SALMONELLA SPP. VIRULENT AND RESISTANT MULTIDRUG RECOVERED FROM CHICKEN CARCASSES IN BRAZIL

ORIGINAL ARTICLE

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ABSTRACT

The aim of this study was to evaluate the biofilm production, the susceptibility profile and the detection of resistance genes present in *Salmonella* spp isolates from fresh chicken carcasses sold in a Brazilian metropolis. From a total of 61 samples of fresh poultry carcasses, 21 were positive for the presence of *Salmonella* spp. Regarding the antimicrobial susceptibility test, (13/21) isolates tested were resistant to at least one antibiotic, corresponding to 61.9%, and 38% (08/21) were Resistant to Multiple Drugs. At least two resistance genes were identified in all isolates, especially the genes related to β-lactamases and Quinolones resistance. It was also observed that some *Salmonella* spp isolates showed identical genetic patterns. And all 21 isolates were able to form biofilm. The identification of *Salmonella* spp. biofilm forming and carrying different β-lactamase genes and determinants of resistance to quinolones demonstrates the capacity of these bacteria to accumulate various mechanisms of virulence and resistance to antimicrobials. Therefore, the spread of different clonal groups of *Salmonella* spp. MDR in poultry meat carcasses expressed in this attest to the need for effective controls to contain this microorganism, which besides being a risk to public health, is also responsible for considerable economic losses.

Keyword: Beta-lactams, Food poisoning, PCR, Public market.

INTRODUCTION

Salmonellosis is a foodborne disease caused by pathogenic *Salmonella* spp strains. Cases of Salmonellosis in humans occur mainly through the consumption of contaminated food or water. In most cases, food of animal origin, especially those from poultry, are the major sources of *Salmonella* spp. infections. The symptoms of the enteric infection are nausea, vomit, non-bloody diarrhea, fever, cold, abdominal pain, myalgia and headaches, and in immunodepressed patients it can lead to bacteremia, endocarditis, and death (GRIMONT and WEILL, 2007).

According to the Centres for diseases, control and prevention (CDC), 1.2 million cases of infections, 23.000 hospitalizations, and 450 deaths per year in the United States of America are caused by *Salmonella* spp, approximately. And the infections related to the ingestion of contaminated food represents 1.0 million of the cases, approximately (CDC, 2020). In Brazil, according to the Ministry of Health, a total of 12.660 cases of foodborne illness was reported between 2000 and 2017, and *Salmonella* spp. was one of the major agents reported in these cases, which represents a total of 35% of the cases reported (BRASIL, 2019).

In most salmonellosis cases, healthy individuals do not need treatment. However, immunodepressed patients need to go through treatment (SERENO *et al.,* 2017). Nevertheless, the indiscriminate use of antimicrobials, especially in animal production has led to an increased number of microorganisms resistant to therapeutic agents, leading to a limited number of antimicrobial options, which can result in treatment failures (ALEKSHUN and LEVY, 2007; PENG *et al.,* 2014). Over the years, there has been an increase in the prevalence of *Salmonella* strains multiple drugs resistant, mainly in poultry products, such as poultry meat (AZEVEDO, 2014; BAPTISTA *et al.,* 2018; DUARTE *et al.,* 2009; FITCH *et al.,* 2016;

BONI *et al.,* 2011; REZENDE *et al.,* 2005; RIBEIRO *et al.,* 2007; THRELFALL, 2002).

Therefore, the emergence of multidrug-resistant *Salmonella* (MDR) is a worldwide concern, due to the increased hospitalization rates and death. This phenomenon is a consequence of the extensive use of antibiotics by humans, and also in animal production.

Moreover, *Salmonella* species have the ability to form biofilm, where cells microorganisms are embedded in an extracellular matrix, so these microorganisms can adhere to biotic or abiotic surfaces (DAVEY and O'TOOLE, 2000; FLEMMING *et al.,* 2016). The complex matrices of the biofilm keep the microbial cells protected from the action of sanitation process and antimicrobial agents, therefore, when pathogenic microorganisms are involved in the biofilm, there is a huge risk to contaminate the food, which leads to a public health problem. (KASNOWSKY *et al.*, 2010).

