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ORIGINAL ARTICLE

Phenotypic and genotypic profile of clinical and animal multidrug-resistant *Salmonella enterica* isolates from Mexico

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Abstract

Aims: The objective of this study was to obtain a phenotypic and genotypic profile of *Salmonella enterica* including multidrug-resistant (MDR) isolates from food-producing animals and clinical isolates, as well as their genetic relatedness in two different States of Mexico (Jalisco and State of Mexico).
Methods and Results: A total of 243 isolates were evaluated in terms of antimicrobial resistance (AMR) and related genes through a disk diffusion method and PCR respectively; we found 16 MDR isolates, all of them harbouring the bla_{CMY} gene but not *qnr* genes, these isolates represent less than 10% of the collection. The pulsed-field gel electrophoresis revealed a higher genotypic similitude within isolates of State of Mexico than Jalisco.
Conclusions: A low percentage of *Salmonella* isolates were resistant to relevant antibiotics in human health, nevertheless, the AMR and involved genes were similar despite the different serovars and origin of the isolates.
Significance and Impact of the Study: This investigation provided an insight of the current status of AMR of *Salmonella* isolates in two States of Mexico and pinpoint the genes involved in AMR and their epidemiological relationship, the information could help to determine an adequate therapy in human and veterinary medicine.

Introduction

Salmonella enterica is one of the most common food-borne pathogen worldwide and more than 2500 serovars are known, however, only some serovars are responsible for the majority of human salmonellosis cases, for example in the Europe Union *Salmonella* Enteritidis, Typhimurium and monophasic *Salmonella* Typhimurium 1,4, [5],12:i:-, have been the three most common serovars for several years representing 69·8%, of confirmed human cases with a known serovar in 2015, while in USA *Salmonella* Enteritidis, Typhimurium and Newport are the three most frequently reported with little variation since 2003 (Agbaje *et al.* 2011; CDC 2016; EFSA 2016). In Mexico, it has been previously reported from different sources including meat and vegetables (Miranda *et al.* 2009; Gómez-Aldapa *et al.* 2013). Also, the increase of antimicrobial resistance (AMR) and emergence of multidrug-resistant (MDR) isolates, mostly of *S. enterica* serovar Typhimurium, are frequent in clinical cases related to intestinal and extra-intestinal illness (Zaidi *et al.* 2007; Wiesner *et al.* 2016) which it is a public health concern.

The World Health Organization (WHO) has highlighted the overuse of antibiotics in food-producing animals which could generate MDR micro-organisms and their possible spread through the food supply. It also includes a ranked list of antibiotics according to their relevance to human health, some cephalosporins (β-lactams), quinolones and aminoglycosides are classified as 'critically' important antibiotics, in the particular

case of *Salmonella* extra-intestinal infections, the use of ceftriaxone and ciprofloxacin is an established therapy protocol, however some genetic mechanisms have favoured the presence of MDR isolates that could limit the use of these antibiotics (Collignon *et al.* 2009).

The quinolone resistance was known to develop through chromosomal mutations, nevertheless the plasmid mediated-quinolone resistance (PMQR) has emerged in recent years and include a set of different mechanisms: the *qnr* genes (*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*) that encode pentapeptide repeat proteins that bind to and protect type II DNA topoisomerases from inhibition by quinolones, the *aac*(6′)-*Ib-cr* (modified acetyltransferase) and the *qepA* (efflux pump) genes. In case of resistance to cephalosporins, β-lactamases constitute the main mechanism of resistance (enzymatic modification), different genes are implicated but the extended-spectrum β-lactamases (ESBL) that include the bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ genes and the AmpC β-lactamases (bla_{CMY} is one of the most common from this group) are considered of special relevance. In Mexico, these genes are present in Gram-negative micro-organisms isolated in the nosocomial environment, thus the antimicrobial therapy employed in case of an infection could be hindered and affect the outcome of the patients (Seiffert *et al.* 2013; Silva-Sánchez *et al.* 2013; Jacoby *et al.* 2014).

Although the presence of MDR *Salmonella* has been described in Mexico, there is yet a lack of information regarding their presence in food-producing animals, their AMR profile and epidemiological relatedness between isolates that could be a risk to human health. Therefore, the aim of this study was to obtain a phenotypic and genotypic profile of *Salmonella* including MDR isolates from different sources (food-producing animals and clinical isolates), as well as their genetic relatedness in two different States of Mexico.

