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Antimicrobial Susceptibility and Biofilm Production by Salmonella sp. Strains Isolated from Frozen Poultry Carcasses

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ABSTRACT

The objectives of this study were to evaluate the antimicrobial resistance and the biofilm-producing ability of *Salmonella* sp. strains isolated from frozen poultry carcasses. Antimicrobial susceptibility was tested by the disk-diffusion method. Biofilm-producing ability was determined in 96-well polystyrene microplates stained with crystal violet at 1%. Out of the 22 strains tested, all were multiresistant, that is, resistant to more than three antimicrobial classes, and 72.7% were able to form biofilms. The highest resistance rates obtained were against sulfonamides, tetracycline, and quinolones. On the other hand, 100% of the strains were sensitive to chloramphenicol. According to the rate of biofilm formation, 3 (13.6%) and 13 (59.1%) strains were classified as moderate and weak biofilm-producers, respectively, and 27.3% did not form biofilms. Biofilms increase the tolerance of microorganisms to stress, reducing their sensitivity to disinfectants and antimicrobials; favor equipment corrosion; and act as substrates for the adhesion of bacteria with lower biofilm-producing capacity. The results of the present study stress the importance of cleaning procedures in food processing plants and highlight the public health risks related to the emergence of multiresistant strains.

INTRODUCTION

Animal products, particularly poultry products, have been reported as the main sources of *Salmonella* sp. foodborne diseases (FD) (Dallal *et al*., 2010). In 2012, about 91,034 cases of salmonellosis were confirmed in humans in Europe (European Food Safety Authority & European Centre for Disease Prevention and Control, 2014), and it is estimated that, in the US, one million people are annually infected by *Salmonella* sp., with an average of 19,000 hospital admittances and 380 deaths (Scallan *et al*., 2011). In Brazil, according to the Health Surveillance Agency of the Ministry of Health, between 2000 and 2014, there were 9,719 FD outbreaks, and *Salmonella* sp. was the most frequent microorganism associated with these outbreaks. This microorganism was identified as the etiological agent of 1,564 outbreaks in the period (Brasil, 2014).

Salmonellosis is generally clinically manifested by to self-limiting gastroenteritis, and does not require treatment in healthy patients. Treatment is only required in cases of immunosuppressed patients, neonates, elderly patients, or those affected by septicemia, as well as in patients with typhoid fever or enteric fever caused by *S.* Typhi or *S.* Paratyphi, respectively. However, the routine and indiscriminate use of antibiotics have favored the emergence of resistant bacterial populations (Brasil, 2012), increasing the possibility of occurrence of treatment failures, and limiting therapeutic options both in human and veterinary medicine, when treatment is necessary (Souza *et al*., 2010a).

Microorganisms may be naturally resistant to a given antimicrobial agent or a specific class of antimicrobials due to the molecular structure of the compound, but resistance may also be acquired. Acquired resistance is a specific property of a bacterial strain and may result from the acquisition of resistance genes, which are frequently located in mobile genetic elements, such as plasmids, transposons, gene cassettes inserted in integrons, genomic islands, or mutations in chromosomal genes (Kadlec *et al*., 2012).

Public health impacts may be more severe when microorganisms, in addition of being resistant to antimicrobials, also have the ability to form biofilms. Biofilms are defined as complex microbial communities embedded in a self-produced extracellular polymeric matrix that attach to surfaces and are the predominant mode of microbial growth in nature (Steenackers *et al*., 2012). Biofilm formation begins with the adhesion of organic or inorganic molecules to surfaces forming a conditioning film. The reversible phase begins with Van der Walls force and electrostatic attraction of planktonic cells to the preformed substrate and the irreversible phase is the result of the extracellular polymeric matrix production, strengthening the bonds between the bacteria and the surface (Oliveira *et al*., 2010).