Hence, the aim of this study was to characterize the determinants of antibiotic resistance, and to evaluate the production of biofilm in *Salmonella* spp. strains recovered from fresh poultry meat sold in a Brazilian metropolis.

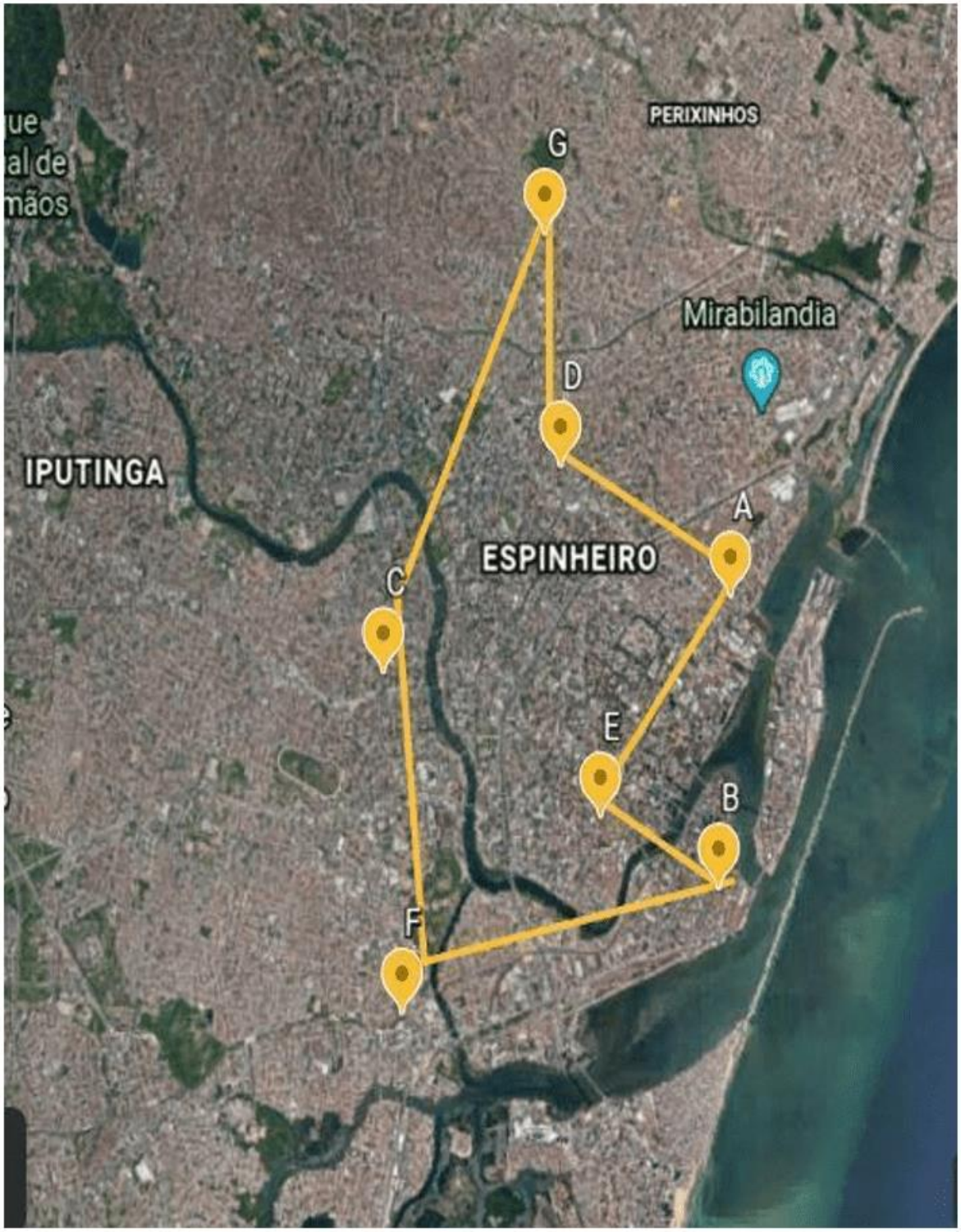
METHODS

Sampling and identification of the poultry carcasses

A total of 61 poultry meat carcasses were purchased from 07 different public markets in the city of Recife in the state of Pernambuco, Brazil, between 2018 to 2019 (figure 1 and table 1). The samples were sent to the Meat and Milk Inspection Laboratory (LICAL), of the Department of Veterinary Medicine in the Federal Rural University of Pernambuco (UFRPE).

At the laboratory, the surface of each poultry carcasses pack was wiped with 70 percent alcohol. Then, 25g of the carcases were randomly taken and placed in individual sterile stomacher bags with 225 ml of Buffered Peptone Water (Kasvi, Brazil), and homogenized in stomacher for 2 min (Kasvi, Brazil) and incubated at 37 °C for 24 hours for the pre-enrichment stage. For the enrichment stage, 0.1 ml of the pre-enriched broth was transferred into 10 ml of Tetrathionate broth (Merck e pais) and then incubated at 41.5 °C for 24 hours, and 1.0 ml of the pre-enriched broth was also transferred into 10 ml of Rappaport and incubated at 37 °C for 24 hours for selective enrichment. After the incubation, one loopful of the TT broth and Rappaport cultures were streaked onto Xylose Lysine Agar (XLD) (Kasvi, Brazil) and Hektoen Enteric Agar (HE) (Kasvi, Brazil) plates and incubated at 37 °C for 24h. Three presumptive *Salmonella* colonies on the plates were selected and streaked onto Nutrient agar (Merck, pais-) and incubated at 37.8 °C for 24 hours. Then, colonies from the Nutrient agar were submitted to biochemical and serological tests (ISO, 657912017).