Material and methods

Bacterial isolates

A total of 243 *S. enterica* isolates from two federal states (Jalisco and State of Mexico) were employed; the isolates were serotyped in the National Laboratory for Diagnosis and Epidemiological Reference (INDRE) (Mexico City, Mexico) following the Kauffmann–White–Le Minor scheme, the collection was obtained from ground beef (n = 135) (Cabrera-Diaz *et al.* 2013), human clinical samples (diarrhoea) (n = 35), swine (n = 53), poultry (n = 8) and bovine carcass (n = 12; Table 2). All isolates were retrieved from frozen stock cultures and grown in Brilliant-Green Agar plates and incubated at 37°C for 24 h, a colony with the typical morphology was selected and harvested in trypticase soy broth for further characterization.

Antimicrobial susceptibility testing

The test was carried out using the disk diffusion method on Mueller–Hinton agar, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2012). The following antimicrobials were used: ampicillin 10 μg (AMP), ceftazidime 30 μg (CAZ), cefotaxime 30 μg (CTX), cefoxitin (FOX), nalidixic acid 30 μg (NAL), ciprofloxacin 5 μg (CIP), tetracycline (TET), gentamicin (GEN) and amikacin (AK) (Becton Dickinson, Sparks, MD, USA). The ATCC 25922 *Escherichia coli* strain was used as quality control micro-organism. Isolates resistant to β-lactam antimicrobials were subjected to ESBL confirmatory test using cefotaxime and ceftazidime on their own and in association with 10 μg clavulanic acid (CLA). All isolates were classified as resistant, intermediate or susceptible as described previously (CLSI 2015). Isolates were considered as MDR when resistance to three or more classes of antimicrobial agents was observed as proposed previously (Magiorakos *et al.* 2011).

Detection of antimicrobial resistance genes

All MDR isolates were subjected to PCR in order to detect the presence of bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{CMY}, *qnrA*, *qnrB* and *qnrS* genes with published primers (Cattoir *et al.* 2007; Dallenne *et al.* 2010). A positive control DNA from previously characterized *E. coli* isolates (bla_{TEM}+, bla_{CMY}+, *qnrA*+, *qnrB*+) (Aguilar-Montes de Oca *et al.* 2015) and a negative control DNA from *Salmonella* Typhimurium (ATCC 14028) were used.

Pulsed-field gel electrophoresis

The clonal relationship of MDR isolates of *Salmonella* was carried out using XbaI restriction enzyme in agarose following the standardized protocol of the CDC, the *Salmonella* Branderup H9812 was used as the reference strain (Ribot *et al.* 2006). Electrophoresis was carried out using a CHEF-DRII chamber and band patterns were analysed using the NTSYSpc ver. 2.2 software, relationships among genotypes were determined using the unweighted pair group method with arithmetic mean (UPGMA) and band similitude was calculated using Dice's coefficient.

Results

Bacterial isolates

A total of 34 serovars and 10 serogroups (partial identification) were distributed among the *Salmonella* isolates. The most frequent serovars were *Salmonella* Typhimurium (17·7%), followed by *Salmonella* Anatum (7%),

Salmonella Agona (5·8%), *Salmonella* Infantis (5·8%), *Salmonella* London (5·8%) and *Salmonella* Group B (5·3%) (Table 1). In virtue of sample kind, *Salmonella* was detected in 208 (14·8%) of the tested samples. Recovery was highest in ground beef (56·7%), followed by swine (16·5%), cattle (3·6%) and poultry carcasses (1·5%). Human samples were not included as they were granted by Dr. Jeannette Barba-León (clinical sampling was not performed as a result no prevalence could be calculated (Table 2).

Antimicrobial resistance

A total of 243 isolates were screened. The highest resistant frequency was TET (41·6%), followed by AMP (24·7%), NAL (21·8%), GEN (9%), CTX, CAZ and FOX (6·6% each), moreover the lowest resistant was observed to AK (0·8%). In case of CIP, only intermediate susceptibility was found (1·2%) (Figure 1). Further characterization was applied to 16 MDR isolates that showed phenotypic resistance to β-lactam antibiotics (specifically to cephalosporins CTX, CAZ and FOX). The ESBL test showed a different profile from the one established by CLSI, since they were resistant to CAZ, CTX and their combination with CLA. A total of 10 MDR patterns were identified among them, the predominant pattern was CTX-CAZ-FOX-AMP-NA-GEN-TET (Table 3).

