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Original Article

High Prevalence of Multidrug-Resistant Nontyphoidal Salmonella Recovered from Broiler Chickens and Chicken Carcasses in Brazil

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Nontyphoidal *Salmonella*, multidrug resistance, poultry industry, Heidelberg, cephalosporins.

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ABSTRACT

The extensive use of antimicrobial agents has contributed to the emergence of antimicrobial resistance and multidrug resistance (MDR) in *Salmonella,* an important zoonotic pathogen that causes outbreaks and sporadic cases of gastroenteritis in humans. The study aimed to investigate the antimicrobial resistance profile of *Salmonella* strains isolated from poultry in Brazil. A total of 230 *Salmonella* strains, isolated from cloacal swabs (n=56) and broiler carcasses swabs (n=174) before and after chilling from slaughterhouses under Federal Inspection Service within the period 2012-2017, were analyzed. Serotyping and antibiotic susceptibility testing were performed on all the isolates. Serotyping results showed that 41% of the strains were *Salmonella* Heidelberg, 29% *S*. Minnesota, 12% *S*. Saintpaul, 6.5% *S*. Enteritidis, 3.9% *S*. Anatum, 2.2% *S*. Cerro, 2.2% *S*. Senftenberg, 1.7% *S*. Newport, 0.4% *S*. Ealing, 0.4% *S*. O:4,5 and 0.4% *S*. O:9,12. MDR rates of the isolates were 67.4%. *S*. Heidelberg 89.5%, *S*. Minnesota 51.5%, *S*. Saintpaul 82.1%, *S.* Anatum 66.7%, *S*. Cerro 60%, *S*. Senftenberg 40%. Out of the 230 strains, 41.3% presented resistance to Penicillins + beta-lactamase inhibitor, Penicillin, 1st and 2nd Generation Cephalosporin, 3rd and 4th Generation Cephalosporin, Tetracycline and Sulfonamide. *Salmonella* Heidelberg, *S*. Saintpaul, *S*. Anatum, *S*. Cerro, *S*. Senftenberg and *S*. Minnesota were isolated after chilling tank highlighting a food safety concern for the industry of poultry and poultry products indicating a risk to collective health. The high prevalence of MDR nontyphoidal *Salmonella* obtained in this study limit the options available to treat infectious disease in humans and animals.

INTRODUCTION

Surveillance of antimicrobial resistance (AMR) can be very valuable for orienting treatment choices, understanding AMR trends, identifying priority zones for interventions and monitor the impact of interventions in order to contain resistance spread. The lack of adequate surveillance in various parts of the world leaves large gaps in existing knowledge of the distribution and dimension of this phenomenon (Prestinaci *et al.*, 2015).

Furthermore, surveillance data in nontyphoidal *Salmonella enterica* is essential to monitor the transmission of resistance from the food chain to humans, and to establishing effective treatment protocols (Neuert *et al*., 2018).

Due to the overuse of antibiotics in animals and humans, the number of foodborne multidrug resistant *Salmonella* isolates has increased rapidly in the last years. These multidrug resistant strains cause a heavy burden on clinical diagnosis and treatment of salmonellosis and have become a major public health issue in Brazil and in several other countries (Zishiri *et al*., 2016; Xu *et al*., 2019).

Salmonella is an important zoonotic pathogen that causes outbreaks and sporadic cases of gastroenteritis in humans all around the world (Zishiri *et al*., 2016). According to "The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017", 91,662 human salmonellosis cases were reported in the European Union and it was identified in 1.241 (24.4%) foodborne outbreaks (FBO), affecting 9.600 people (EFSA; ECDC, 2018).

As reported by Centers for Disease Control and Prevention (CDC), *Salmonella* was the second most common cause of confirmed, single-etiology outbreaks, accounting for 132 (33%) outbreaks and 3,047 (33%) illnesses in the United States. Among the 125 confirmed *Salmonella* outbreaks with a serotype reported, Enteritidis was the most common (21 outbreaks, 17%), followed by I 4,[5],12:i:- (13, 10%), Newport (13, 10%), and Typhimurium (13, 10%). Concerning confirmed, single-etiology outbreaks, *Salmonella* caused the most outbreak-associated hospitalizations (456 hospitalizations, 56%) and among the 17 deaths reported, three were attributed to *Salmonella* (CDC, 2018).

