Salmonella spp. prevalence, antimicrobial resistance and risk factor determination in Colombian swine farms

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To determine Salmonella spp. prevalence/seroprevalence, antimicrobial resistance patterns and risk factor identification associated with its presence in Colombian swine farms. 504 samples (Faeces, swabs and environment samples) were obtained from 21 farms distributed in four geographical regions in Colombia. Salmonella spp. microbiological and molecular detection were determined by two Salmonella spp. MDS3M™ and MALDI-TOF MS assays, respectively. In addition, for serological evaluation 231 serum samples were analyzed employing ELISA Salmonella Pigtype™-Salmonella Ab (QUIAGEN®). Additionally, 41 isolates were tested for antimicrobial susceptibility using broth microdilution technique (Panel B1016-180 Beckman Coulter NC72™) and verified with WHONET 2016 software. Risk factors were assessed from a survey and analyzed for statistical significance by U Mann-Whitney test. An 8.9% prevalence (n=45) and 38.1% (n=88) seroprevalence were determined. All isolates presented 100% antimicrobial susceptibility against amikacin. However, resistance against penicillin, tetracycline, cefuroxime and trimethoprim/sulfamethoxazole was present in more than 50% of evaluated strains. Risk factors associated with Salmonella spp. presence were surface water use, rough-surfaced on floors, presence of hoppers as feeders and worker's boots. Bacteria were present in animals and environmental samples from evaluated farms. Animal contact and/or exposure with the microorganism were also evident in obtained serological response. Bacteria presence depended on management practices and infrastructure, likewise antibiotic use, supplemented in the diet may have induced an increase in Salmonella spp. antimicrobial resistance.

INDEX TERMS: Salmonella spp., antimicrobial resistance, risk factors, swine farm, prevalence, seroprevalence, susceptibility test, pigs, Colombia.
MDS3M™ e MALDI-TOF MS, respectively. Além disso, para avaliação sorológica, foram analisadas 231 amostras de soro empregando ELISA Salmonella Pigtype® - Salmonella Ab (QUIAGEN®). Além disso, 41 isolados foram testados quanto à suscetibilidade antimicrobiana usando a técnica de microdiluição em caldo (Painel B 1016-180 Beckman Coulter NC72™) e verificados com o software WHONET 2016. Os fatores de risco foram avaliados em uma pesquisa e analisados quanto à significância estatística pelo teste U Mann-Whitney. Foram determinadas prevalências de 8,9% (n=45) e 38,1% (n=88). Todos os isolados apresentaram 100% de suscetibilidade antimicrobiana à amicacina. No entanto, resistência à penicilina, tetraciclina, cefuroxima e trimetoprim/sulfametoxazol estava presente em mais de 50% das cepas avaliadas. Fatores de risco associados à Salmonella spp., presença de uso de água de superfície, superfície áspera no chão, presença de tremoanas como alimentadores e botas de trabalho. Bactérias estavam presentes em animais e amostras ambientais de fazendas avaliadas. O contato animal e/ou a exposição ao microrganismo também foram evidentes na resposta sorológica obtida. A presença de bactérias dependia de práticas de manejo e infraestrutura, assim como o uso de antibióticos suplementados na dieta pode ter induzido um aumento de Salmonella spp. resistência antimicrobiana.

**INTRODUCTION**

Swine production is a growing industry in Colombia. It is reflected by the 1,064,555 heads of pigs sacrificed during the third trimester of 2017 (DANE 2018). Additionally, consumption of pork meat "per capita" is estimated at 9.3kg/year (Porkcolombia 2018). Moreover, swine population increased to 5,327,460 animals, mainly located in the departments of Antioquia (34.53%), Cundinamarca (9.24%), Córdoba (6.9%), Valle del Cauca (5.82%) and Meta (4.19%) (ICA 2017).

Swine production is likely affected by different pathogens, such as viruses, parasites and bacteria. Pathogens generate serious health problems and economic losses resulting from growth rate variation, decrease in weight gain, meat quality alteration, and occasional animal death. In addition, veterinary medical care costs, required diagnosis tests and treatment costs (Hurd et al. 2002).