Figure 1: Public markets where the samples were acquired



Source: Google Earth.

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Table 1: Number of samples acquired in different public markets in the city of Recife – Pernambuco, Brazil

Public Market	Samples acquired
A	1, 2, 3 22, 23, 24, 25, 26
B	4, 5, 6, 7, 8, 9, 27, 28, 29
C	30, 31, 32
D	10, 33, 34, 35, 36,
E	11, 37, 38, 39, 40, 41
F	12, 13, 14, 15, 16, 17, 18, 45, 46,47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61
G	18, 20, 21, 42, 43, 44

Source: Authors.

Susceptibility profile to antimicrobials

The susceptibility profile test was done by the agar disc-diffusion method with the following antimicrobial agents: ampicillin, chloramphenicol, ciprofloxacin, ceftriaxone, sulfamethoxazole-trimethoprim, imipenem, amoxicillin-clavulanic acid, ceftazidime, cefotaxime and aztreonam, according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2018).

Detection of resistance genes

The DNA used in the molecular analyses was obtained from bacterial suspensions made up of fresh colonies, which were previously grown for 16-18 hours in Luria Bertani agar and inoculated in approximately 300 µL ultrapure water free of nuclease homogenized with the aid of a vortex tube shaker (Vision Scientific).

The investigation of genes to β-lactams and quinolones resistance was performed by PCR. The thermal cycling conditions were those described by the authors listed in the primer table.

Table 2: Primers used to detect the β-lactamases genes

Primer		**5' 3' Sequences**	**Target Gene**	**Product**	**References**
KPC	F	TGTCACTGTATCGCCGTC	*bla*$_{KPC}$	1011pb	(Bratu et al., 2005)