Most people recover from illness without antibiotic treatment. In rare cases, when the infection spreads from the gut to the bloodstream it may lead to hospitalization, antibiotic treatment and even to death, (CDC, 2016). A rapid increase of resistance to extended-spectrum cephalosporins, including the third and fourth generation cephalosporins, poses a significant threat to public health worldwide (Nguyen *et al*., 2016).

The aim of this study was to characterize the phenotypic profile of antimicrobial resistance and establish multidrug resistance (MDR) patterns of *Salmonella* strains isolated from broiler chickens and carcasses obtained from slaughterhouses in Brazil.

MATERIALS AND METHODS

Sample collection, Isolation and Serotyping

A total of 230 *Salmonella* strains previously isolated, recovered from cloacal swabs (n=56) and broiler carcasses swabs (n=174) before and after chilling, from slaughterhouses under Federal Inspection Service (West-Center, Southeast and South regions of Brazil) between the year of 2012 and 2017 were received by the Poultry Health Laboratory/Universidade Federal Fluminense and stored in Nutrient Agar (Merck). After reactivation, the isolates were sent to serotyping at the Enterobacteria Laboratory, Oswaldo Cruz Foundation, in Rio de Janeiro, Brazil.

Antimicrobial susceptibility

Disk Diffusion Test was performed for 19 antimicrobials using Mueller Hinton Agar (Merck) and breakpoints were defined by Clinical and Laboratory Standards Institute (CLSI, 2013). The antimicrobial categories tested were Aminoglycosides (Gentamicin-10µg), Carbapenems (Ertapenem-10 µg, Imipenem-10 µg, Meropenem-10 µg), 1st and 2nd Generation Cephalosporins (Cephalexin-30µg, Cephalothin-30µg, Cefoxitin-30µg), 3rd and 4th Generation Cephalosporins (Cefotaxime-30µg, Ceftazidime-30µg, Ceftiofur-30µg, Cefepime-30µg), Penicillins (Ampicillin-10µg), Penicillins + b-lactamase inhibitors (Amoxicillin+clavulanate-20/10µg), Monobactams (Aztreonam-30µg), Fluoroquinolones (Ciprofloxacin-5µg, Enrofloxacin-5µg), Phenicols (Chloramphenicol-30µg), Tetracyclines (Tetracycline-30µg) and Sulfonamides (300µg).

Multidrug Resistance

Antimicrobial susceptibility patterns were evaluated by the disk diffusion method and the strains classified as resistant to at least one antimicrobial in three or more categories was considered multidrug resistant, as defined by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) (Gieraltowski *et al*., 2016; Magiorakos *et al*., 2012).

RESULTS

The most frequently isolated serotypes were Heidelberg (41%, n=95), Minnesota, (29%, n=66), Saintpaul (12%, n=28) and Enteritidis (6.5%, n=15). The results are shown in Table 1.

The isolates presented variable resistance levels to 16 out of the 19 tested antibiotics; only Ertapenem, Imipenem, Meropenem totally inhibited the growth of tested strains. All *S*. Newport, *S*. Ealing and *S*. Enteritidis strains were fully susceptible to the antimicrobials tested. The results in table 2 shows the highest resistances observed among the evaluated categories.

In this context, 155 (67.4%) isolates presented MDR pattern, with *Salmonella* Heidelberg as the most frequent, accounting for 54.8% of the isolates (85/155), followed by *S*. Minnesota, 22% (34/155), and *S*. Saintpaul, 14.8% (23/155). The results in Table

Table 1 – Serotype distribution of *Salmonella enterica*, isolated from cloacal and broiler carcass swab samples before and after chilling from slaughterhouses in Brazil.

Salmonella serotypes	Sources			
	Cloaca	broiler carcasses before chilling	broiler carcasses after chilling	Total
	n (%)	n (%)	n (%)	n (%)
Heidelberg	18 (19)	-	77 (81)	95 (41)
Minnesota	20 (30)	33 (50)	13 (20)	66 (29)
Saintpaul	-	-	28 (100)	28 (12)
Enteritids	15 (100)	-	-	15 (6.5)
Anatum	-	-	9 (100)	9 (3.9)
Cerro	-	-	5 (100)	5 (2.2)
Senftenberg	-	-	5 (100)	5 (2.2)
Newport	-	4 (100)	-	4 (1.7)
Ealing	1 (100)	-	-	1 (0.4)
O:4,5	1 (100)	-	-	1 (0.4)
O:9,12	1 (100)	-	-	1 (0.4)
Total	56 (24.3)	37 (16.1)	137 (59.6)	230 (100)

Table 2 – Antimicrobial resistance rates of *Salmonella* isolates from cloacal and broiler carcasses swabs before and after chilling from slaughterhouses in Brazil.

