5. *S. Typhimurium* already showed a mean of 0.17 MDRI, ranging from 0 to 0.4. And *S. Enteritidis* was isolated with less MDRI (0.12), varying between 0 and 0.4 shown in Table 4.

Discussion

The results of the resistance profile analyzes of isolates of *S. Heidelberg*, *S. Enteritidis* and *S. typhimurum* front to the ten showed incidence of antimicrobial resistant strains. It is possible to see that isolates of *Salmonella* spp. studied showed 100% susceptibility only to antibiotics belonging to the class of fluoroquinolones (enrofloxacin, ciprofloxacin and norfloxacin). These results were expected, since enrofloxacin is an antibiotic of the family of fluoroquinolones, is considered a second generation quinolone. Therefore, it is an antibiotic that was developed before the high resistance levels observed in the first generation quinolones, both synthetic antibiotics and obtained from nalidixic acid, which currently also demonstrates high levels of resistance. Corroborating the results obtained by Seyfarth, Wegener and Frimodt-moller et al. [13], Vaz et al. [14] and Duarte et al. [15] in four independent studies also isolates of Salmonella poultry origin. For many years nalidixic acid has been used for therapy in birds in Brazil [13]. This study showed that 68% of the isolates were resistant to this antibiotic. The high level of resistance observed is mainly due to resistance among isolates of *S. Typhimurium* and *S. Enteritidis* (68.5%), however resistance to nalidixic acid among isolates of *S. Heidelberg* was also high (66.7%). Different than was found by Medeiros et al. [16] and Vaz et al. [14], which respectively 26.01% and 14.56% of the isolates were resistant to nalidixic acid. According Malorny et al. [17], an increased incidence of resistant strains to quinolones in animals was associated with use of nalidixic acid in livestock. Tetracycline had been extensively used as a growth promoter in animal production, when its use was banned in Brazil in 1998 but it is still being used therapeutically. This could be an explanation to the high resistance levels for tetracycline found on this study (47%). This resistance observed is mainly due to *S. Heidelberg* isolates (64.8%), followed by *S. Typhimuirum* (63%) and *S. Enteritidis* (13%). Similar results were found by other researchers, with resistance levels ranging from 78% to 100% [18,19]. Gentamicin is an amino glycoside commonly used in veterinary medicine to treat infections of farm animals. Busani [18] and Cortez et al. [20] found lower gentamicin resistance levels (<3.45%), unlike what was found in this study, where 14% of the isolates were resistant. The observed resistance is primarily due to the isolates of *S. Typhimuirum* (31.5%). Since Ribeiro et al. [21] demonstrated more results similar to ours (22.8%). According Mion et al. [22] we studied the resistance of 20 isolates of *S. Heidelberg* gentamicin found that 50% of the isolates were resistant to the drug. In the present study of 54 isolates of *S. Heidelberg* reviews 7.4% were resistant to gentamicin. Third generation cephalosporin's are used to treat *Salmonella* spp. infections in animals, are also used to treat this infection especially in children [9]. The findings of this study showed lower ceftiofur resistance levels in isolates of *S. Typhimurium* (1.9%) and *S. Enteritidis* (3.7%), in agreement with those developed by Palmeira [23]. Higher resistance values (18.1%) were found by Biffi et al. [11] evaluating isolates of poultry origin. While analyzing the data regarding *S. Heidelberg,* there was 31.5% of the isolates were resistant to ceftiofur. Resistance values were well above that of the isolates of *S. Enteritidis* and *S.*

Table 4: Multiple Drug Resistance Standard (MDR) and the Multiple Antibiotic Resistance Index (IRMA) of isolates from *S. Heidelberg (SH)*, *S. Enteritidis (SE)* and *S. Typhimurium (ST)* of poultry origin to the antimicrobials studied.

Isolated	MDR Padrão	ID do Padrão	IRMA	Total (n)	Percentage (%)
SH	Ceftiofur - Nalidixic acid - Tetracycline	A	0.3	12	7
SH	Ceftiofur - Ceftriaxone - Nalidixic acid	B	0.3	1	1
SH(1), SE(2), ST(14)	Gentamicin - Nalidixic acid - Tetracycline	C	0.3	17	10
SH(1), SE(1)	Ceftiofur - Ceftriaxone - Nalidixic Acid-Tetracycline	D	0.4	2	1
ST	Ceftiofur - Gentamicin - Nalidixic Acid-Tetracycline	E	0.4	1	1
SH(3), SE(1)	Ceftiofur - Gentamicin-Ceftriaxone- Nalidixic Acid-Tetracycline	F	0.5	4	2
Total multidrug-resistant island				**37**	**23**

