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Original Article

Extended Spectrum Beta-Lactamase Production and Biofilm Formation in Salmonella Serovars Resistant to Antimicrobial Agents

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ABSTRACT

Antimicrobial resistance is a serious public health problem and *Salmonella* spp. is highly resistant to antimicrobial agents. Biofilms are important in the food industry due to their formation on products, utensils, and surfaces and the difficulty in their removal. The objective of this study was to assess extended spectrum beta-lactamase (ESBL) production, antimicrobial resistance, and biofilm production of *Salmonella* isolated from poultry slaughterhouses. Antimicrobial susceptibility was assessed by the disk diffusion assay and ESBL by double diffusion disk assay using the beta-lactamase inhibitor (amoxicillin+clavulanate). The antimicrobials tested were: ampicillin, amoxicillin+clavulanate, aztreonam, ceftazidime, cefotaxime, chloramphenicol, gentamicin, enrofloxacin, sulfonamide, and tetracycline. Serovars Infantis, Panamá, and Tennessee were found to produce ESBL. All serovars were sensitive to tetracycline, and *S*. Brandenburg was sensitive to all drugs tested. Serovars Panamá, Anatum, Infantis, and Schwarzengrund were moderate biofilm producers at 3 ºC and 9 ºC±1 ºC, respectively, showing possible adaptation of these serovars to these temperatures. Antimicrobials should be used with caution because of the levels of resistance observed and because of ESBL production, and hygiene and sanitary measures should be enhanced to minimize the adhesion of biofilm-forming *Salmonella* serovars at refrigeration temperatures.

INTRODUCTION

Salmonella strains adapt to adverse environmental conditions and can survive in the environment for long periods, colonizing domestic animals and humans. The most common sources of infection among humans are products of poultry origin, dairy products, and surfaces in contact with contaminated products reused without proper disinfection, which could stimulate biofilm formation (Murray *et al*., 2015). Biofilm promotes bacteria survival in stressful environments, like slaughterhouses and food-processing plants (Steenackers *et al.*, 2012). Biofilms favor greater resistance to antimicrobials and disinfectants (Steenackers *et al.*, 2012). Antimicrobial resistance is one of the major public health problems worldwide and microorganisms isolated from foods, especially *Salmonella* spp., show high resistance to these agents (Markle *et al*., 2015). A global increase in extended spectrum beta-lactamase (ESBL) producing is likely to be occurring (Ziech, 2015; ECDC, 2016).

Therefore, the present study assessed antimicrobial resistance, ESBL production, and biofilm formation by *Salmonella* serovars isolated from poultry slaughterhouses.

MATERIALS AND METHODS

Salmonella strains

The tested *Salmonella* serovars were previously isolated (Santos *et al.*, 2015; Mion *et al.*, 2016) from ten poultry slaughterhouses under federal inspection system between 2012 and 2014 from the northern region of the state of Rio Grande do Sul (Table 1). The samples were stored in brain heart infusion broth (Oxoid®, United Kingdom) and frozen at -18ºC.

Table 1 – *Salmonella* serovars and source of poultry slaughterhouse isolates.

Salmonella serovars	Source
S. Agona	Transport cages after cleaning
S. Anatum	Cloacal swabs
S. Brandenburg	Chilled broiler carcasses
S. Bredeney	Cloacal swabs
S. Infantis	Transport cages after cleaning
S. Lexington	Transport cages after cleaning
S. Panamá	Cloacal swabs
S. Rissen	Cloacal swabs
S. Schwarzengrund	Cloacal swabs
S. Tennessee	Chilled broiler carcasses

Antimicrobial susceptibility and ESBL production tests

Antimicrobial susceptibility was assessed by the disk diffusion assay following the Clinical and Laboratory Standards Institute (CLSI, 2015) and the following antibiotics: ampicillin (10µg), amoxicillin+clavulanate (20/10µg), aztreonam (30µg), ceftazidime (30µg), cefotaxime (30 µg), chloramphenicol (30µg), gentamicin (10µg), enrofloxacin (5µg), sulfonamide (300 µg), and tetracycline (30 µg). Interpretation was performed using the criteria described in the approved standards VET01-S2 (CLSI, 2014) and M100-S25 (CLSI, 2015). All strains classified as being moderately susceptible were considered non-susceptible.