Detection of antimicrobial resistance genes

The MDR isolates were screened for different β-lactamases families and *qnr* genes, the CMY-2 group was detected through all isolates, also in three isolates the co-presence with *bla*TEM genes was found. None of the isolates was positive for *bla*SHV, *bla*OXA, *bla*FOX and *qnr* genes (Table 3).

Pulsed-field gel electrophoresis

The 16 MDR isolates were selected to study their clonal relationship using pulsed field gel electrophoresis (PFGE). The constructed dendogram shows the different isolates and some characteristics of these (Figure 2).

Discussion

In Mexico, the presence of *S. enterica* in different food continues to be a Public health concern, in case of meat products the exposition′s risk to *Salmonella* is high if we consider that livestock animals are reservoirs of foodborne pathogens and the carcass can be contaminated during the harvest (Gonzales-Barron *et al.* 2014).

Another complication is the possible cross-contamination with other raw meats (chicken, pork) or deficient sanitation of equipment, utensils, food handlers and storage at butcher′s shops or supermarkets it should be taken into account (Carrasco *et al.* 2012).

The information published in Mexico regarding the presence of *S. enterica* is limited and in general, there are

Table 1 *Salmonella* isolates employed in this study (n = 243)

Salmonella serovars	No. (%) of isolates	Origin
Adelaide	1 (0·4)	GB
Agona	14 (5·8)	GB, CL, SW
Albany	3 (1·2)	GB, CL
Anatum	17 (7·0)	GB, CL, SW
Azteca	2 (0·8)	GB, CL
Braenderup	2 (0·8)	GB
Brandenburg	4 (1·6)	GB
Bredeney	7 (2·9)	GB, CL, SW
Cannstatt	1 (0·4)	GB
Derby	7 (2·9)	GB
Duesseldorf	1 (0·4)	CL
Enteritidis	6 (2·5)	CL, SW
Give	4 (1·6)	GB
Havana	8 (3·3)	GB
Infantis	14 (5·8)	GB, CL, SW
Javiana	1 (0·4)	CL
Kentucky	2 (0·8)	GB
Lockleaze	2 (0·4)	GB
London	14 (5·8)	CL, SW
Manhattan	1 (0·4)	CL
Montevideo	2 (0·8)	GB, CL
Muenchen	6 (2·5)	GB, CL
Muenster	1 (0·4)	GB
Panama	5 (2·1)	GB
Reading	2 (0·8)	GB, SW
Rissen	4 (1·6)	GB
Saintpaul	5 (2·1)	GB, CL
Senftenberg	3 (1·2)	SW
Sinstorf	6 (2·5)	GB
Tennesse	1 (0·4)	SW
Typhi	3 (1·2)	BV
Typhimurium	43 (17·7)	GB, CL, SW, BV, PY
Urbana	1 (0·4)	CL
Worthington	2 (0·8)	GB
Partially serotyped		
Group B	13 (5·3)	GB
Group B monophasic	2 (0·8)	SW
Group C1	3 (1·2)	GB, CL
Group D	3 (1·2)	CL
Group E1	6 (2·5)	GB
Group E1 monophasic	8 (3·3)	GB
Group G1	1 (0·4)	GB
Group G2	2 (0·8)	GB
Group G2 monophasic	1 (0·4)	GB
G18	1 (0·4)	GB
NT	8 (3·3)	GB, SW, BV, CL
Total	243 (100)	

GB, ground beef; CL, clinical isolates; SW, swine; BV, bovine; PY, poultry; NT, nontypeable.

Table 2 Distribution of *Salmonella* isolates

Sample type	Sample size	Positive samples (%)
Swine	320	53 (16·6)
Bovine	327	12 (3·7)
Ground beef	238	135 (56·7)
Poultry	520	8 (1·5)
Total	1405	208 (14·8)