Pathogen presence may represent a public health risk, since some cause zoonoses. Additionally, certain pathogens can increase human mortality rate, where Salmonella spp. is the main microorganism related with this problem (Baer et al. 2013). The United States presents approximately 80.3 million cases of salmonellosis per year, resulting in 150,000 deaths approximately (Majowicz et al. 2010). On the other hand, the European Food Safety Authority (EFSA) reported that in Europe 8.9% of salmonellosis cases were attributed to pork consumption (EFSA 2016). In Colombia, from 2003 the "Instituto Nacional de Salud" (INS) obtained 7,424 Salmonella spp., isolates from human clinical samples (97.2%) and water and food samples (2.8%), none the less the exact source of origin was not specified (INS 2011).

Salmonella spp. is a pathogen that has the ability to persist in the environment for long periods of time, causing asymptomatic infections. Therefore, infected animals can be a source of contamination for the healthy population of the herd and even in slaughterhouses (Martín-Peláez et al. 2010, Baer et al. 2013). Then, one strategy is to interrupt dissemination, using prevention and control measures, since the first source of infection is the farm (EFSA 2006). Furthermore, frequently microorganisms express resistance to antibiotics, specifically Salmonella spp. strains isolated from the animal productive chain (Gomes-Neves et al. 2014).

In Colombia, Salmonella spp. presence has been shown in porcine meat juice; in slaughterhouse in Bogotá, with a 27.2% prevalence, whereas in the department of Tolima reported pork meat prevalence has been of 4.3% (Mora 2003, Ávila et al. 2013). Previous findings by GBAI research group to antibiotics, specifically Salmonella spp. strains isolated from human clinical samples (97.2%) and water and food samples (2.8%), none the less the exact source of origin was not specified (INS 2011).

**MATERIALS AND METHODS**

Animal care and welfare. This study was performed under field conditions in commercial farms. Its housing and care were adequate. This project was approved by the institutional animal care Document No.C-077-16 (FUA 038-16) in "Pontificia Universidad Javeriana".

Pig farms and population of study. According to the 2016 porcine census farms were sampled for breeding (19%) and full production cycle (replacement-females, pregnant sows, lactating sows, weaning, nursery, farrowing, grow finish or fattening pigs, and boars) located in areas of highest porcine production in the country Antioquia, Valle del Cauca, Eje Cafetero and Cundinamarca-Meta (Table 1).

**Questionnaire survey.** This study applied a survey to assess each farm on general farm conditions, pens, biosecurity measures,
sanitary and environmental aspects, animal care, pig feeding and worker’s occupational health among other aspects.

Population of study and sample size. Sampling was carried-out in a stratified manner and by proportional sampling for participating departments, using program sample size 1.5, with an estimated prevalence of 50%. Sample size was 395, however, 504 samples were obtained, after determining ideal sample size. Additionally, four farms volunteered to participate in the project. Therefore, a total of 21 farms enrolled in the study. The Table 2 details the type of samples obtained. Samples were obtained by veterinarian, following biosecurity measures and guidelines established by the world organization for animal health manual regarding land animals (chapter 1.1.1, section A, numeral 1). Also, packing and transport recommendation was followed as described in section D chapter 1.1.1 of the same manual (OE 2004).

Fecal samples in pens. Five pens housing growing finish pigs were selected at random and feces were collected to pool approximately 25g of feces (Rajic et al. 2005).

Rectal swabbing. Obtained from pigs at different stages of production through the use of culturettes (Transystem®) with Clary-Blair transport medium (Wilkins et al. 2010).

Environmental swabbing. For each farm samples were obtained from a sponge, previously dampened in 15mL buffered peptone water (BPW) and rubbed over surfaces to obtain the sample (workers boots, pig pen drains and empty pens after cleaning and disinfection processes). In addition, approximately 500mL of water supply system was collected (Wilkins et al. 2010).

