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Article

Antimicrobial resistance in *Salmonella* spp. isolated from pig carcasses in two slaughterhouse types in Jalisco, Mexico

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Abstract:

Salmonella is one of the main bacteria causing foodborne illness. Research into antimicrobial resistance in *Salmonella* is increasingly important as treatment of salmonellosis becomes more difficult. An analysis was done of samples from pig carcasses in two slaughterhouse types (federal-inspected and municipal) in the state of Jalisco, Mexico. Thirty-eight *Salmonella* strains were isolated, with fewer ($P<0.05$) strains (n= 1) in the federal-inspected slaughterhouse than in the municipal one (n= 37). This difference is probably due to stricter sanitation measures in the federal-inspected

slaughterhouse. The main identified *Salmonella* serotypes were London (44.7 %), Anatum (15.8 %), and Agona, Muenchen and Typhimurium (7.9 %). Resistance was broadest against aminoglycosides (100 %), tetracyclines (73.7 %) and ciprofloxacin (44.7 %). Most (66.6 %) of the strains were resistant to three or four different antimicrobial classes. Presence of the gene coding for integrase 1 was confirmed. In the sampled slaughterhouses *Salmonella* strains have acquired genetic elements promoting resistance to different antimicrobial classes, potentially complicating treatment of infections caused by them. Implementation of better practices and compliance with existing regulations could contribute to reducing the frequency of *Salmonella* isolates in the sampled slaughterhouses.

Key words: Slaughterhouses, *Salmonella*, Pig carcasses, Serotypes, Antimicrobial resistance.

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Introduction

The advent of antimicrobial-resistant pathogenic bacteria drives research into the frequency of these bacteria in food and their resistance[1]. In 2018, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) established five priority areas of surveillance. Primary among them was the presence of resistant bacteria, toxic residues and resistance genes in food production environments. Another area of concern was frequent use of antimicrobials in food production and its relationship with the development of resistant bacteria. And emphasis was placed on the need for integrated monitoring systems to control processes in all stages of food production and distribution[2]. The United States of America and the European Union work towards food safety through supervision systems such as the National Antimicrobial Resistance Monitoring System (NARMS) and the European Food Safety Authority (EFSA)[3,4]. Pathogenic and resistant bacteria in food is a worldwide problem. The number of resistant pathogenic strains can differ between regions, but is related to implementation and monitoring of control measures in food production processes[4-6].

Pork consumption in Mexico has increased as its price becomes competitive with that of other meats such as poultry or beef. In 2017, pork consumption in Mexico was 19

kg/person/yr[7], and overall consumption in 2018 was estimated at 1.5 million tons, making Mexico the tenth largest consumer of pork worldwide. Annual pork production in 2018 was predicted to grow by 2.3 % in 2018, with a total production of 113.5 million tons. The states of Jalisco, Sonora, Puebla, Yucatan and Veracruz account for 69.4 % of national production[8].

Two types of slaughterhouses operate in Mexico: Federal Inspection Type (Tipo Inspección Federal - TIF) and un-certified municipal (rastros no certificados - RNC)[9]. Those certified as TIF process animals to supply meat products manufacturers and are subject to permanent sanitary inspection by the Ministry of Agriculture and Rural Development (Secretaría de Agricultura y Desarrollo Rural - SADER); they guarantee products of optimum sanitary quality[10]. The RNC slaughterhouse is municipality-owned, slaughter animals for general use and must comply with federal regulation NOM-194-SSA-2004[11].

The present study objective was to isolate *Salmonella enterica* from pig carcasses processed in TIF or RNC slaughterhouses, identify the strains present and analyze their resistance to ten antimicrobials used in humans and animals.