Serotypes	AMC	AMP	CFL	CTX	GEN	ENO	CLO	TET	SUL
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Heidelberg	82 (86.3)	83 (87)	82 (86)	80 (84.2)	6 (6.3)	13 (13.7)	0	87 (92)	95 (100)
Minnesota	19 (28.8)	29 (44)	28 (42)	32 (48.5)	0	0	1 (1.5)	65 (98)	65 (98)
Saintpaul	7 (25)	23 (82)	23 (82)	23 (82.1)	21 (75)	1 (3.6)	0	2 (7.1)	25 (89)
Enteritids	0	0	0	0	0	0	0	0	0
Anatum	1 (11)	6 (67)	6 (67)	6 (66.7)	5 (56)	0	0	0	6 (67)
Cerro	1 (20)	3 (60)	3 (60)	4 (80)	2 (40)	1 (20)	0	1 (20)	5 (100)
Senftenberg	1 (20)	2 (40)	2 (40)	3 (60)	1 (20)	0	0	1 (20)	4 (80)
Newport	0	0	0	0	0	0	0	0	0
Ealing	0	0	0	0	0	0	0	0	0
O:4,5	0	1 (100)	1 (100)	1 (100)	0	1 (100)	0	1 (100)	1 (100)
O:9,12	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	0	1 (100)	1 (100)
Total	112 (48.7)	148 (64)	146 (63)	150 (65.2)	35 (15)	17 (7.4)	1 (0.4)	158 (69)	202 (88)

AMC,Amoxicillin-clavulanate; AMP,Ampicillin; CFL,Cephalothin; CTX,Cefotaxime; GEN,Gentamicin; ENO,Enrofloxacin; CLO, Chloramphenicol; TET,Tetracyclines; SUL,Sulfonamide.

3 shows the MDR patterns (n=21) *Salmonella* isolates presented. *Salmonella* Heidelberg strains were resistant to various combinations of Amoxicillin-clavulanate, Ampicillin, Cephalexin, Cephalothin, Cefoxitin, Ceftazidime, Cefotaxime, Ceftiofur, Tetracycline and Sulfonamide. In relation to the source of the isolates, 43.2% (16/37) of Salmonella strains isolated before chilling presented multidrug resistance, 81% (111/137) after chilling and 50% (28/56) were from cloacal swabs.

DISCUSSION

Several studies have found high prevalence of *Salmonella* Heidelberg in Brazil (Borges *et al*., 2019; Neves *et al*., 2016; Webber *et al*., 2019), corroborating with this study, where *S*. Heidelberg accounted for 41% of isolated serotypes. The high prevalence of this serotype has a great zoonotic importance, since *S*. Heidelberg has been shown to be more invasive compared to Typhimurium and Enteritidis in a study about *Salmonella* surveillance data from FoodNet collected during 1996-2006 in the United States (Jones *et al*., 2008). In the present study, we observed serotypes that have recently been reported worldwide, as *S*. Enteritidis (6.5%) and *S*. Newport (1.7%) (CDC, 2018; EFSA; ECDC, 2018), besides others not so frequently reported, as *S*. Senftenberg (2.2%) and *S*. Cerro (2.2%) (Baptista *et al*., 2018). Knowledge of serotypes distribution constitutes an important epidemiological tool for the country, especially when it comes to nontyphoidal serotypes, important zoonotic pathogens that can cause gastroenteritis in humans worldwide (Prestinaci *et al*., 2015).

The poultry industry has been frequently implicated in *Salmonella* outbreaks, with reports of human pathogenic *S. enterica* serotypes in poultry products representing a major food safety concern for the

Table 3 – Multidrug resistance patterns observed in *Salmonella* serotypes isolated from cloacal and broiler carcasses swabs in Brazil.