Total blood. Jugular vein blood samples were collected from animals at different phases of their productive cycle, using vacutainer system in tubes without anticoagulant (PuthVacumine®) (van der Wolf et al. 1999).

Sample processing. For Salmonella spp. isolation and identification, samples were microbiologically enriched in BPW. A 1/10 BPW dilution was performed for subsequent molecular detection.

Fecal samples in pens. 10g sample was weighed and 90mL BPW was added. Sample was incubated at 35°C for 18 to 24 hours (Wilkins et al. 2010).

Rectal swabbing. Initial non-selective processed in 5mL of BPW and incubated at 35°C for 18 to 24 hours.

Environmental swabbing. BPW damped sponges rubbed against surfaces (workers boots, empty pens and pig pen drains) were initially nonselectively pre-enriched in 60mL BPW and incubated at 35°C for 18 to 24 hours. Collected watersamples were processed according to the American Public Health Association (APHA) protocol section 9260b. As previously indicated, microbiological enrichment processed molecular detection using Molecular detection assay 2 Salmonella spp. (MDS™).

Serology. Collected blood samples were centrifuged at 3,000rpm for 5min to obtain serum. Serum were processed using indirect ELISA employing the Salmonella kit Pigtype®-Salmonella Ab (QUIAGEN®) following manufacturer’s instructions.

Identification. Positive samples obtained from the molecular detection system were recovered in agar Chromagar® Salmonella. Identification was confirmed by MALDI-TOF MS methodology (Bruker Daltonics).

Antimicrobial susceptibility testing. Taking into account the microorganism’s zoonotic potential, antimicrobial susceptibility was evaluated against antibiotics used in human therapy. To this end, broth microdilution technique was used using Panel B1016-180 (Beckman Coulter, Negative Combo 72, NC72), as recommended by the Clinical and Laboratory Standards Institute (CLSI) M100-S27 (CLSI 2017). For data analysis Who-Net 2016 program was used.

Data analysis. Data was analyzed using descriptive statistics. Serological tests were performed following manufacturer’s indications. Asample was considered positive when CP OD ≥0.7 and M/P Relation ≥0.3. To establish the relationship between prevalence data and identified determinants factors in the questionnaire, a Mann-Whitney U Test was carried-out using SPSS® software (IBM Company). Additionally, susceptibility tests were interpreted based on cut-off points by Who-Net program (Table 3).

RESULTS

Of the 504 analyzed samples 8.9% (n=45) were positive for Salmonella spp., as assessed by microbiological isolation and molecular identification. From the 231 serums evaluated a general seroprevalence of 38.1% (n=89) was evident. Prevalence

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Table 2. Type of sample, sample quantity and type of analysis

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Sample quantity</th>
<th>Type of analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal samples in pens</td>
<td>5</td>
<td>Detection</td>
</tr>
<tr>
<td>Rectal swabbing</td>
<td>11</td>
<td>Microbiological/molecular (n=504) and Susceptibility test (n=41)</td>
</tr>
<tr>
<td>Empty pen swabbing</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Worker boots swabbing</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pig pen drain swabbing</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1 1 (500ml collecting area)</td>
<td></td>
</tr>
<tr>
<td>Total blood</td>
<td>1 1 (500ml feed water system)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Serology (ELISA) (n=231)</td>
</tr>
</tbody>
</table>