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	R	CTCAGTGCTCTACAGAAAACC			
CTX-M	F	SCSATGTGCAGYACCAGTAA	*bla*$_{CTXM}$	500pb	(Cao et al., 2002)
	R	CCGCRATATGRTTGGTGGTG			
TEM	F	TCGGGGAAATGTGCGCG	*bla*$_{TEM}$	700pb	
	R	TGCTTAATCAGTGAGGCACC			
SHV	F	TTATCTCCCTGTTAGCCACC	*bla*$_{SHV}$	900pb	
	R	GATTTGCTGATTTCGCTCGG			
	R	AAGCAGACTTGACCTGA			
qnrA	F	AGA GGA TTT CTC ACG CCA GG	*qnrA*	580pb	(Cattoir et al., 2007).
	R	TGC CAG GCA CAG ATC TTG AC			
qnrB	F	GGM ATH GAA ATT CGC CAC T	*qnrB*	264pb	
	R	TTT GCY GYY CGC CAG TCG AA			
qnrC	F	GGG TTG TAC ATT TAT TGA ATC	*qnrC*	447pb	(Wang et al., 2009)
	R	TCC ACT TTA CGA GGT TCT			
qnrD	F	CGA GAT CAA TTT ACG GGG AAT A	*qnrD*	582pb	(Cavaco et al., 2009).

Source: Authors.

Analysis of the genetic profile of the isolates

The comparison and identification of genetic variations in the content of bacterial strains was performed by ERIC-PCR. The primers used for the reaction were ERIC-1R (5' ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'AAGTAAGTGACTGGGGGGGCCG-3') (Sigma Aldrich) according to (VERSALOVIC *et al.,* 1991), with modification described by (FENDRI *et al.,* 2013).

Biofilm production capacity

The biofilm forming capacity was evaluated according to the methodology proposed by (STEPANOVIĆ *et al.,* 2000; STEPANOVIĆ *et al.,* 2007). Each strain was diluted to 108CFU/mL (0.5 in the MacFarland scale) using Trypticase Soy Broth (TSB) (Merck), and 200µL were cultured in three wells of the 96-well flat-bottom polystyrene microplate (Nest®). A total of 69 wells were used to test 21 strains; the other 3 wells received the *Salmonella* Typhimurim ATCC 14028 as positive control, and 3 wells received the negative control (non-inoculated culture medium). The plates containing *Salmonella* spp. strains and controls were incubated at 35ºC for

96 hours. 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 wavelength (SERENO *et al.,* 2017).

The optical density (OD) of each *Salmonella* spp. strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (ODnc). After that, the strains were classified in no biofilm producer (OD≤ODnc); weak biofilm producer (ODnc<ODs≤2.ODnc); moderate biofilm producer (2.ODnc<ODs≤4.ODnc); and strong biofilm production (4.ODnc<ODs) classification given according to (STEPANOVIĆ *et al.,* 200; STEPANOVIĆ *et al.,* 2007).

RESULTS

Prevalence of *Salmonella* spp. in poultry meat carcass samples

From the 61 samples of poultry meat carcasses collected in the public markets, 34% (21) were contaminated by *Salmonella* spp. The prevalence of *Salmonella* spp. on each public market is expressed in figure 3, and the data is detailed in table 3.

Figure 2: Prevalence of *Salmonella* spp. isolates from each public market in the city of Recife – Pernambuco, Brazil