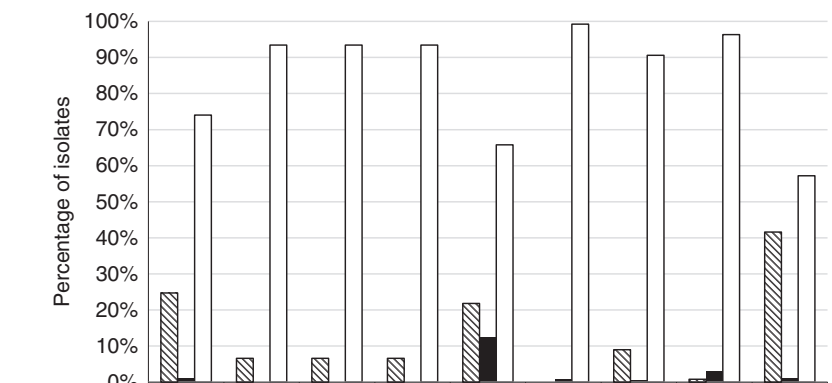
more data on beef compared with pork or poultry meat. We found an overall of 14·8% of *Salmonella* in different type of samples, a study carried out in another federal state of Mexico found 21·1% (from 441 samples) of *Salmonella*-positive isolates in a wide variety of raw food samples from supermarkets and retail stores, the investigation included meat from different species, no positive samples were obtained from fish, followed by beef (15·1%), pork (17·3%) and the highest frequency for chicken meat (35·3%) (Miranda *et al.* 2009), this contrasts with our study, where *Salmonella* was most frequent in ground beef (56·7%), the difference suggests regional variations in the distribution, however comparison among studies should be done cautiously due to disparity in sample type, as well as, sampling and isolation procedures; more comparative studies across the country are necessary to ensure differences or similarities in different areas of Mexico.

In the particular case of cattle, the study realized by Narvaez-Bravo *et al.* (2013) found 52·5% of *Salmonella* isolates (from 1695 samples) in cattle carcass, the major serovars found were *Salmonella* Anatum, Montevideo, Kentucky, Muenster, Give, Reading, Tennessee, Mbandaka, Meleagridis, and Fresno, curiously the first six serovars were also found in samples of ground beef in the present study but not in bovine carcass. Unfortunately, the research did not include the AMR profile for comparative purposes.

In the current study, *Salmonella* Typhimurium was the most frequent serotype (17·7%) (Table 1), this result agrees with previous publications where this serotype it is one of the main agents of several outbreaks and in some cases, MDR isolates have been implicated (Laufer *et al.* 2014; Andino and Hanning 2015). The dissemination of this serotype could be explained by the complex repertoire of genes involved in the colonization of their host and some of them are essential for infection across different food-producing animals, followed by colonization of human gut and finally develop the fitness of particular strains which allow them to be predominant in a geographical zone, for example the emerging of *Salmonella* Typhimurium genotype ST213 in Mexico (Chaudhuri *et al.* 2013; Wiesner *et al.* 2016).

An interesting finding was the presence of *Salmonella* Typhi in three samples of cattle carcass, a host-restricted pathogen and the causative agent of typhoid fever in humans, it′s probably that the meat could be contaminated by food handlers, since thyphoidal *Salmonella* is related to asymptomatic carriers and poor hygiene while handling food (Gebreyesus *et al.* 2014).

In general, little information has been published concerning the presence of *S. enterica* in beef meat or other farm animals in Mexico and most research published only include information locally and are not realized continuously through the years, hence is difficult to ascertain an overview of distribution and frequency of this pathogen. This information is necessary in order to establish measures to control the spread of *Salmonella* and cross-contamination as well as know the success or failure of these approaches; a national surveillance system like the Food-borne Diseases Active Surveillance Network



	AMP	FOX	CAZ	CTX	NAL	CIP	GEN	AK	TET
▧ Resistant	24·7%	6·6%	6·6%	6·6%	21·8%	0	9·0%	0·8%	41·6%
■ Intermediate	1·2%	0	0	0	12·4%	0·8%	0·4%	2·9%	1·2%
□ Susceptible	74·0%	93·4%	93·4%	93·4%	65·8%	99·2%	90·6%	96·3%	57·2%

Figure 1 Frequency of antimicrobial resistance in *Salmonella enterica* (n = 243) isolates from Mexico. Antimicrobial agents: AMP, ampicillin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; AK, amikacin.

Table 3 MDR profile of *Salmonella* serovars (n = 16)

Salmonella serotypes	Number of isolates	Origin	Resistance phenotypes	Resistance genotypes
Typhimurium	3	PY	CTX-CAZ-FOX-AMP-NA-GEN-TET	bla_{CMY}, bla_{TEM}*
Bredeney	1	SW		
NT	1	SW		
Typhimurium	1	GB	CTX-CAZ-FOX-AMP-NA-TET	bla_{CMY}
Typhimurium	1	SW	CTX-CAZ-FOX-AMP-TET	bla_{CMY}
Typhimurium	2	SW	CTX-CAZ-FOX-AMP- GEN-TET	bla_{CMY}
London	1	SW		
Infantis	1	CL	CTX-CAZ-FOX-AMP-NA	bla_{CMY}
Typhimurium	1	CL	CTX-CAZ-AMP-NA-GEN-AK†-TET	bla_{CMY}
Typhimurium	1	CL	CTX-CAZ-FOX-AMP-NA-CIP†-GE-TET	bla_{CMY}
Urbana	1	CL	CTX-CAZ-AMP-AK	bla_{CMY}
NT	1	CL	CTX-CAZ-FOX-AMP-NA-CIP†	bla_{CMY}
Group B	1	GB	CTX-CAZ-FOX-AMP-NA-GEN-AK†-TET	bla_{CMY}