Biofilms on equipment and tools used in food processing are reservoirs of pathogenic and spoilage microorganisms, increasing the risk of microbial contamination in food processing plants (Shi & Zhu, 2009; Xu *et al*., 2010; Hasegawa *et al*., 2011; Wang *et al*., 2013). Bacteria in biofilms are protected from damaging environmental agents, such as disinfectants. Consequently, they are extremely difficult to eliminate, contributing for the resistance and persistence of these bacteria in these sites (Shi& Zhu, 2009).

The objective of the present study was to evaluate the susceptibility pattern of *Salmonella* sp. strains isolated from frozen poultry carcasses to antimicrobials, and to test their ability to produce biofilms.

MATERIAL AND METHODS

The *Salmonella* sp. strains tested in the present study were obtained from two previous studies on the prevalence of this microorganism in 80 poultry carcasses sold in the western region of the state of Paraná, Brazil (Sereno *et al*., 2012; Druziani *et al*., 2013). In those studies, only frozen carcasses with their packages intact, bearing the Federal Inspection Service stamp, and before the expiration date were acquired. Samples were plated on selective and differential media, and typical *Salmonella* colonies were submitted to phenotypical and serological confirmation. Out of each of the 22 positive carcasses obtained by Sereno *et al*. (2012) and Druziani *et al*. (2013), a typical *Salmonella* colony was randomly selected and subjected to the antimicrobial susceptibility test and the evaluation of biofilm-producing ability.

Antimicrobial susceptibility was tested by the disk diffusion method, according to the recommendations of the National Committee for Clinical Laboratory Standards (Clinical and Laboratory Standards Institute, 2011), against 11 agents of nine different classes. (1) aminoglycosides: streptomycin (STR - 10 µg); (2) quinolones: nalidixic acid (NAL - 30 µg) and enrofloxacin (ENR - 5 µg); (3) cephalosporins: Cephalotin (CF - 30 µg) and ceftiofur (CFT - 30 µg); (4) phenicols: chloramphenicol (C - 30 µg); (5) sulfonamides: sulfonamide (SSS - 300 µg); (6) penicillins: ampicillin (AMP - 30 µg); (7) monobactams: aztreonam (AZT - 30 µg); (8) tetracyclines: tetracycline (TET - 30 µg); (9) sulfonamides and pyrimidines: sulfametoxazole-trimetoprim (SXT - 23.75 µg/1.25). For the interpretation of results, strains that show halos in the resistance and intermediate zones were considered resistant. Strains that were resistant to at least three antimicrobial classes were considered multiresistant (Ngoi & Thong, 2013).

The biofilm-producing test applied the methodology described by Stepanović *et al*. (2000), with some adaptations according to Steenackers *et al*. (2012). Each strain was diluted to 10^8 CFU/mL (0.5 in the MacFarland scale) using Luria-Bertani LB broth (Difco™), and 200µL were cultured in four wells of the 96-well flat-bottom polystyrene microplate (Nest®). A total of 88 wells were used to test 22 strains; the other 4 wells received the *Salmonella* Typhimurim ATCC 14028 as positive control (Oliveira *et al*., 2014), and 4 wells received the negative control (non-inoculated culture medium). The plates containing *Salmonella* sp. strains and controls were incubated at 35ºC for 96h. The plate was washed three times with phosphate buffered saline (PBS pH 7.2) and stained with crystal violet at 1% for 15 minutes. After washing three times with distilled water and drying at room temperature, absorbance was read in a Polaris (Celer®) microplate reader at 492 nm wave length.

In order to determine absorbance results, optical density of each sample (OD) was compared with the three standard deviations above the mean OD of the negative control (OD_c); to determine biofilm-producing

rate, the following classification was used: non-producer ($OD \leq OD_c$), weak biofilm-producer ($OD_c < OD \leq 2xOD_c$), moderate biofilm-producer ($2xOD_c < OD \leq 4XOD_c$), and strong biofilm-producer ($4xOD_c < OD$). All tests were carried out two times and the results were averaged.