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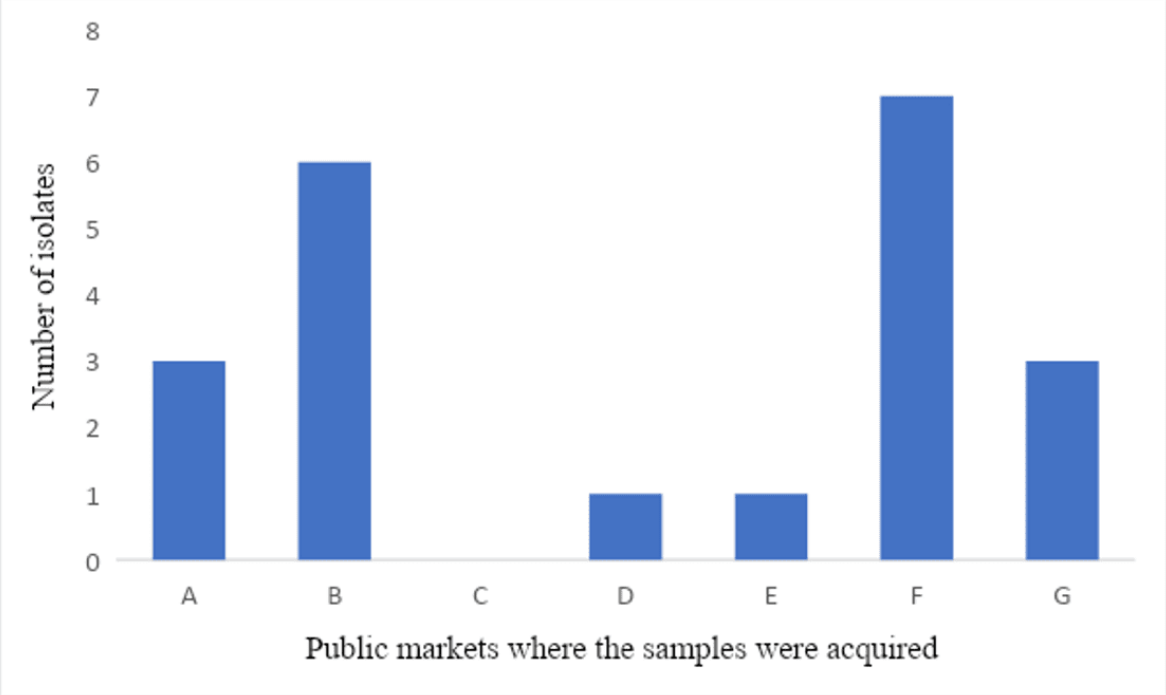

Source: Authors.

Table 3: *Salmonella* spp. isolates from each public market in the city of Recife – Pernambuco, Brazil

Isolates	**Public market**	**Year**
1	A	2018
2		2019
3		2019
4	B	2018
5		2018
6		2018
7		2018
8		2018
9		2018
10	D	2019
11	E	2019
12	F	2019
13		2019
14		2019
15		2019
16		2019
17		2019
18		2019
19	G	2018
20		2019
21		2019

Source: Authors.

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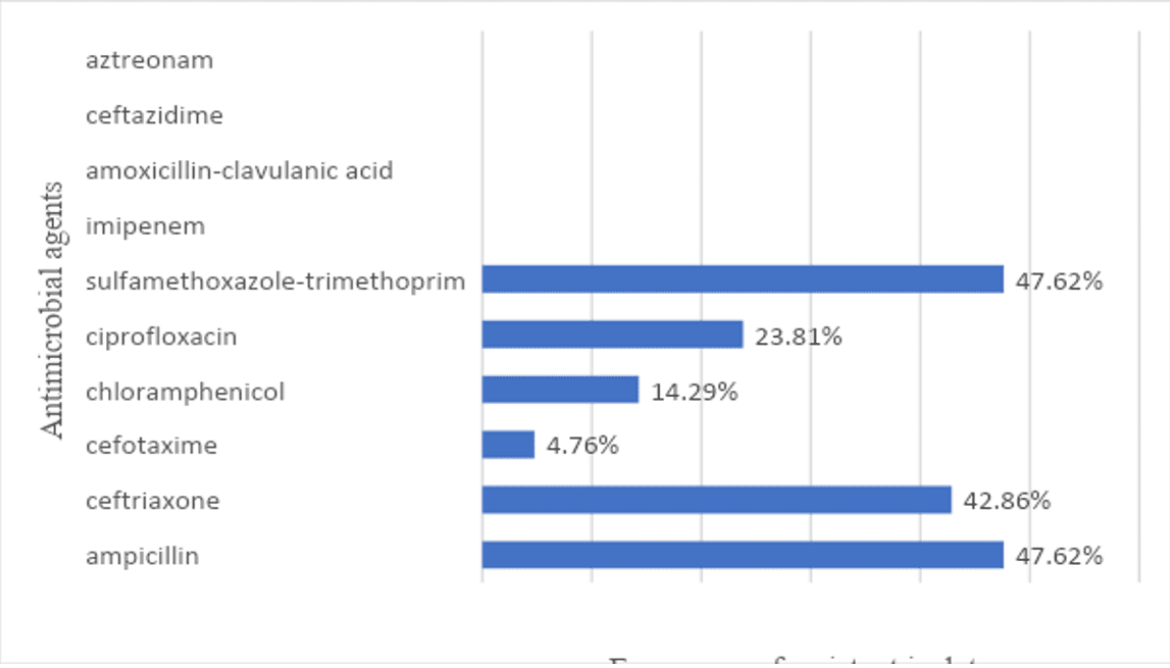
Figure 3: Antimicrobial resistance to individual antimicrobial agents among *Salmonella* spp. isolates from poultry carcasses sold in public markets in the city of Recife – Brazil