Table 3. Cut off points detected in antimicrobial susceptibility tests to cefotaxime (CTX), ampicillin (AMP), ciprofloxacin (CIP), colistin (COL) and trimethoprim/sulfamethoxazole (SXT)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Cut off points</th>
<th>%R</th>
<th>%I</th>
<th>%S</th>
<th>%R 95%L.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX</td>
<td>≤1 ≥8</td>
<td>5.6</td>
<td>25</td>
<td>69.4</td>
<td>1.0 - 20.1</td>
</tr>
<tr>
<td>COL</td>
<td>≤2 ≥8</td>
<td>27.8</td>
<td>8.3</td>
<td>63.9</td>
<td>14.8 - 45.5</td>
</tr>
<tr>
<td>AMP</td>
<td>≤8 ≥32</td>
<td>33.3</td>
<td>0</td>
<td>66.7</td>
<td>19.1 - 51.0</td>
</tr>
<tr>
<td>CIP</td>
<td>≤0.64 ≥1</td>
<td>11.1</td>
<td>5.6</td>
<td>83.3</td>
<td>3.6 - 27.0</td>
</tr>
<tr>
<td>SXT</td>
<td>≤2 ≥4</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>33.2 - 66.8</td>
</tr>
</tbody>
</table>
and seroprevalence for each region analyzed was determined (Fig.1), as well as for each productive stage (Fig.2).

According to sample type, total prevalence in feces was 7.6% (8/105). The lowest prevalence observed was from rectal swabbing with 8.7% (8/231), followed by its presence in workers boots (9.5%) and pig pen drains (11.9%) (5/42). The highest observed prevalence came from water samples 14.3% (3/21).

Positive samples, 45 in total were confirmed by molecular system detection, however the microbiological recovery was possible in 41 samples (91%). From these, antimicrobial susceptibility tests showed that 100% (41) of the strains were susceptible to amikacin, while 94.4% (39) were resistant to penicillin (P4), 94.4% to tetracycline (39), followed by 87.8% to cefuroxime (36), 52.8% to cephalothin (21), 48.7% (20) to trimethoprim/sulfamethoxazole and 11.2% to ciprofloxacin (5). Of the positive strains 12% displayed multi-resistance, i.e. resistance to at least minimum four antibiotics.

Risk factors in the farm associated with presence of Salmonella spp. were: the source of water (surface water type) (P<.005), type of floor as a porous surface in pig pens (P<.030), hopper type of feeder (P<.005) and workers boots type) (P<.005), type of floor as a porous surface in pig pens (P<.005). Of the positive strains 12% displayed multi-resistance, i.e. resistance to at least minimum four antibiotics.

According to Funk et al. (2001) the productive stage influences pathogen presence variation. It has been reported that Salmonella spp. prevalence increases along with the growth stages, until reaching the fattening (grow-finish) stage (Funk et al. 2001). Several reports revealed a higher prevalence (57%) during fattening; however, results obtained in this study did not detect bacteria presence in this stage (Korsak et al. 2003, Dorr et al. 2009). Prevalence in pregnant sows and piglets was 7% and 4%, respectively. Different authors agree that pregnant sows are often more vulnerable to infection compared with piglets (Wilkins et al. 2010). Wilkins et al. (2010) reported prevalence differences between pregnant sows and piglets, where 59% pregnant females were positive, while only 32% were positive; data differing from the observed in this work for the two age groups. Nevertheless, it is important to take into account sample size difference, and the type of farms analyzed. It should be noted that although in some stages of the productive cycle isolates were not achieved, presence of serologically detected antibodies suggest females could be in contact with the microorganism at some point of the cycle. Nevertheless, it might be indicative of effective control strategies implemented in evaluated farms at the particular stage of the cycle.

Differences in prevalence values of each stage of production might be justified by multiple variables, such as the geographical region where sampling took place, automation degree, production system and management practices in each farm (Baer et al. 2013). The most common implemented breeding management practices for swine production farms are All-In/All-Out (AIAO) system and Three-site swine production system (Dors et al. 2015). The first practice is used in Colombia, specifically in the farms analyzed. Thus, in agreement with Dors et al., implementation of this pig farm management method is a factor reducing Salmonella spp. presence (Dors et al. 2015).