Materials and methods

A total of 159 samples were collected from the surface of pig carcasses. Of these, 79 were from a RNC slaughterhouse in the central highlands of the state of Jalisco, and 80 were from a TIF slaughterhouse in the southern highlands of Jalisco. Between October 2013 and May 2014 eight samples from each slaughterhouse were processed every fifteen days. Samples were collected from the surface of carcasses selected randomly on each visit. A sterile sponge moistened with 10 ml buffered peptone water (APA, DB) was wiped over 100 cm^2 each of the belly, the ham and the jowl regions, resulting in a total sampled area of 300 cm^2 per carcass. After wiping, each sponge was placed in a sterile bag and placed in a cooler with refrigerants for transport to the Food Safety Laboratory of the University of Guadalajara, where they were analyzed according to the MLG 4.04 technique[12].

Isolation of *Salmonella* spp. from the collected sponges was done by adding 50 ml APA for a total volume of 60 ml. Each sample was homogenized in a peristaltic homogenizer (BagMixer®) for 1 min and incubated at 35 ± 2 °C for 20 to 24 h. From each homogenized sample a 0.1 ml subsample was transferred into 9.9 ml tetrathionate broth (TTB)(BD), and a 1 ml subsample was transferred into 9 ml modified Rappaport Vassiliadis broth (mRV)(BD). These selective broths were incubated at 42 ± 1 °C for 24 h. Sample

discrimination was done using polymerase chain reaction (PCR) to amplify the *invA* and *fimA* genes following previously described conditions[13]. After incubation, 500 µl TTB and 500 µl mRV were taken for DNA purification with the Wizard commercial kit (PROMEGA) following manufacturer instructions. Samples exhibiting *invA* and *fimA* amplification were subjected to selective isolation in brilliant green sulfa agar (BGS, BD) and xylose-lysine-tergitol agar (XLT4, DB). The BGS and XLT4 plates were incubated at 35 ± 2 °C for 24 to 48 h. Three colonies with typical *Salmonella* morphology were selected from each agar and analyzed biochemically on triple sugar and iron agar and on iron and lysine agar (BD) at 35 ± 2 °C for 24 h. Isolates exhibiting a typical *Salmonella* biochemical profile were confirmed by amplification of *invA* and *fimA* with PCR[13]. Fifty (50) strains with typical *Salmonella* morphology and biochemical profile confirmed via PCR were randomly selected assuming the presence of one presumptive positive strain per sample. The strains were sent to the Epidemiological Diagnosis and Reference Institute (Instituto de Diagnóstico y Referencia Epidemiológicos - InDRE) for confirmation and serotyping using the Kauffmann method[14]. The number of *Salmonella* isolates confirmed by InDRE in each slaughterhouse by month was compared with an analysis of variance (ANOVA) applied with the GraphPad Prism8 statistical program[15].

The susceptibility profile of the *Salmonella* isolates to ten antimicrobial agents was generated following the Kirby-Bauer technique, standardized by the Clinical and Laboratory Standards Institute[16]. The ten evaluated antimicrobials were ampicillin (AM, 10 µg); nalidixic acid (NA, 30 µg); cephalothin (CF, 30 µg); ceftriaxone (CRO, 30 µg); ciprofloxacin (CIP, 30 µg); chloramphenicol (C, 30 µg); streptomycin (S, 10 µg); gentamicin (GM, 10 µg); kanamycin (K, 30 µg); tetracycline (TE, 30 µg); and trimethoprim-sulfamethoxazole (SXT, 1.25 and 23.75 µg). Inhibition halo measurement (mm) was interpreted using CLSI tables[17], and *Escherichia coli* ATCC 25922 was used as a control.

Presence of the *tetA* and *tetB* resistance genes[18] was analyzed by PCR in all isolates exhibiting TE resistance. The same was done for integrases 1 (*intI1*) and 2 (*intI2*) in strains resistant to three or more antimicrobial classes[17,18].

