Salmonella Heidelberg Isolated from Poultry Shows a Novel Resistance Profile

Gabriella Bassi das Neves[2], Lenita Moura Stefani[1,2], Eduarda Pick[1], Denise Nunes Araujo[1,2], Jéssica Giuriatti[2], Cleverson Percio[1] & Maiara Cristiane Brisola[2]

ABSTRACT

Background: The development of antimicrobial resistance in bacteria is a serious public health issue worldwide. *Salmonella* spp. is considered a leader cause of gastrointestinal disease in animals and humans, and poultry products have been reported as an important reservoir of the bacterium. *S*. Heidelberg became lately one of the most prevalent serovars found in several countries. However, hardly any information is available about the epidemiology and the resistance profile of it. Therefore, the objective of this study was to determine the antibiotic resistance profile of *S*. Heidelberg (SH) and to compare to *S*. Enteritidis (SE) and *S*. Typhimurium (ST) isolated from the Southern part of Brazil.

Materials, Methods & Results: A total of 162 *Salmonella* isolates of poultry origin serotyped as SH (54), SE (54), and ST (54) were submitted to the disk-diffusion test with disks containing 10 antibiotics of 7 different classes, routinely used in veterinary and human medicine such as: enrofloxacin, ciprofloxacin, norfloxacin, gentamicin, ceftiofur, ceftriaxone, nalidixic acid, tetracycline, sulfamethoxazole-trimethoprim, and chloramphenicol. In addition, the Multi-drug Resistance Pattern (MDRP) and the Multiple-Drug Resistance Index (MDRI) were determined. The Chi-square (χ^2) test with 1% of significance level was used to statistically evaluate the results. All isolates were susceptible to norfloxacin, enrofloxacin, and ciprofloxacin. The majority of the isolates were resistant to the quinolone class (68%), more specifically to nalidixic acid, which is considered a synthetic quinolone, followed by penicillin (47%) and cephalosporin (16%). Overall, SH isolates showed higher resistance compared to ST and SE (18, 16.5, and 9.6%, respectively). SH isolated from field samples, mainly drag swabs, showed higher resistance levels (24.2%) than those isolated from slaughterhouses (5.6%). SH showed the highest percentage of resistance to ceftiofur (31.5%), ceftriaxone (9.3%), and tetracycline (64.8%) in comparison to the other two serotypes. Most of the SH isolates were resistant to at least two (66.7%), three or more antibiotics (33.3%). A different scenario was observed for ST and SE, where 25.9 and 9.3% were susceptible to at least one drug, respectively. The most common pattern of resistance (MDRP) was C (gentamicin - nalidixic acid - tetracycline) for 14 SH isolates, and A (ceftiofur - nalidixic acid - tetracycline) for 12 SH isolates. MDRI indicated that 22.8% of all isolates were multidrug resistant. SH was the isolate with the largest variety of resistance patterns compared to ST and SE, where 11.7% of the isolates were resistant to more than three antibiotics. In addition, SH showed the greatest MDRI (0.25) ranging from 0.2 to 0.5.

Discussion: SH was resistant to almost all antibiotics tested and showed multi-drug resistant profile, therefores, it showed a potential for horizontal transmission of resistance genes. Additionally, SH showed a higher resistance profile for ceftiofur, an important antibiotic used in poultry, which can cross-resist to ceftriaxone, commonly used to treat salmonellosis in children. Our results showed that SH is a real challenge regarding antimicrobial resistance. This scenario leads to the need for rational and judicious use of antimicrobials in poultry and, as an alert to the medical community.

Keywords: antibiotics, multidrug resistance, public health, resistance, *Salmonella*.

INTRODUCTION

Salmonellosis is a gastrointestinal disease caused by a bacterium of the genus *Salmonella* spp., belonging to the family *Enterobacteriaceae* [6,13]. It is considered an important zoonotic disease worldwide with significant impact in public health. The ingestion of contaminated food is the main source of human contamination. In addition, poultry products, mainly eggs, are considered important reservoirs for *Salmonella* [3,19].