Multidrug resistance patterns	*S*. Heidelberg	*S*. Minnesota	*S*. Saintpaul	*S*. Anatum	*S*. Cerro	*S*. Senftenberg	O:4,5	O:9,12	Total
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
3-4GC; T, S	0	5 (7.6)	0	0	0	0	0	0	5 (2.2)
A, F, T, S	2 (2.1)	0	0	0	0	0	0	0	2 (0.9)
Pe, 1-2GC, 3-4GC, S	0	0	2 (7.1)	3 (33.3)	0	1 (20)	0	0	6 (2.6)
Pe, 1-2GC, T, S	0	1 (1.5)	0	0	0	0	0	0	1 (0.4)
Pe, 3-4GC, T, S	0	1 (1.5)	0	0	0	0	0	0	1 (0.4)
Pe, 1-2GC, 3-4GC, T	0	1 (1.5)	0	0	0	0	0	0	1 (0.4)
Pe, Pe+in, F, T, S	1 (1)	0	0	0	0	0	0	0	1 (0.4)
Pe, Pe+in, 1-2GC, T, S	2 (2.1)	1 (1.5)	0	0	0	0	0	0	3 (1.3)
Pe, Pe+in, 1-2GC, 3-4GC, S	1 (1)	0	0	0	0	0	0	0	1 (0.4)
Pe, 1-2GC, 3-4GC, A, S	0	0	14 (50)	2 (22.2)	2 (40)	0	0	0	18 (7.8)
Pe, 1-2GC, 3-4GC, T, S	0	7 (10.6)	0	0	0	0	0	0	7 (3)
Pe, 1-2GC, 3-4GC, M, T, S	1 (1)	0	0	0	0	0	0	0	1 (0.4)
Pe, Pe+in, 1-2GC, 3-4GC, T, S	46 (48.4)	17 (25.7)	1 (3.6)	0	0	0	0	0	64 (27.8)
Pe, Pe+in, 1-2GC, 3-4GC, M, S	1 (1)	0	0	0	0	0	0	0	1 (0.4)
Pe, Pe+in, 1-2GC, 3-4GC, A, S	0	0	5 (17.8)	1 (11.1)	0	1 (20)	0	0	7 (3)
Pe, 1-2GC, 3-4GC, F, T, S	0	0	0	0	0	0	1 (100)	0	1 (0.4)
Pe, Pe+in, 1-2GC, 3-4GC, F, T, S	11 (11.6)	0	1 (3.6)	0	1 (20)	0	0	1 (100)	14 (6.1)
Pe, Pe+in, 1-2GC, 3-4GC, M, T, S	15 (15.8)	0	0	0	0	0	0	0	15 (6.5)
Pe, Pe+in, 1-2GC, 3-4GC, M, A, S	4 (4.2)	0	0	0	0	0	0	0	4 (1.7)
Pe, Pe+in, 1-2GC, 3-4GC, Ph, T, S	0	1 (1.5)	0	0	0	0	0	0	1 (0.4)
Pe, Pe+in, 1-2GC, 3-4GC, M, F, T, S	1 (1)	0	0	0	0	0	0	0	1 (0.4)
Total	85 (89.5)	34 (51.5)	23 (82.1)	6 (66.7)	3 (60)	2 (40)	1 (100)	1 (100)	155 (67.4)

A, Aminoglycoside; 1-2GC, 1st and 2nd Generation Cephalosporin; ; 3-4GC, 3rd and 4th Generation Cephalosporin; Pe, Penicillin; Pe+in, Penicillin + betalactamase inhibitors; M, Monobactam; F, Fluoroquinolone; Ph, Phenicol; T, Tetracycline; S, Sulfonamide.

industry (Neves *et al*., 2016; Rothrock Jr *et al*., 2015). Each official poultry slaughter establishment must ensure that all poultry carcasses, parts, and giblets will be chilled immediately after slaughter operations so that there is no outgrowth of pathogens (USDA-FSIS, 2014). Nevertheless, in the current study, most of the strains (59.6%, n=137/230), were recovered after being in the chilling tank, where contact between the carcasses processed during the day occur, being *S.* Heidelberg the most frequent followed by *S*. Saintpaul, *S*. Anatum, *S*. Cerro, *S*. Senftenberg and *S*. Minnesota. These findings are worrisome, since cold water used for chilling the carcasses can act as a cross-contamination vehicle among them (Demirok *et al*., 2013).