Typhimurium. According Mion et al. [22], who studied the resistance of 20 isolates of *S. Heidelberg* in the periods of 2005 and 2009, found 38.4% of the isolates were resistant in 2005 to ceftiofur, agreeing with the results here found. However, in the year 2009, 100% of the samples were resistant to the drug. Already second Medeiros et al. [24] and Nunes et al. [25] smaller ceftriaxone resistance levels were observed, agreeing with the findings reported here (4%). This observed resistance value is mainly due to isolates of *S. Heidelberg* (9.3%) and *S. Enteritidis* (3.7%), since they were not identified isolates of S. Typhimurium resistant to ceftriaxone. Increased resistance to broad-spectrum cephalosporins (ceftiofur and ceftriaxone) among *S. Heidelberg,* as observed in this study, is of significant interest to public health because ceftriaxone is an important drug for the treatment of children with severe form of salmonellosis. Considering that the microorganisms resistant to ceftiofur are cross-resistant to ceftriaxone, the use of this antimicrobial agent in food animals is under increased scrutiny, as a potential agent responsible for the emergence and spread of resistance to ceftriaxone in *Salmonella* spp. and other enteric pathogens [7,9]. Frye & Cray et al. [9] reported the growing global concern in the emergence of multi-drug resistant strains. The isolated is considered multi-drug resistant when it shows a pattern of resistance to three or more antibiotics. Medeiros et al. [26] also evaluated isolates of *Salmonella* spp. presenting a pattern of resistance to one or two drugs (36.1%) and three or more drugs (63.9%). When considering the multi-drug resistance, this study found that 35.8% of the isolates were resistant to one or two drugs and 20.4% of the isolates were resistant to three or more drugs. Studies by Zhao et al. [27] aiming to identify the number of isolates with multi-drug resistance pattern showed that of the 298 isolates of *S. Heidelberg* analyzed, 67% were resistant to at least one drug, different results obtained in this study, which were not *S. Heidelberg* identified isolates resistant to one drug but rather at least two drugs. Again showing the potential of multi-drug resistant isolates of *S. Heidelberg,* and this setting was not observed for *S. Enteritidis* and *S. Typhimurium* that were resistant to at least one drug (9.3% and 25.9 %, respectively). In assessing the multi-drug resistance of 16 isolates of *S. Typhimurium,* Medeiros et al. [26] detected 55.6% of isolates resistant to one or both drugs and 44.4% of isolates resistant to more than three drugs. These results are well above those, since 15.4% of the isolates were resistant to *S. Typhimurium* one or two drug and 9.3% were resistant to three or more drugs. According Busani et al. [18] multiple resistances have been more commonly observed in *S. Typhimurium.* Different than was observed in this study, where the *S. Heidelberg* had a greater number of multiresistant isolates (11.72%). It can be seen that the tested strains present a degree of multi-drug resistance worrying which can hinder the treatment of cases of salmonellosis in poultry. Studies conducted by Krewer [28] by analyzing the susceptibility of *E. coli* isolates from pig farms in the states of Rio Grande do Sul and Santa Catarina, found that the average MDRI for these isolates was 0.52, ranging from 0.11 to 1, while the majority of the isolates showed rates above 0.52. These results are higher than that found in the present study, since the average MDRI, considering the isolates of *S. Enteritidis, S. Heidelberg* and *S. Typhimurium,* was 0.2 and ranged from 0.1 to 0.5. Have to analyze the isolates of *S. Heidelberg* alone, the MDRI observed was 0.3 and ranged from 0.3 to 0.5, showing that the isolates of *S. Heidelberg* major concern to public health. Carvalho et al. [29] which analyzed 23 isolates of Salmonella spp from shrimp found only 3 isolates with superior MDRI 0.29, MDRI results similar to those found in this work. In addition to the significant number of isolates of *S. Heidelberg* that were resistant to ceftiofur, antibiotic of interest for animal production, isolate of *S. Heidelberg* was the main serovar responsible for the high levels of resistance observed between them antimicrobial class. Moreover, these isolates of *S. Heidelberg* showed MDRI above 0.3, indicating the multi-drug resistance as well as their potential for horizontal transfer of resistance genes. According to Krumperman [26], MDRI high values indicate a risk to public health and may hinder the treatment of animal and human diseases, warning of the need for rational use of these drugs.

Conclusion

This study found higher percentage of isolates of *S. Heidelberg* resistant to ceftiofur, different from that observed for isolates of *S. Enteritidis* and *S. Typhimurium.* In addition, the greater number of the larger and multiresistant isolates MDRI values was also identified among the isolates of *S. Heidelberg.* The results of this study make it clear that antibiotics should be used judiciously, caution is necessary when deciding which treatment to be adopted. It is worth stressing the need for continued monitoring of these resistant pathogens, both in the poultry industry, as in hospitals and other environments.

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