Source: Authors.

Susceptibility perfil

From the 21 isolates, 13 (61,9%) were resistant to at least one antimicrobial agent. The antimicrobial resistance to individual antimicrobial agents among *Salmonella* isolates is expressed in figure.3. The detailed data from the antimicrobial susceptibility test is expressed in table 4.

Table 4. Antimicrobial susceptibility profile of *Salmonella* spp. isolates from chicken carcasses acquired in public markets in the city of Recife - PE, between 2018 and 2019

Isolate	AMP	AMC	CRO	CTX	CAZ	IPM	ATM	CLO	CIP	SUT
1	R	S	R	S	S	S	S	S	R	R
2	S	S	S	S	S	S	S	S	I	S
3	S	S	S	S	S	S	S	S	R	R
4	R	S	I	S	S	S	S	S	S	S
5	S	S	S	S	S	S	S	S	I	S
6	S	S	I	S	S	S	S	S	R	S
7	S	S	I	S	S	S	S	S	I	S

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8	S	S	S	S	S	S	S	S	R	S
9	S	S	I	S	S	S	S	S	I	S
10	S	S	S	S	S	S	S	S	I	S
11	S	S	S	S	S	S	S	S	I	S
12	R	S	R	S	S	S	S	S	I	R
13	R	S	R	S	S	S	S	S	I	R
14	S	S	S	S	S	S	S	S	I	S
15	R	S	R	S	S	S	S	R	S	R
16	R	S	R	S	S	S	S	R	S	R
17	R	S	R	S	S	S	S	R	S	R
18	R	S	R	S	S	S	S	S	S	R
19	S	S	S	S	S	S	S	S	S	S
20	R	S	R	S	S	S	S	S	S	R
21	R	S	R	R	S	S	S	S	R	R

AMP - Ampicillin, CLO - Chloramphenicol, CIP - Ciprofloxacin, CRO- Ceftriaxone, SUT - Sulfamethoxazole-trimethoprim, IPM - Imipenem, AMC - amoxicillin-clavulanic acid, CAZ - Ceftazidime, CTX- Cefotaxime and ATM - Aztreonam, S – Sensitive; R- Resistant; I-. Intermediate. Source: Authors.

At least two resistance genes were identified in all isolates, among which the presence of resistance genes encoding β-lactamases (bla) and resistance genes encoding quinolones (qnr) were observed in the molecular tests carried out in the present study, and the results are detailed in table 3.

Table 5: Resistance genes detected in each *Salmonella* spp isolate, recovered from poultry carcasses acquired in public markets in the city of Recife - Pernambuco between the years 2018 and 2019

β-lactams resistant					**Quinolones resistants**			
Isolates	*Bla$_{TEM-like}$*	*Bla$_{SHV-like}$*	*Bla$_{CTX-M-like}$*	*Bla$_{KPC-2}$*	*qnrA*	*qnrB*	*qnrC*	*qnrD*
1	+	-	-	-	+	+	-	-
2	+	-	-	-	+	+	-	-
3	+	-	-	-	+	+	-	+
4	+	-	-	-	+	+	-	-
5	+	-	-	-	+	+	-	+
6	+	-	-	-	+	+	-	+
7	+	-	-	-	+	+	-	+
8	+	-	-	-	+	+	-	+
9	+	-	-	-	+	+	-	+
10	+	-	+	-	-	+	-	-
11	+	-	-	-	-	+	-	-
12	+	-	+	-	-	+	-	-
13	+	-	+	-	-	+	-	-
14	+	-	+	-	-	+	-	-
15	+	-	+	-	-	+	-	-
16	+	-	+	-	-	+	-	+
17	-	-	+	-	-	+	-	+