RESULTS AND DISCUSSION

All 22 strains tested were considered multiresistant in the antimicrobial susceptibility test, with 100% resistant to quinolones, sulfonamide, and tetracycline; 81.8% resistant to cephalosporins; and 77.3% resistant to penicillins. Only 36.4% strains were resistant to aminoglycosides, and 13.6% to monobactams and sulfonamides associated with pyrimidines (Table 1). Among these agents, quinolones, third-generation cephalosporins, penicillins, and monobactams are considered critically important antimicrobials in human medicine, according to the World Health Organization (World Health Organization, 2012).

Table 1 – Sensitivity and resistance of each of the 22 strains of *Salmonella* sp. to each antimicrobial tested.

Antimicrobial Class	Antimicrobial Tested	Strains Tested	
		Resistant (n/%)	Sensitive (n/%)
Aminoglycosides	STR	8/36.4	14/63.6
Quinolones	NAL	22/100	0/0
	ENR	2/9.1	20/90.9
Cephalosporins	CF	18/81.8	4/18.2
	CFT	15/68.2	7/31.8
Phenicols	C	0/0	22/100
Sulfonamides	SSS	22/100	0/0
Penicillins	AMP	17/77.3	5/22.7
Monobactams	AZT	3/13.6	19/86.4
Tetracyclines	TET	22/100	0/0
Sulfonamides/ Pyrimidines	SXT	3/13.6	19/86.4

STR –Streptomycin; NAL – Nalidixic acid; ENR – Enrofloxacin; CF – Cephalotin; CFT – Ceftiofur; C – Chloramphenicol; SSS – Sulfonamide; AMP – Ampicillin; AZT – Aztreonam; TET – Tetracycline; SXT – Sulfametoxazole-trimetoprim.

The obtained results are in agreement with those of Cortez *et al*. (2006), Dallal *et al*. (2010), and Wang *et al*. (2013), who reported tetracycline resistance rates of 72.4%, 69%, and 70%, respectively. Tetracycline was widely used in Brazil as a prophylactic agent in poultry feed until it was banned (Brasil, 2009). The high rates of tetracycline resistance observed may be related to the transference of several *tet* genes that confer resistance to *Salmonella* sp. (Pezzella *et al*., 2004). Other results presented by Cortez *et al*. (2006), in Brazil, and Wang *et al*. (2013) in China, showed that 86.2% and 78% of the isolated strains, respectively, were also resistant to ampicillin, as also detected in the present study. Bacci *et al*. (2012) in Italy, observed that only 33.3% of the *Salmonella* strains isolated from chicken carcasses were resistant to ampicillin.

Among the tested quinolones, 100% of the strains showed resistance to nalidixic acid, and among cephalosporins, 68.2% showed resistance to ceftiofur (Table 1). Ceftiofur is a third-generation cephalosporin, and these high levels of resistance represent a public health concern, as after the emergence of strains resistant to chloramphenicol around the 1990s, quinolones and third-generation cephalosporins started to be the antibiotics of choice for the treatment of salmonellosis in humans (Pokharel *et al*., 2006; Souza *et al*., 2010b; Lopes 2014).

On the other hand, none of the strains was resistant to chloramphenicol (Table 1), as previously reported by Cardoso *et al.* (2006), Lima *et al.* (2009), Begum *et al.* (2010), and Galdino *et al.* (2013). These authors also did not find any resistance to norfloxacin in *Salmonella* strains isolated from chicken products. According to Lima *et al*. (2009), the sensitivity to chloramphenicol is partially due to the fact that the use of this antimicrobial, both as growth promoter and therapeutic agent, has been banned from animal production since the 1970s, thereby reducing the exposure of bacteria to this active principle.