The *S*. Heidelberg (SH) is among the most common serovar isolated from people infected with salmonellosis in many parts of the world. Despite that, little is known about its epidemiology and resistance profile. Therefore, the objective of this study was to determine the resistance and multidrug resistance profile of isolates of SH in comparison to *S*. Enteritidis (SE) and *S*. Typhimurium (ST) isolated from poultry meat plants and farms of few states of Southern Brazil.

MATERIALS AND METHODS

Salmonella *isolates*

All *Salmonella* spp. samples used in this study were of poultry origin (132 field samples and 30 slaugtherhouses) as described in Table 1. It were analyzed 162 isolates of *S*. Enteritidis, *S*. Typhimurium and *S*. Heidelberg, reaching a total of 54 isolates for each sorovar. They were isolated in 2009 to 2013 in three Brazilian Southern states (Paraná, Santa Catarina, and Rio Grande do Sul) where approximately 73% of all exported meat is produced [2]. Samples were isolated by two laboratories: a private veterinary laboratory accredited by the Ministry of Agriculture, Livestock and Food Supply (MAPA), and a laboratory at the State Agricultural Foundation (FEPAGRO) in the state of Rio Grande do Sul. Later, these isolates were serotyped by the Oswaldo Cruz Foundation (FIOCRUZ). At the Laboratory of Molecular Biology, Immunology and Microbiology (LABMIM) of the State University of Santa Catarina (UDESC) all samples were subcultured following the methodology of conservation and maintenance for the microorganism [16].

Disk-diffusion tests

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 [1]. Antimicrobial discs[1] used 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), sulfamethoxazole-trimethoprim (25 µg) and chloramphenicol (30 µg). *Escherichia coli* ATCC® 25922[2] was used as a reference strain. Isolates were classified as sensitive or resistant.

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

Field		Total	Number of samples (SH/SE/ST)*
Broiler houses	Cupboard boxes	2	0/2/0
	Drag swabs	78	28/13/37
	Meconium	2	0/2/0
	Feces	1	0/1/0
Hatcheries	Pipped eggs	2	0/2/0
	Yolk sacs	8	0/4/4
	Feathers	1	0/1/0
Feedmills	Feed paper bags	1	0/1/0
	Bonemeal	1	0/0/1
Organs from broilers at necropsy	Dead broilers	6	2/4/0
	Cecum	2	0/1/1
	Spleen	1	0/0/1
	Intestines	3	0/3/0
	Crop	1	0/0/1
	Bursa of Fabricius	1	0/0/1
	Culture (miscellaneous)	22	6/9/7
Field total		132	36/43/53
Slaughterhouse			
Poultry products	Liver/heart	1	0/1/0
	Neck	1	0/1/0
	Broiler meat (parts)	24	18/6/0
	Wings	1	0/1/0
	Meat mechanically separated (CMS)	1	0/1/0
	Whole carcass	1	0/0/1
	Broiler thighs	1	0/1/0
Slaugtherhouse total		30	18/11/1
TOTAL		162	54/54/54

*SH: *S*. Heidelberg; SE: *S*. Enteritidis; ST: *S*. Typhimurium.

Multi-drug Resistance Pattern (MDRP)

According to the disk-diffusion data, it was possible to determine the number of isolates that were resistant to three or more antibiotics, e.g. those truly

multi-drug resistant [5]. Resistant samples were classified into six patterns:

ceftiofur - nalidixic acid - tetracycline (A);
ceftiofur - ceftriaxone - nalidixic acid (B);
gentamicin - nalidixic acid - tetracycline (C);
ceftiofur - ceftriaxone - nalidixic acid - tetracycline (D);
ceftiofur - gentamicin - nalidixic acid - tetracycline (E);
ceftiofur - gentamicin - ceftriaxone - nalidixic acid - tetracycline (F).

Multiple-drug Resistance Index (MDRI)

MDRI was calculated according to the methodology described by Krumperman [7] considering the ratio between the number of drugs that each isolate was resistant and the total number of antimicrobials tested.