Results and discussion

A *Salmonella* strain was isolated from 1.3% (1/80) of the TIF samples and 46.8% (37/79) of the RNC samples. The number of strains isolated differed ($P<0.05$) between the months of October, January and February, but not between March and April (Table 1). The sanitation measures applied in the TIF slaughterhouse apparently control the growth of *Salmonella* isolates in the carcasses processed there, where only pigs are processed. This

coincides with EFSA recommendations about that implementation of control measures in the slaughtering of pigs greatly reduces the presence of pathogenic bacteria associated with gastroenteritis in humans[4]. The high number of strains isolated from carcasses in the RNC slaughterhouse may be related to failures in the evisceration process. Carcass enterobacteria contamination increases during evisceration of pigs but differs between portions of the carcass[5]. Contamination increases in the anterior and ventral portions of the carcass when the tonsils are cut during viscera removal, but can decrease when the tools are sanitized after tonsil removal[5]. The contamination documented in the RNC slaughterhouse may also originate in cross contamination between the bacteria present in pigs and cattle, since both are processed in this slaughterhouse[19].

Table 1: Serotype, month isolated and resistance profile of 38 *Salmonella enterica* strains isolated from pork carcasses in two types of slaughterhouses

Type	Serotype	Month isolated	Resistance profile
TIF	Typhimurium	April	GM, K, S, C, NA, TE
RNC	Agona	February	K, S
		February	K
		February	K
	Anatum	January	K, NA, STX, TE
		October	K, SXT, TE
		January	K, NA, TE
		January	K, NA
		January	K, S
		January	K
	Bovismorbificans	October	K
	Bredeney	October	K, S, C, TE
	Derby	October	K
		October	K
	London	February	K, S, AM, C, TE
		February	K, S, NA, TE
		October	K, NA, TE
		October	K, NA, TE
		October	K, NA, TE
		January	K, NA, TE
		January	K, NA, TE
		January	K, NA, TE
		February	K, NA, TE
		February	K, NA, TE
		March	K, NA, TE
		October	K, TE
		January	K, TE
		January	K, TE
		January	K, TE
		February	K, TE
		February	K, TE

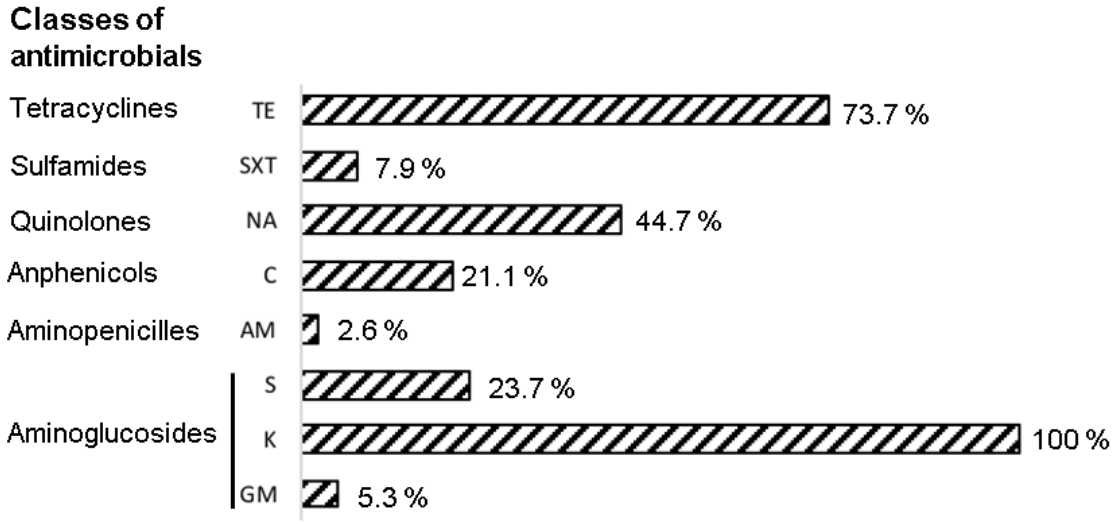
Montevideo	October	K
Muenchen	February	K, C, NA, TE
	January	K, S, C, TE
	January	K, NA, TE
Typhimurium	October	GM, K, S, C, NA, TE
	October	K, S, C, SXT, TE
Senftenberg	October	K, C, TE

TIF= Federal Inspection Type slaughterhouse; RNC= Uncertified municipal slaughterhouse; AM= ampicillin, C= chloramphenicol, GM= gentamicin, K= kanamycin, NA= nalidixic acid, S = streptomycin, TE= tetracycline, SXT= trimethoprim-sulfamethoxazole.