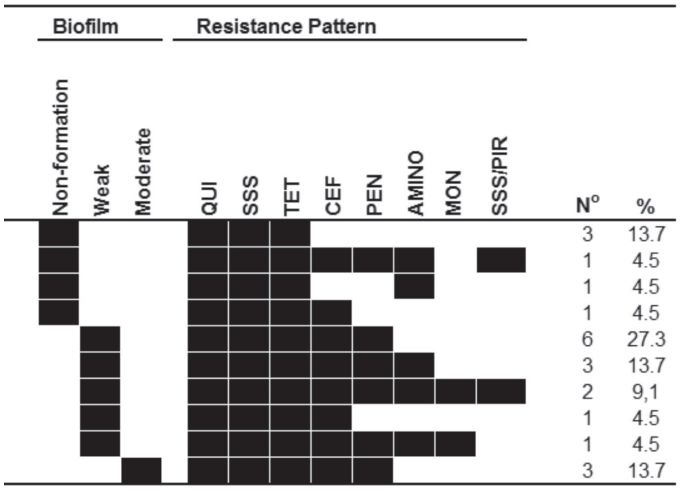
As for the assessment of biofilm production, 72.7% of the strains tested were able to form biofilms on polystyrene microplates. Ziech *et al*. (2016a) reported that 100% of *Salmonella* sp. strains isolated in poultry slaughterhouses and processing plants in Brazil were able to produce biofilm. Oliveira *et al*. (2014), in Brazil, and Solano *et al*. (2002), in Spain, found rates of 98.3% and 97%, respectively. According to the classification by biofilm production rate, in the present study, 13.6% isolates were moderate producers, 59.1% were weak biofilm-producers, and 27.3% did not form biofilm (Table 2).

Table 2 – Classification of the 22 strains of *Salmonella* sp. according to the rate of biofilm production.

Rate of biofilm production	Strains tested	
	Nº.	%
No formation	6	27,3
Weak	13	59.1
Moderate	3	13.6
Strong	0	0
Total of strains	22	100

According to Gilbert *et al*. (2002), the resistance of microbial biofilms to a wide variety of antimicrobial agents is related with the organization of bacterial cells inside the polymer matrix, which hinders the penetration of antimicrobial agents into the biofilm. In addition, cells under stress, showing slow growth rate and undergoing restricted access to nutrients, may express phenotypes related to mechanisms of resistance to antimicrobials. This is due to the exchange of genetic material, especially of genes that encode resistance, among bacteria within the biofilm, where bacterial multiplication is slow, but conjugation rates are fast (Ghigo, 2001).

In the present study, the evaluated strains showed multiresistance to antibiotics, and most were able to form biofilms to some degree. Wang *et al*. (2013), analyzing *Salmonella* sp. isolated from chicken carcasses and from contact surfaces of processing plants, did not find any positive correlation between biofilm formation and antimicrobial resistance. In the present study, the most frequent profile of the isolates was weak biofilm formation and resistance to quinolones, sulfonamides, tetracyclines, cephalosporin, and penicillins (Figure 1). On the other hand, Ziech *et al*. (2016b), evaluating *Salmonella* sp. strains isolated in broiler processing plants, detected 86% multiresistant strains, and the most frequent profile was weak biofilm formation, production of wide-spectrumbeta-lactamase, and resistance to beta-lactams, quinolones, and tetracyclines.



QUI - Quinolones; SSS - Sulfonamides; TET - Tetracyclines; CEF - Cephalosporins; PEN - Penicillins; AMINO - Aminoglycosides; MON - Monobactams; SSS/PIR - Sulfonamides/Pyrimidines

Figure 1 – Distribution of resistance pattern and ability to produce biofilm in the 22 strains of *Salmonella* sp. that were tested.

CONCLUSIONS

Data obtained in the present study suggest that poultry carcasses, particularly those found in retail stores, show a high prevalence of *Salmonella* sp. resistant to the antimicrobials commonly used in human medicine to control severe infections. These results point out to an alarming public health situation.

In addition to the fact that the strains tested showed high resistance to antimicrobials, a high percentage presented the ability to form biofilms. Biofilms increase the tolerance of microorganisms to stress, reducing their sensitivity to disinfectants and antimicrobials; favor equipment corrosion; and aid the adhesion of bacteria that are less able to form biofilms, as these act as substrates. Consequently, biofilms contribute to the persistence of resistant microorganisms both in the food industry environment and in processed foods, mainly due to cross contamination.

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