Statistical analysis

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

RESULTS

Disk-diffusion test

The number and the percentage of resistant isolates of SH, SE, and ST are shown in Table 2. All isolates were susceptible to norfloxacin, enrofloxacin, and ciprofloxacin. The highest resistance percentage was observed for the nalidixic acid reaching 100% for SH isolated in the field, followed by tetracycline. Overall, SH isolates showed higher resistance compared to ST and SE (18, 16.5, and 9.6%, respectively). SH isolated from field samples showed almost five times more resistance (24.2%) than those isolated from slaughterhouses (5.6%). SH showed the highest percentage of resistance to tetracycline (64.8%), ceftiofur (31.5%), and ceftriaxone (9.3%) in comparison to the other two serotypes.

All antibiotics were grouped into seven different classes of antibiotics: cephalosporin, fluoroquinolone, aminoglycoside, penicillin, quinolone, chloramphenicol, sulfa-trimethoprim. Figure 1 shows the results of antibiotic resistance for all three sorovars according to the class of antibiotic used. The majority of the isolates were resistant to the quinolone class (68%), more specifically to nalidixic acid, which is considered a synthetic quinolone, followed by penicillin and cephalosporin.

Table 2. Resistance profile according to the disk-diffusion testof *S.* Heidelberg (SH), *S.* Enteritidis (SE), and *S.* Typhimurium (ST) isolated from poultry farms and poultry slaughterhouses.

Antibiotics	Field and Slaughterhouse			Field			Slaughterhouse		
	SH (%)	ST (%)	SE (%)	SH (%)	ST (%)	SE (%)	SH (%)	ST (%)	SE (%)
Ceftiofur	17/54 (31.5)	1/54 (1.85)	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 (5.5)	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 (1.85)	0/36	0/53	0/43	0/18	0/1	1/11 (9.1)
Sulfa-trimethoprim	0/54	0/54	1/54 (1.85)	0/36	0/53	1/43 (2.3)	0/18	0/1	0/11
Tetracycline	35/54 (64.8)	34/54 (63,0)	7/54 (13.0)	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	16.5	9.6	24.2	16.4	11.6	5.6	20	1.8

In all cases, there was a significant difference between the resistance profile and the antibiotic tested ($P < 0.01$), except ($P = 0.3505$) for SE and ST isolated from chicken meat (slaughterhouses).

Multi-drug Resistance Pattern (MDRP)

Table 3 shows the percentage of all three isolates of *Salmonella* resistant to one, two, three or more antibiotics. All SH isolates showed some percentage of resistance to one or more antibiotics, and most of the SH isolates were resistant to two (66.7%), three or more antibiotics (33.3%). A different scenario was observed for ST and SE, where 25.9 and 9.3% was susceptible to at least one drug, respectively.

Table 4 shows the MDRP for SH, SE, and ST to three, four and five antibiotics, as well as their MDRI.

Of all the isolates, 30 were resistant to three drugs (18.5%), 3 were resistant to four drugs, 4 were resistant to five drugs, and none were resistant to more than five antibiotics. The most common profile of resistance found was C (gentamicin - nalidixic acid - tetracycline) and A (ceftiofur - nalidixic acid - tetracycline). SH was the isolate with the largest variety of resistance patterns, compared to ST and SE.

Multiple-drug Resistance Index (MDRI)

The mean MDRI for SE, ST and SH isolates was 0.2. Considering each individual isolate, SH showed the greatest MDRI (0.25), ranging from 0.2 to 0.5. ST showed mean of 0.17 MDRI, ranging from 0 to 0.4, and SE was the isolated with lower MDRI (0.12), varying from 0 to 0.4.
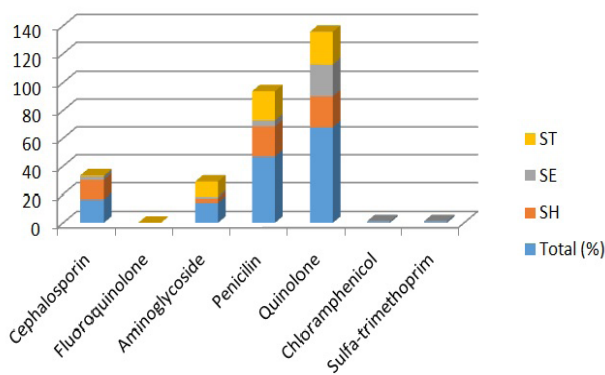
Figure 1. Percentage of resistant *S.* Heidelberg, *S.* Enteritidis and *S.* Typhimurium (Y axis) related to the class of antimicrobials (X axis).