Ten *Salmonella* serotypes were isolated from carcasses in the RNC slaughterhouse: London (45.9 %, 17/37); Anatum (16.2 %, 6/37); Agona and Muenchen (8.1 % each, 3/37); Derby and Typhimurium (5.4 %, 2/37); and Bredeney, Bovismorficans, Montevideo and Senftenberg (2.7 % each, 1/37). Only one serotype (*S.* Typhimurium) was isolated from carcasses in the TIF slaughterhouse (Table 1). In previous reports the predominant serotypes of *Salmonella* isolates are diverse, depending on the year and place if isolation, as well as the type of food sampled. In samples from pig carcasses from the United States collected from 2003 to 2015, the five predominant serotypes were *S.* Typhimurium monophasic (4,(5),12:i:-), Infantis, Johannesburg, Typhimurium and Derby[20]. In Belgium, a study carried out between October 2015 and February 2016 identified *S.* Typhimurium, Derby, Livingstone, Rissen and Bredeney as the prevalent serotypes[5]. In contrast, a study done in Spain between November 2012 and May 2014, found *S.* Rissen, Typhimurium, Panama and Brandenburg[6]. A study done in China using samples collected from pig carcasses from October 2012 to July 2013 identified *S.* Saintpaul, Agona, Give and Corvallis, as well as Derby and Infantis to a lesser extent[21]. The *S.* Agona, Typhimurium and Derby serotypes isolated in the present study coincide with those recovered from pig carcasses in the aforementioned studies.

Study of *Salmonella* resistance to antimicrobials has been deemed of worldwide importance[2], making the resistance profiles produced in the present study of interest. The 38 strains isolated here (37 from RNC, and 1 from TIF) were challenged against ten antimicrobial agents. All 38 isolates were resistant to K, but susceptible to CIP and the two evaluated cephalosporins (CF and CRO); this highlights that 100 % were resistant to at least one class of antimicrobials. Resistance was also present in 73.7% (28/38) of the isolates against TE, 44.7 % against NA (17/38), 23.7 % against S (9/38), 21.1 % against C (8/38), 7.9 % against SXT (3/38), 5.3 % against GM (2/38) and 2.6 % against AM (1/38). The *S.* Derby, Bovismorficans and Montevideo isolates only exhibited resistance against K (Figure 1).

Figure 1: Percentage of *Salmonella enterica* strains isolated from pig carcasses exhibiting resistance to different antimicrobial classes



Some of the *S.* Agona (3), Anatum (4), Bovismorbificans (1), Derby (2), London (6) and Montevideo (1) isolates showed resistance to one and/or two classes of antimicrobials. In contrast, other *S.* Anatum (3), Bredeney (1), London (11), Muenchen (3), Typhimurium (3) and Senftenberg (1) isolates showed resistance to three and/or four classes (Table 2). Resistance was broadest against the aminoglycosides and tetracyclines classes, as well as ciprofloxacin (Figure 1). The NARMS has reported greater resistance to fluoroquinolones (CIP), third-generation cephalosporins (CF) and macrolides (azithromycin), which are frequently used in treating *S.* Typhi, Paratyphi and nontyphoidal *Salmonella* infections[3]. Isolates from pork and beef sold in stores in China, however, were resistant to tetracyclines, aminopenicillins (AM), sulfamides (SXT) and aminoglycosides (S)[21]. Antimicrobial resistance is difficult to compare between studies because it is influenced by the type of antimicrobials used in growing pigs in a given region[6], as well as by the presence of antimicrobial residues in water, farm soil and slaughterhouses where animals are grown or processed[2].