The disk diffusion assay was used for testing ESBL production (CLSI, 2015), using amoxicillin+clavulanate placed at the center of the Mueller Hinton and three other disks were placed within a 20 mm radius of the first one: aztreonam, cefotaxime and ceftazidime. ESBL production was considered positive when the formation of "inhibition zones" or an increase in inhibition halo size was observed. The quality control of antimicrobials was made with *E. coli* ATCC 25922 and the positive control for ESBL was a *K. pneumoniae* ATCC 700603 strain.

Biofilm-forming assay

Biofilm-forming ability was assessed on polystyrene plates (Rodrigues *et al.*, 2009) at the following incubation temperatures: 3±1ºC (temperature used for refrigeration of meat cuts), 9±1ºC (cutting room temperature for export to the European Union), 25±1ºC (ambient temperature), 36±1ºC (optimal pattern for the growth of mesophilic), and 42 ºC±1ºC (temperature for selective enrichment due to thermotolerance of *Salmonella*). The arithmetic mean of the absorbance values for each sample (ODa) was compared with the average absorbance of the wells containing sterile TSB (OD) and the following classification was used to determine the level of adhesion: non-adherent (ODa≤OD), weakly adherent (OD<ODa≤2OD), moderately adherent (2OD<ODA≤4OD), strongly adherent (4OD<Oda).

RESULTS AND DISCUSSION

Table 2 displays the susceptibility profiles of *Salmonella* serovars, ESBL production, and their capacity to adhere to polystyrene at different incubation temperatures.

Eight out of the 10 serovars were resistant to sulfonamides. High resistance to these drugs is probably related to their extensive use, leading to an increase in the selective pressure on resistant strains and in the dissemination of resistance genes (Machinski Junior *et al.*, 2005; WHO, 2011; ECDC, 2016). Also, 40% of serovars were resistant to enrofloxacin, which belongs to the class of fluoroquinolones and is exclusively used in veterinary medicine; however, despite its restricted use, it has been shown that enrofloxacin-resistant bacteria can develop resistance to ciprofloxacin. Fluoroquinolones and quinolones play a crucial role in human clinical practice, as they are used in the treatment of severe infections and are the main treatment option against salmonellosis (Souza *et al.*, 2010; WHO, 2011).

Resistance to chloramphenicol was found to be 20%, even though it cannot be used in animal production in Brazil since 2003 (Brasil, 2003). As this antimicrobial was used for many years in veterinary medicine, resistance to it might not have been reversed yet, or else, florphenicol, which is exclusively used by veterinarians, could be favoring the transmission of resistance genes that are common to these two antimicrobials (Nógrády *et al.*, 2012; Mattiello *et al.*, 2015).

Table 2 – Sensitivity of *Salmonella* serovars, ESBL production, and adhesion to polystyrene at different incubation temperatures.

Salmonella serovars	Antimicrobial resistance	ESBL production	Adhesion to polystyrene				
			3°C±1°C	9°C±1°C	25°C±1°C	36°C±1°C	42°C±1°C
S. Agona	ENO, SUT	-	Non-adherent	Non-adherent	Weak	Non-adherent	Non-adherent
S. Anatum	SUT	-	Weak	Moderate	Weak	Weak	Moderate
S. Brandenburg	-	-	Weak	Weak	Moderate	Weak	Weak
S. Bredeney	CLO, SUT	-	Weak	Weak	Moderate	Weak	Weak
S. Infantis	ENO, GEN, SUT	Yes	Weak	Moderate	Moderate	Weak	Weak
S. Lexington	SUT	-	Non-adherent	Non-adherent	Weak	Non-adherent	Weak
S. Panamá	AMP, ENO, SUT	Yes	Moderate	Weak	Moderate	Weak	Non-adherent
S. Rissen	SUT	-	Non-adherent	Non-adherent	Weak	Non-adherent	Weak
S. Schwarzengrund	CLO, SUT	-	Weak	Moderate	Moderate	Weak	Weak
S. Tennessee	ENO, GEN	Yes	Weak	Weak	Moderate	Weak	Weak