With regards to general seroprevalence determined in the present work (38.1%) it was different from that reported in Mexico, Italy, USA and Spain, where lower seroprevalences were determined (28.7%, 19.3%, 5% and 4%, respectively) (Vicente et al. 2002, Montagnaro et al. 2010, Thakur et al. 2011, Pérez-Rivera et al. 2017). Nevertheless, for Cundinamarca-Meta, seroprevalence was 36.4%, a value close to 40% the seroprevalence reported by Pulido-Villamarín et al. (2016). In relation to results obtained for each stage group, through microscopic agglutination test (MAT) in South Korea a seropositivity of 46.7% was detected in females, 6.7% on farrowing and 3% in nursery (Vicente et al. 2002), similar values were observed in different female groups in this work. Nevertheless, this was not the case for nursery animals and...
farrowing, where very dissimilar values were observed compared with those reported by the Korean report. This may be related with differences in biosecurity measures and management for each country, given sociocultural, economic and even environmental conditions specific to each one, as well as the methodologies used in each work.

In relation to the clinical samples, 7.6% prevalence was observed for fecal samples and 8.7% from rectal swabbing samples. Data was in agreement with reports in Canada, where a similar work detected the pathogen on the same type of sample, yet with an average prevalence of 25.2% (Rajic et al. 2005, Wilkins et al. 2010). Canadian reported prevalence was significantly higher compared with the value observed in the present work. Even though it has been established that bacteria isolates from animal clinical samples can be difficult to obtain given low concentrations, competition and overgrowth by other bacteria of the Enterobacteriaceae family, collectively it was possible to isolate the microorganism from this type of sample.

Presence of bacteria in the environment may be associated with the capacity to survive and persist for long periods of time (Baer et al. 2013). Some environmental samples, such as empty pens, drains and workers boots can harbor microorganisms, and might be an important source of indirect contamination within the farm (Wilkins et al. 2010). Furthermore, surface water presented the greatest number of isolates, suggesting a possible pathogen dissemination source within farms, since surface water is used for different activities, such as cleaning facilities, farm equipment, and drinking water, among others. This result may be associated with Salmonella spp. capacity to establish biofilms and colonize pipes for water distribution to different sites at the farm (Yang et al. 2015).

González et al. (2015) claimed presence of this microorganism in water drinkers is associated with deficiencies in cleaning and disinfection procedures, hence promoting dissemination to the non-infected population. It is also important to consider at the moment of washing the pens, some fecal material contained within the pen can splash and contaminate drinkers. Therefore, other aspects to consider are the design of drinkers. It has been described that basin type drinkers reduce the risk of Salmonella spp. infection compared with fountain type or wells (Bahnson et al. 2006). However, in this work all the farms evaluated (100%) employ nipple drinkers. Hence, it is possible microorganism presence resulted from contaminated water, together with ineffective maintenance and deficiencies in disinfection protocols, contributing with pathogen dissemination and persistence. Salmonella spp. dissemination through water increases when water is obtained from wells and surface water without purification, such waters can be contaminated by healthy carriers wildlife passing, from nearby farms and even from the same farm, as could be the case for farms evaluated in this study (Mejía et al. 2006), where 76% of the farms are located near other livestock farms, mostly cattle, species known to carry bacteria and thus contaminate water bodies.

Another variable resulting in Salmonella spp. presence and dissemination in the farm are pen drainage systems, the 11.9% bacteria presence was detected, where contamination was attributed to sewage water, which can contain infected pig feces remaining in the drainage system. Additionally, these are contamination sites, since they attract insects and rodents, which can be bacteria carriers and disseminators, allowing their continuous presence and cross-contamination in the pen. On the other hand, 7.1% bacteria were also detected in previously disinfected empty pens, which could indicate deficiencies in the procedure. Among the many causes could be the chemical properties of the disinfectant used, its concentration, contact time, pen surface or type of floor. In any case, failures in cleaning processes could have favored inactivation of the product, even the use of high pressure hoses can generate aerosol, disseminating the pathogen within the facilities (Dors et al. 2015, Montagnaro et al. 2010). In turn, farms where empty pens were only cleaned with water, a direct correlation was observed between the type of floor and Salmonella spp. presence (P<0.05), specifically bacteria can adhere to the floor surface or lodge in floor pores, without achieving an effective pathogen elimination, given the lack of appropriate disinfectants that favor microorganism elimination.