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18	+	-	+	-	-	+	-	+
19	+	-	-	-	-	+	-	+
20	+	-	-	-	-	+	-	+
21	+	-	-	-	+	+	-	+

Source: Authors.

Clonal relationship

The isolates showed a pattern ranging from 3 to 9 strips, which revealed the presence of different genotypes pattern indistinguishable from each other and others whose standard is different for only one or two bands. Some isolates of *Salmonella* spp. showed identical genetic patterns (2, 3, 4, 5, 6 and 7). Samples 9, 11, 19, 20 and 21 were closely related genetically, as they have a similar pattern of strips, differing only in one (9, 21) or two strips (11 and 20).

Biofilm forming

All the 21 *Salmonella* spp. isolates tested were biofilm producers, and these isolates were classified as weak biofilm producers according to (STEPANOVIĆ *et al.,* 2000; STEPANOVIĆ *et al.,* 2007).

DISCUSSION

This work identified *Salmonella* spp. contaminating chicken carcasses sold in public markets in a Brazilian metropolis. In addition, we detected the resistance mechanisms present in these bacterial isolates and confirmed that they were all biofilm producers. The results that are presented here showed several isolates with a genetic proximity and revealed that the others are clones.

Isolates 2, 3, 4 and 5 have an identical genetic profile, although they were collected in two different markets, Market A and Market B, which are about 5 km apart from each other. This finding indicates that the sellers may have the same supplier, where the contamination by *Salmonella* MDR started. Market F presented a greater number of strains characterised as clones (12, 14, 15, 16 and 18). In addition to the possibility

of the same supplier, according to a set of failures in hygienic conditions, such as the exposure of products to dust and insects and the inappropriate sharing of utensils such as knives and containers between traders, they can contribute to the contamination of the carcasses, which results in the dissemination of isogenic isolates (AZEVEDO, 2014).

The presence of *Salmonella* spp. in poultry meat was also observed in the studies done by (BAPTISTA *et al.,* 2018; MELO *et al.,* 2020; YIN *et al.,* 2021). However, our findings differ from these studies due to the higher prevalence of *Salmonella* spp isolates.

The contamination by *Salmonella* spp. detected in the samples may have been due to previous contamination in the slaughterhouse itself, because of failures in the process of slaughtering (LOPES *et al.,* 2007; VON RÜCKERT *et al.,* 2009). It was also observed that traders removed the carcasses from the original packaging and placed them in plastic bowls and later sold them at room temperature (88 ºF), which is considered a fraud. This temperature promotes the multiplication of *Salmonella* spp., as these microorganisms can multiply at temperatures around 5°C to 45°C (MAHMOUD, 2012) This problem related to the temperature was also noticed in the studies done by (KHAN *et al.,* 2018; JARQUIN *et al.,* 2015).

Therefore, this practice increases the risk of product contamination, mainly due to inadequate handling practices and inadequate sanitary hygienic conditions and to what is observed in these public markets, as it was found that tables and benches were not cleaned in 59% (36/61) of the places. Regarding the handlers, it was observed that 100% did not have their nails well cut and clean. In addition, the same employee who took care of the meat was the same one in the checkout counter. Consequently, these flaws in the processes of good handling practices observed in these markets can contribute to the contamination and spread of *Salmonella* spp. in this product. This transference can occur between various interactions between

man, food and the environment. Therefore, cross-contamination is the basis for the spread of *Salmonella* spp.