Legend: AMP = Ampicillin 10 µg, CLO = Chloramphenicol 30 µg, GEN = Gentamicin 10 µg, ENO = Enrofloxacin 5 µg, SUT = Sulfonamides 300 µg, TET = Tetracycline 30 µg. ESBL = Amoxicillin+Clavulanate 20/10 µg, Aztreonam 30 µg, Ceftazidime 30 µg, Cefotaxime 30 µg.

All tested serovars were sensitive to tetracycline. Serovar Brandenburg was sensitive to all tested drugs. Serovars Anatum, Lexington, and Panamá; Agona and Tennessee; Schwarzengrund; and Infantis were considered moderately resistant to sulfonamides, enrofloxacin, chloramphenicol, and gentamicin, respectively. These are alarming findings as the assessed antimicrobials are widely used in veterinary and human medicine and also because the use of drugs with moderate resistance could result in the selection of a resistant bacterial population (Machinski Junior *et al*., 2005).

The number of *Salmonella* serotypes showing ESBL production in the current study was lower than in previous studies (Gelinski *et al.*, 2014). Only serovars Infantis, Panamá, and Tennessee phenotypically demonstrated ESBL production, accounting for 30% of the assessed serovars. The prevalence of ESBL production strains varies worldwide. However, the incidence has increased greatly in recent years in several countries. In the study undertaken by Ziech (2015) in Brazilian samples, 45% (44/98) of *Salmonella* isolates collected from birds were ESBL producers. ESBL production has emerged in serovar Infantis in Italy and Switzerland (Franco *et al.*, 2015; Hindermann *et al.*, 2017). Serovars Panama and Tennessee have also been reported as ESBL producers (Weill *et al.*, 2004; Gelinski *et al.*, 2014). ESBL-producing strains have also been described in serovars Heidelberg, Senftenberg, Newport, Enteritidis, Weltevreden, Indiana, Typhimurium, Litchfield, Oranienburg and Typhi (Jure *et al.*, 2010; González-López *et al.*, 2014; Bae *et al.*, 2015; Djeffal *et al.*, 2017).

Salmonella serovars can adhere to polystyrene, leading to biofilm formation. It is of public health concern, since strains which remain in slaughter plants and in food products could produce biofilms and favor dissemination through breakage of these structures and consequent release of pathogenic microorganisms.

Note that serovars Panamá; Anatum, Infantis and Schwarzengrund were moderate biofilm producers at 3°C and 9°C, respectively, demonstrating a possible adaptation of these serovars to these temperatures. These findings are in line with those of Rodrigues *et al*. (2017), who observed biofilm formation at these temperatures, with strongly adherent *S.* Enteritidis strains. These findings are relevant because these serovars adhered moderately to polystyrene at unfavorable temperatures for the multiplication of *Salmonella* spp., which is recommended as a conventional method for food preservation under refrigeration (Gast, 2008).

The results obtained in the present study allow inferring that even if the recommended temperatures are complied with, there could be multiplication of *Salmonella* spp. and biofilm formation in slaughterhouses and consequent contamination of the final products, which may lead to foodborne diseases. In addition, serovars Infantis, Panamá, and Tennessee were found to produce ESBL. This is a public health problem, which highlights the need of caution in the use of antimicrobials.

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