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

		Percentual of multi-drug resistance							
	Total	none		1 atb		2 atbs		≥ 3 atbs	
		n	%	n	%	n	%	n	%
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	18	33,3
Total	162	19	11.7	48	29.6	58	35.8	37	22.8

Table 4. Multi-drug resistant profile (MDRP) and Multi-drug resistance index (MDRI) of *S.* Heidelberg (SH), *S.* Enteritidis (SE) and *S.* Typhimurium (ST) isolated from poultry.

Isolate	MDRP	Pattern	MDRI	Total
SH(12)	Ceftiofur-Nal. acid-Tetra	A	0.3	12
SH(1)	Ceftiofur-Ceftriaxone-Nal acid	B	0.3	1
SH(1), SE(2), ST(14)	Gentamicin-Nal. acid-Tetra	C	0.3	17
SH(1), SE(1)	Ceftiofur-Ceftriaxone-Nal. acid-Tetra	D	0.4	2
ST(1)	Ceftiofur-Gentamicin-Nal. acid-Tetra	E	0.4	1
SH(3), SE(1)	Ceftiofur-Gentamicin-Ceftriaxone-Nal. acid-Tetra	F	0.5	4
Total of multiresistant isolates				37

DISCUSSION

There is a growing global concern in the emergence of multidrug-resistant strains [5]. Our study found that SH of poultry origin has an important profile of resistance compared to the other two serotypes. SH was the main serovar responsible for high levels of resistance observed for cephalosporin, especially ceftiofur. Moreover, these SH isolates showed MDRI above 0.3, indicating a multidrug resistance profile and a great potential for horizontal transmission of resistance genes to other hosts. High IRMA indicates a risk to public health and may hinder the treatment of animal and human diseases, warning for the need of rational use of these drugs [10].

Regarding fluoroquinolones, this study has showed that they could be used to treat salmonellosis. These results were expected, since enrofloxacin is a fluoroquinolone, considered a second generation of quinolones. Our results are corroborated by many authors while evaluating isolates of Salmonella of poultry origin [4,14,17], despite the lack of information specifically for SH.

For many years, nalidixic acid has been used in poultry flocks in Brazil [14]. This study showed that 68% of the isolates were resistants to this antibiotic, which is different from what was found by other authors that have reported only 26.01% and 14.56% of resistance [9, 17]. An increased incidence of resistance for quinolones in animals has been associated with the use of nalidixic acid in livestock [8]. If only SH was considered, 31.5% of them were found to be resistant to ceftiofur, a much higher level compared to other study (12.19%) [18]. A study on 20 isolates of SH between 2005 and 2009 reported that in 2005, 38.4% of the isolates were resistant to ceftiofur but in 2009, 100% of the samples were resistant to the drug [11]. Moreover, lower resistance levels of ceftriaxone were observed, agreeing with our findings [9,12].

Increased resistance to broad-spectrum cephalosporins (ceftiofur and ceftriaxone) among SH, as observed in this study, is of significant interest to public health, since ceftriaxone is an important third generation cephalosporin used to treat children with severe forms 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 [5,15]. It can be seen that the tested strains showed a worrying degree of multidrug resistance, which can hinder the treatment of salmonellosis in poultry and humans.

CONCLUSION

Our results showed a higher percentage of SH isolates resistant to many antibiotics compared to SE and ST. This profile of multi-drug resistance, including to antibiotics used in humans, requires judicious care by doctors and veterinarians while using these drugs. It is important to point out the need for continued studies on SH, in order to better monitor and control this pathogen that lately is commonly present in the worldwide agrobusiness.

MANUFACTURERS

[1]Laborclin Produtos para Laboratórios Ltda. Pinhais, PR, Brazil.

[2]Newprov Produtos para Laboratórios. Pinhais, PR, Brazil.

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