NT, nontypeable.
*Only the three isolates of *Salmonella* Typhimurium.
†Intermediate resistance.

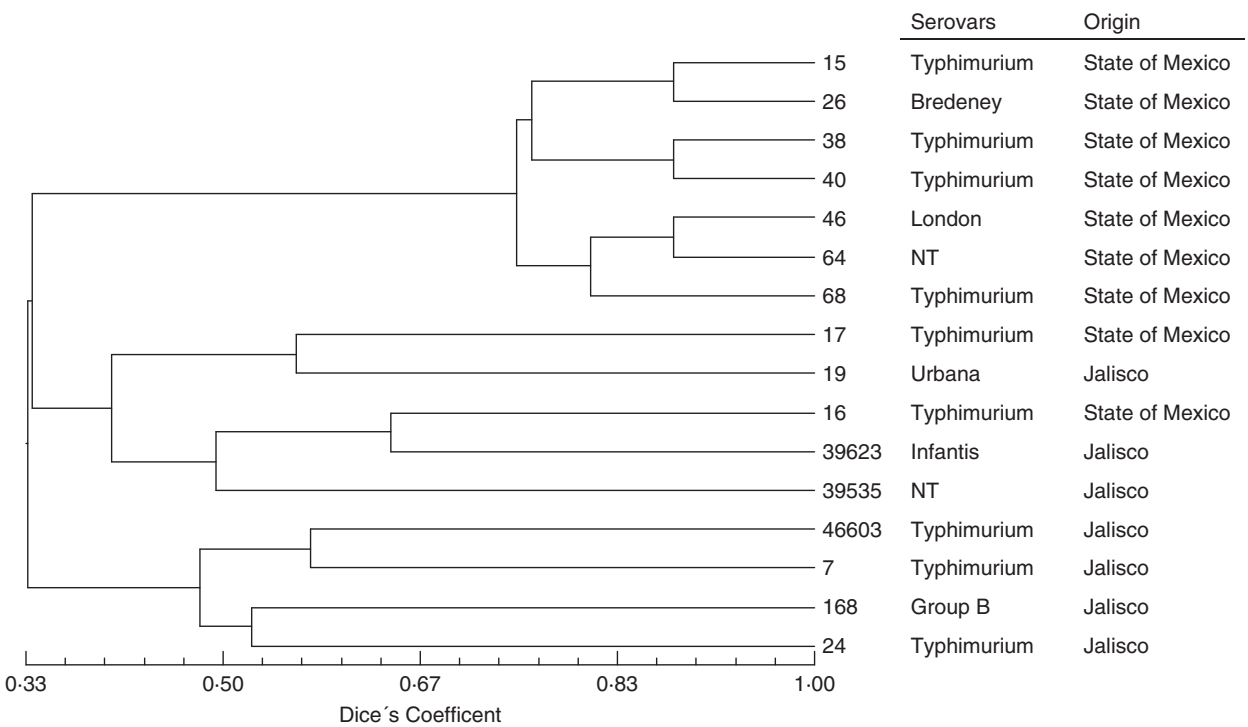


Figure 2 Dendogram representing genetic relationships among *Salmonella enterica* isolates.

(FoodNet, GA, USA) could be adequate to accomplish this.

The third generation cephalosporins, aminoglycosides and quinolones included in this study has been categorized as 'critically' important in human medicine by the WHO (Collignon *et al.* 2009), our finding of an *in vitro* susceptibility above 90% for these antibiotics (except NAL) indicates still an acceptable effectiveness, however, is very likely that the percentage of AMR could increase steadily due to mobile genetic elements like plasmids, transposons or integrons and subsequently their dissemination through animal-origin food, this scenario is observed in other countries, especially from Asia, where *S. enterica* isolates from pork and chicken meat have a

higher resistant rate for all the antibiotic tested in comparison with our work (up to 39% for CIP, 10% for CTX and 40% for GEN) (Lin *et al.* 2015).