From the results from the susceptibility test against antimicrobials agents in the present study, it was observed that 38% (08/21) of the *Salmonella* spp strains were Resistant to Multiple Drugs (MDR), that is, bacteria that are resistant to at least three classes of antimicrobial agents (MAGIORAKOS *et al.*, 2011).

The occurrence of *Salmonella* spp strains resistant to multiple antimicrobials in poultry meat in Brazil is a growing reality, due to the data reported in the present study, and other researches done by (ZIECH, 2015; BAPTISTA *et al.,* 2018). It might happen because of the intensive use of antimicrobial agents in the animal production to treat the animals, and also to improve the production.

The greatest implication regarding the dissemination of multidrug resistant zoonotic strains is related to therapeutic failures in the face of several conditions, both in human and animal cases. In addition, this reality leads to losses in animal herds, since when present, these resistant strains are difficult to eliminate. The permanence of pathogenic and multidrug resistant strains in the poultry production is even more worrying, since the animal density is higher, and consequently, there is a greater spread of the microorganisms in a shorter period of time.

It was observed that only 4.28% (03/21) of the isolates were resistant to Chloramphenicol. This result may reflect the ban on the use of this agent as a therapeutic agent and growth promoter in animal production in Brazil (PACHECO-SILVA *et al.*, 2014).

On the other hand, 42.85% (09/21) of the isolates were resistant to Ceftriaxone, 3rd generation cephalosporin. The use of this drug in animal production is prohibited in Brazil (MION *et al*., 2014). Therefore, this result indicates that this drug or others from the same antimicrobial class is still being used in animal production in Brazil.

In addition, 42.85 (9/21) of the isolates were resistant to three or more antimicrobial drugs. Then, when comparing the resistant profile with the markets, it was noticed that isolates number 13, 15, 16, 17, 18, which were resistant to up to three drugs were from the same public market (public market F). It suggests that there may have been cross contamination at the market, or that these chicken carcasses may have suffered previous contamination in the poultry slaughterhouse.

Hence, the occurrence of *Salmonella* MDR strains in food animal origin is increasing in Brazil (BAPTISTA *et al.,* 2018; MELO *et al.,* 2020; VOSS-RECH *et al.,* 2015). Infections related to multidrug resistant strains are associated with high morbidity and mortality, compared to the sensitive ones, since these microorganisms represent a barrier in the treatment of human and animal diseases.

The presence of such resistance genes detected in *Salmonella* spp. isolates from food of animal origin represents a serious threat to public health, since the horizontal transmission of resistance genes occurs mainly by plasmids encoding β-lactamases (GYLES, 2008). In addition, these genes are considered from community origin, and are common in hospital environments (SUN *et al.,* 2013). Thus, the dissemination of resistant strains points out the importance of the *Salmonella* spp control in poultry meat (BORGES *et al.,* 2019; SIVASANKAR *et al.,* 2020).

The presence of biofilm-forming strains of *Salmonella* spp. observed in the present study points out the risk that this finding may bring to the food industry and the public health. The ability to form biofilm protected the microorganisms inside the biofilm, and thus less susceptible to external factors, such as the action of antimicrobial agents (ZIECH, 2015; SERENO *et al.,* 2017; BORGES *et al.,* 2018).

In addition, biofilms promote the permanence of pathogenic and spoilage microorganisms, which cause illnesses to consumers, and the depreciation of the final product due to physical-chemical and sensory changes (KASNOWSKY *et al.*, 2010; SINGH *et al.,* 2017).

Finally, this investigation showed that the identification of *Salmonella* spp. producer of biofilm and carrying different β-lactamase genes and determinants of resistance to quinolones demonstrates the ability of these bacteria to accumulate various mechanisms of virulence and resistance to antimicrobials. The selective pressure is exerted by the indiscriminate use of antibiotics in agriculture is an important factor for the acquisition of different mechanisms of resistance and dissemination of these strains. Furthermore, the spread of different clonal groups of *Salmonella* spp. MDR, in chicken carcasses, shown in this study attests to the need for effective controls to contain this microorganism, which besides being a risk to public health, is also responsible for considerable economic losses.

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