In this work 66.7% of the farms had mixed floors and 28.7% cement floors. However, the type of floor changes according to production stage. This findings, are in agreement with Andres & Davies (2015) report in who claim that floor design influences the way waste can be removed, and microorganism persistence after cleaning and disinfection processes (Andres & Davies 2015). Even though these risks are a fact, procedures, such as flaming and rotation of disinfectants could prevent bacteria from attaching. Although some farms in this work carry out such procedures, untreated water is still used, thus favoring pathogen presence.

Furthermore, worker’s boots can be cross-contamination agents, as was detected in this work, since bacteria presence in these elements was 9.5%. All evaluated farms in this study had footbaths for boot disinfection. However, previous unsuitable cleaning results in feces collection in sole furrows, and contact with this organic matter with products, inactivates the disinfectant (Amass et al. 2000, Pritchard et al. 2005, Wales et al. 2011, Rabie et al. 2015).

In agreement with microbiological findings, one management variable associated with seropositivity was the frequency with which manure was collected. This task is performed on a daily basis, being the most frequent activity. According with reports, it is essential to remove feces from the environment, since Salmonella spp. is spread by the fecal-oral route. Therefore, farm hygiene practices are directly related with bacteria permanence in the environment, as well as increase in infected animals (Xiao et al. 2005, Nielsen 2013). Although manure collection contributes with bacterial load reduction, use of untreated water, allows pathogen recirculation. Another statically significant variable observed in this study was the floor pit, which is a source of attraction for flies that become vectors of the microorganism (Béjar et al. 2006).

In relation to antimicrobial resistance from the isolates obtained, it was established that 57% of analyzed farms added antibiotics and other supplements to food. As was reported by Diaz et al. (2011) our findings are consistent with their report stating in Colombia is common to formulate concentrate with antibiotics in swine farms. Moreover, according to the "Instituto Colombiano Agropecuario” (ICA) reports pigs are treated with ciprofloxacin, ampicillin and trimethoprim/sulfamethoxazole for different purposes including preventive. These same products are used therapeutically in human salmonellosis.
Hence, resistance to the antimicrobial by the microorganism can arise, due to prolonged and often exposure to the antibiotic.

According to Butaye et al. (2003) the main cause of resistance in isolate strains from farms is attributed to indiscriminate and excessive use of antibiotics in animal rations, using them as growth promoters, thus inducing multiresistance. Additionally, plasmid transfer among bacteria found in swine gastrointestinal tract is facilitated. Multiresistance data obtained in this work is in agreement with Gomes-Neves et al. (2014) report who observed 63% of analyzed isolates were multiresistant. Furthermore, as aforementioned it is necessary to emphasize the resistance to first and second choice antibiotics used in human therapy against trimethoprim/sulfamethoxazole, ampicillin, colistin, cefatoxime, and ciprofloxacin, of which in the present investigation a significant percentage of resistance was obtained against trimethoprim/sulfamethoxazole and to ciprofloxacin, this added to the fact that 5.6% of the isolates showed shared resistance to both antibiotics.

Finally, data obtained for antimicrobial resistance in farms included in this work, revealed the presence of *Salmonella* spp. strains resistant to first line antibiotics and multidrug-resistance strains, which can have a negative impact on national public health. Therefore, it is necessary to establish an integrated monitoring program for epidemiological surveillance, with the objective to decrease zoonotic risk.

**CONCLUSIONS**

Although in Colombia good porcine practices are established for porcine production, it is evident bacteria were present in animals and environmental samples of analyzed farms.

Animal contact/exposure to the microorganism was also evidenced by serological response obtained.

The presence of these bacteria was influenced by management practice (type of water used, feeders-hopper type, handling by workers) and infrastructure (porous floor) in analyzed farms.

Use of antibiotic supplements in food could be inducing high antimicrobial resistance against *Salmonella* spp.

Emerge of multiresistance strains is becoming more frequent and with a tendency to increase.

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**Conflict of interest statement.** The authors declare no conflict of interests.

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