The use of antimicrobial agents in food-producing animals as growth promoters or veterinary therapy is reported in countries from the Europe Union or from USA, albeit the majority of countries either do not collect or do not release data on veterinary antimicrobial consumption (FDA 2015; EMA 2016). In Mexico, some cephalosporins (ceftriaxone), aminoglycosides (amikacin, gentamicin) and quinolones (ciprofloxacin, enrofloxacin) that are relevant in human medicine are approved to be used in food-producing animals by the government agency (NOM-040-Z00-1995 1996), however, an official document related to the sales of veterinary antimicrobials is not published, nevertheless a study estimated that Mexico was the top five country with the largest shares of global antimicrobial consumption in food animal production in 2010 (Boeckel *et al.* 2015). A detailed summary of specific antibiotics and their consumption per year in the principal food-producing animals (swine, poultry and cattle) is necessary to assess a correlation between the use of antimicrobials and the presence of AMR as this has been observed previously (Chantziaras *et al.* 2014).

Nowadays, ceftriaxone, ciprofloxacin and azithromycin are the antibiotics of choice for invasive *Salmonella* infections, our finding of practically nonexistent CIP resistance, implies that this antimicrobial agent could be adequate when dealing with infections (Wong *et al.* 2014). In contrast, a study employed 112 ESBL-producing micro-organisms (*Klebsiella pneumoniae*, *E. coli* and *Enterobacter cloacae*) also found PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac*(6′)-*Ib-cr* and *qepA*) in 36 of them (Silva-Sánchez *et al.* 2013). The co-presence of ESBL and PMQR genes in clinical isolates could lead to failure of antimicrobial therapy, furthermore, data regarding the susceptibility to azithromycin are lacking and should be considered in future investigations to determine his actual utility in Mexico.

The bla_{CMY} gene had been described to be widespread in this country (Zaidi *et al.* 2007), in this work it was present in poultry, swine, ground beef and clinical MDR isolates, supporting this statement. Additionally, we found this gene present in *Salmonella* Bredeney, Infantis, London, Urbana and *Salmonella* Group B (Table 3). This could indicate the dissemination through mobile genetic elements between different *Salmonella* serotypes, although in less quantity than *Salmonella* Typhimurium.

In case of AMR well-known surveillance programs like the National Antimicrobial Resistance Monitoring System (NARMS, USA), the Japanese Veterinary Antimicrobial Resistance Monitoring System (JVARMS, Japan) for the presence of food-borne pathogens and AMR could give a general view of the status of this and others food-borne pathogens. In Mexico′s case, the national and international information network has made more progress in comparison of the investigation of food-borne pathogens however, the bulk of information embraces nosocomial pathogens from ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) and set aside food-borne micro-organisms like *E. coli* and *Salmonella*. An exception is the Integrated food chain surveillance system (IFCSs) established in four states of Mexico (Zaidi *et al.* 2008), which is focused in *S. enterica* from human and animal origin and includes information on both, contamination of food and AMR status.

The PFGE profile generated clades with an evident differentiation between isolates from State of Mexico and Jalisco, the clade from State of Mexico showed a major relatedness, even two isolates showed a similarity of 100%, which could indicate clonal relationship, while the isolates from Jalisco clustered in another group with more distant relatedness between them suggesting major diversity of genotypes from ground beef and clinical isolates. Due to the amount of analysed isolates was low (16), we could not establish a linkage between animal and human isolates and the possibility should not be discarded. Establishment of causal relationships in these cases will require the use of additional subtyping methods such as MLVA.

We determinated the AMR profile of 34 serotypes and 10 serogroups present in different kind of samples from two States of Mexico and found high levels of resistance to TET, AMP and NAL, in contrast, the resistance to 'critically' important antibiotics like cephalosporins, CIP and GE were lower than 10%, moreover, the principal mechanism of resistance to cephalosporins was bla_{CMY} and could represent a risk against therapy with these kind of antibiotics; while non *qnr* genes were found. The PFGE profile showed a closest relationship in isolates from the State of Mexico than Jalisco and could be related to farming and production practices, however mobile genetic elements could play an important role in the transmission of resistance genes to others *Salmonella* serovars, a continuous surveillance is necessary to monitor the AMR and, if possible take control measures in the use of antibiotics in food-producing animals to ensure a low transmission of resistance genes.

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Conflict of Interest

No conflict of interest is declared by the authors.

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