The most observed MDR pattern in S. Heidelberg and S. Minnesota was Penicillin + betalactamase inhibitor, Penicillin, 1st and 2nd Generation Cephalosporin, 3rd and 4th Generation Cephalosporin, Tetracycline and Sulfonamide, 54.1% (n=46/85) and 50% (n=17/34) respectively. The threat of gram-negative bacteria resistant to multiple antimicrobials including to cephalosporins (WHO, 2017) is a growing global concern. These results show that *Salmonella* spp. is a challenge regarding AMR because of the frequent use of the 3rd generation cephalosporins (3GC) in salmonellosis treatment in human medicine, and because of the frequent use of ceftiofur in food animals (Neves *et al.*, 2016; Nguyen *et al*., 2016). Cephalosporin use, specifically ceftiofur, the only 3GC available for animal food, is considered a major driver of selection and development of 3CG resistance in animal food (Carson *et al.*, 2019). Microorganism resistant to ceftiofur are cross-resistant to ceftriaxone, important antimicrobial agent used to treat children with severe salmonellosis (Neves *et al*., 2016). Among *S*. Heidelberg, the dissemination of AMR determinants is likely due to plasmid-mediated conjugative transfer and by transposons (Deblais *et al.*, 2018). *S.* Heidelberg has a propensity to acquire and disseminate multiple plasmids encoding for MDR (Foley *et al.*, 2011). Besides that, it was observed in the present study an increased resistance to tetracyclines and sulfonamides, the oldest agents used in the treatment of bacterial infections, both in human and in veterinary medicine. Until 1998, these antimicrobial agents were used as additives in animal feeds in Brazil, when their use was restricted to therapeutic purposes. Nevertheless, these drugs still exert selection pressure on the microorganisms. In Brazil, manufacturing, handling, fractionation, marketing, import and the use of chloramphenicol for veterinary use is also prohibited (Brasil, 2003), even so, we observed a high susceptibility to this agent in the current study.

The concern about MDR was also reported by Borges *et al.*, 2019, who studied *Samonella enterica* serotypes isolated from poultry sources in Brazil and 18% were classified as multidrug resistant strains. Neves *et al*., 2016 found MDR strains in 22.8% of *Samonella enterica* serotypes (including Heidelberg) from poultry origin in Brazil, being the most common resistance pattern gentamicin, nalidixic acid and tetracycline for 14 *Salmonella* Heidelberg isolates, followed by ceftiofur, nalidixic acid and tetracycline for 12 *Salmonella* Heidelberg isolates. Jeon *et al*., 2018 analyzed *Salmonella enterica* serotypes from chicken meat in Korea and 50.9% were multidrug resistant strains, being the most common resistance profiles ampicillin, chloramphenicol, nalidixic acid, trimethoprim-sulfamethoxazole, tetracycline and ampicillin, chloramphenicol, nalidixic acid, trimethoprim-sulfamethoxazole for 5 isolates each. These authors found a lower frequency of MDR isolates compared to this study, where 67.4% of the strains were classified as multidrug resistant. Moreover, their resistance patterns were also different, as Table 3 shows that 27.8% (n=64/230) of *Salmonella* strains presented resistance to Penicillin, Penicillin + betalactamase inhibitors, 1st and 2nd Generation Cephalosporin, 3rd and 4th Generation Cephalosporin, Tetracycline, Sulfonamide and 7.8% (n=18/230) to Penicillin, 1st and 2nd Generation Cephalosporin, 3rd and 4th Generation Cephalosporin, Aminoglycoside, Sulfonamide.

A study to evaluate the population dynamics and AMR pattern of the most prevalent poultry-associated *Salmonella* serotypes from the United States in the years 2002 to 2012, suggested that serotypes such as Heidelberg, Typhimurium, Kentucky, and Sentfenberg are more likely to be multidrug resistant whereas Enteritidis, Montevideo, Schwarzengrund, Hadar, Infantis, Thompson, and Mbandaka are generally pan-susceptible or display resistance to fewer antimicrobials (Shah *et al*., 2017). These results corroborate with our findings, where 89.5% of S. Heidelberg and 40% of S. Senftenberg were multidrug resistant, and all strains of *S. Enteritidis* were susceptible to all antimicrobials. However, it is unclear why *S.* Enteritidis isolated from the United States poultry have remained recalcitrant to acquisition of MDR. A recent emergence MDR *S*. Enteritidis from other parts of the world raised a possibility that if clonal expansion and international spread of such MDR clones occurs this may pose a significant public health concern worldwide (Shah *et al*., 2017).

Considering the widespread degree of resistance observed in this and other studies related to nontyphoidal *Salmonella*, caution should be exercised when using antimicrobials that are still efficient to human salmonellosis treatment, in order to avoid or at least delay the resistance and, consequently, the appearance of new super-resistant bacterial strains.

The data from this study reinforce the importance of epidemiological surveillance and the need for improved communication between veterinarians and producers.

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