Table 2: Number of antimicrobial-resistant isolates within *Salmonella enterica* serotypes isolated from pig carcasses in two types of slaughterhouses

			No. Antimicrobial classes			
Serotype	**No.**	**%**	**1**	**2**	**3**	**4**
Agona	3	7.9	3			
Anatum	6	15.8	2	1	2	1
Bovismorbificans	1	2.6	1			
Bredeney	1	2.6			1	
Derby	2	5.3		2		
London	17	44.7		6	10	1
Montevideo	1	2.6	1			
Muenchen	3	7.9			2	1
Typhimurium	3	7.9				3
Senftenberg	1	2.6			1	
TOTAL	38	100	7	9	16	6

A majority (73.7 %, 28/38) of the isolates were resistant to tetracycline, therefore the presence of *tetA* and *tetB*, two genes that confer TE resistance, was tested by PCR[6]. However, neither gene was amplified from any of the TE-resistant isolates. Many (44.7 %) of the isolates were resistant to NA. Resistance to NA can reduce susceptibility to CIP, which is commonly used in treating salmonellosis[3]. No isolates were found to be resistant to AM-C-S-SXT-TE (ACSSuT phenotype) or AM-S-SXT-TE (ASSuT phenotype). Both are commonly sought in non-typhoidal *Salmonella* because they can cause complications in treatment of salmonellosis[3]. Although no *Salmonella* isolates with these specific phenotypes were identified in the present study, there were strains resistant to four antimicrobial classes, which can pose serious problems when treating salmonellosis in humans (Tables 1 and 2).

Resistance elements are acquired through horizontal transfer of genes or groups of genes and is mediated by mobile genetic elements (integrons, plasmids or transposons)[6,22]. Integrons are recombination systems consisting mainly of an integrase, a recombination site, and a strong promoter. Three integron classes are associated with resistance cassette acquisition in the Enterobacteriaceae, but Class 1 is associated with resistance to most of the known β-lactams, as well as aminoglycosides, trimethoprim, rifampicin, chloramphenicol, quinolones, erythromycin, and quaternary ammonium compounds[22]. Six of the isolates exhibiting resistance to three or four antimicrobial classes were selected for detection of class 1 and 2 integrons. The *intI1* gene (integron class 1) was amplified in four isolates: Anatum (1), London (1) and Typhimurium (2). Integron class 1 is one of the most frequently reported in isolates from resistant pathogens such as *Klebsiella*, *Salmonella*, *Shigella* and *Yersinia*[22]. No amplification band for the *intI1* or *intI2* genes

was observed in isolates from the Bredeny and Muenchen serotypes (Table 3), so their resistance may be due to a class 3 integron, or some other mobile element.

Table 3: Amplification of *intl1* and *intl2* genes in isolates of *Salmonella enterica* from pig carcasses in two types of slaughterhouses.

Source	Serotype	Antimicrobial resistence profile	*intI1*	*intI2*
TIF	Typhimurium	GM, K, S, C, NA, TE	+	-
RNC	Anatum	K, NA, SXT, TE	+	-
	Bredeney	K, S, C, TE	-	-
	London	K, S, AM, C, TE	+	-
	Muenchen	K, C, NA, TE	-	-
	Typhimurium	K, S, C, SXT, TE	+	-

TIF= Federal Inspection Type; RNC= Uncertified municipal slaughterhouse; AM= ampicillin, C= chloramphenicol, GM= gentamicin, K= kanamycin, NA= nalidixic acid, S= streptomycin, TE= tetracycline, SXT= trimethoprim-sulfamethoxazole.

Conclusions and implications

Salmonella strains resistant to different antimicrobial classes were identified in samples from pig carcasses. The majority were from samples taken in the RNC slaughterhouse. The presence of these multiresistant strains in pork intended for public consumption is a potential public health problem, because if the pork is not adequately prepared the ensuing *Salmonella* infections can be challenging to treat. The results also highlight the importance of implementing good practices and control measures during slaughter and processing to reduce the presence of *Salmonella* and the likelihood of disseminating mobile elements carrying resistance genes to other species of Enterobacteriaceae. Of note is that the present findings are limited in terms of sanitation evaluation and verification at each slaughterhouse. The statement that implementation of good practices reduces the presence of *Salmonella* is made based on previous studies[23]. The results are valid for the sampled slaughterhouses and do not constitute sufficient data to represent RNC and TIF slaughterhouses in the study area or Mexico as a whole.

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