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Alteration of the Reproductive Indicators by the Presence of *Leptospira* spp. in Sows of Swine Farms

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ABSTRACT

***Background*:** Leptospirosis is a worldwide disease that impacts health, welfare and animal production. Manifestations in swine range from subclinical to severe cases of reproductive failure, generating abortions, embryonic resorption, litters with few piglets, and piglets born weak or dead, which causes great economic losses. Tropical conditions in Colombia favor transmission and maintenance of *Leptospira*, highlighting the importance of implementing direct diagnostic techniques such as isolation through culture to reach a definitive diagnosis. The objective of the present study was to relate reproductive indicators behavior with the presence of *Leptospira* spp. in two pig farms in Cundinamarca, Colombia.

***Materials, Methods & Results*:** Sows in reproductive stage in two pig farms were selected. A clinical examination of the animals was performed to demonstrate the presence of signs suggestive of Leptospirosis, also the sow records were reviewed to find reports of any of these clinical manifestations, and the average of the reproductive indicators was calculated to set which were altered in the sows. Blood and urine samples were obtained and analyzed by microscopic agglutination test (MAT) and isolation through culture, respectively. Among the altered reproductive indicators were identified: total pigs born (TPB) in 72.5%, stillbirths (SB) by 70%, mummified pigs (MUM) in a 52.5%, pre-weaning death (PWD) by 40% and the 24 h mortality (M24h) in the 20%. The 77.5% of the sows were positive by MAT. The predominant serovars of *Leptospira* spp. included Grippotyphosa (67.5%), Canicola (22.5%), Icterohaemorrhagiae (20%), Hardjo (17.5%) and Pomona (12.5%). The bacterium was isolated in 32.5% of the analyzed urine samples. There is increased risk of alteration in the indicators M24h (1.27), TPB (1.08), SB (1.15) and MUM (1.27) with the presence of *Leptospira* by isolation through culture.

***Discussion*:** The birth of weak piglets and the alteration of indicators such as SB were the most common findings in this study, which are of the major alterations caused by the bacteria because *Leptospira* can be located in the uterus. Positive cultures, 32.5% (13/40), indicate a high percentage of positive animals in the population. The total of positive culture results reveal that humans, pigs and other animal species from the farms and surrounding areas, are at risk of exposure to the bacteria, because these positive sows are eliminating the microorganism through urine to the environment, representing a problem for public health. This is why it is important to perform the identification of bacteria in urine. It establishes whether the animal is a carrier, although the non-detection of the microorganism in the urine does not rule out that this is a chronic renal carrier because it may indicate that at the moment of the test the animal was not excreting detectable amounts of the bacteria. The total of positive sera (77.5%) indicates a high seropositivity of swine leptospirosis in the population. Regarding serovars of *Leptospira* spp. identified, Grippotyphosa has the largest presentation (67.5%), therefore, as pigs are not maintenance hosts of this serovar, the results of this study suggest that synanthropic rodents that are found on farms may be transmitting the bacteria to pigs. The reproductive indicators related to the *Leptospira* serovars by X^2 test, demonstrated significant association between the average of SB and the serovar Pomona, which has as reservoir the swine species and it is related with the production of piglets born dead, while through the Pearson correlation coefficient it was found that the greater the number of positive samples to serovar Pomona there is a greater presentation of weak piglets, and also was demonstrated that animals with *Leptospira* spp. have a higher risk to present alterations of the M24h, the average of TPB, SB and MUM.

Keywords: Leptospirosis, pig industry, culture, reproductive failure, MAT, zoonoses.

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INTRODUCTION

Leptospirosis can alter the reproductive indicators in the swine industry and lead to large economic losses for pig farmers [2,17,28]. This disease is a zoonotic risk to humans, but unfortunately in Colombia it is not a notifiable disease in animals, even with a 77% seroprevalence in humans and of 86.6% seroprevalence in swine [3,29,38].

The disease is subdiagnosed; reports in humans are scarce due the lack of knowledge, the need of accurate diagnostic methods and the similarity of clinical signs with common diseases in the tropics such as Zika virus, yellow fever, dengue and malaria, among others [7,13,26].

Reports of *Leptospira* spp. isolation in pigs through urine culture are scarce in Colombia [14], although the isolation is primordial to provide a definitive diagnosis of the disease, contribute to the development of programs for prevention and control of leptospirosis, generate new researches that demonstrate the diversity of strains, serogroups and serovars, and start creating native panels to improve diagnosis of the disease from circulating strains in the country [11,42].

Globally, *Leptospira* isolation from pig urine has only been performed in countries like Brazil [19,27], Tanzania [24] and Thailand [25]. The main objective of this study was to relate the behavior of the reproductive indicators with the presence of *Leptospira* spp. in two pig farms on Cundinamarca, Colombia.

MATERIALS AND METHODS

Study location

This study was conducted at two swine farms in the department of Cundinamarca, Colombia. Farm 1 located in the municipality of Choachí, situated at 4° 31'52" north latitude and 73° 55'33'' west longitude, with an average temperature of 18° C and an altitude of 1923 masl [33]; and farm 2 located in the municipality of San Antonio del Tequendama, at 4° 37' latitude and 74° 21' length, whose average temperature is 18° C, and an altitude of 1540 masl [34]. The size of the herd on the farm 1 was 98 and 119 in the farm 2. The two farms had the following common features: open farm, drinking water came from a natural source, direct contact between sows, service method by natural mating, and reproductive failures in recent years, corresponding to risk factors associated with Leptospirosis.

Study type and sample selection

Cross-sectional study, whose population corresponded to 40 sows. It was a convenience sampling, and the sample size was determined using the Win Episcope 2.0 software with a confidence level of 95% and a prevalence of 30% [1]. The following were the inclusion criteria: sows in reproductive stage with an age range between 7 and 40 months, non vaccinated against leptospirosis for at least 6 months and without antibiotic therapy minimun 2 weeks before sampling. The selection of breeding sows was performed based on an analysis of clinical observations of suspected cases of leptospirosis at the population level [43]. Additionally, a clinical examination of the animals was performed to demonstrate the presence of signs suggestive of the disease, and also the sow records were reviewed to find reports of any of these clinical manifestations [8]. The average of the reproductive indicators was compared with the goals set by Porkcolombia, the Colombian association of pork producers, to set which of the next the parameters were altered in the reproductive sows: Pre-weaning death (PWD), mortality in the first 24 hours (M24h), mummified pigs (MUM), stillbirths (SB), total pigs born (TPB).

Urine sample and culture

Urine collection was made by spontaneous voiding of the 40 selected sows, 30 of farm 1 and 10 of farm 2, corresponding to 29.4% and 11.9% of the population of each farm, respectively. Urine was filtered and stored in Falcon® tubes containing phosphate buffer solution (PBS), in order to maintain the viability of the bacteria and increase the probability of isolation through culture. Subsequently, a drop of urine was placed in a tube with Ellinghausen-McCullough-Johnson-Harris (EMJH)[1] semisolid medium with antibiotic (5-fluorouracil and fosfomycin) and in another tube with EMJH semisolid medium without antibiotic. The samples were transported refrigerated at 4°C to the laboratory, and were subsequently centrifuged for 15 min at 3,500 x *g*. The pellet was resuspended in a tube with EMJH liquid medium with antibiotic and in another without antibiotic, and these were incubated in dark at 28°C [20]. Cultures were reviewed by dark field microscopy every 72 h, subcultures were made and those with greater amount of contaminating microorganisms were filtered to promote the viability of *Leptospira* spp. and examine its growth. Positive samples were stored at -70°C [21].

Sampling and serological testing

Blood samples of 40 sows were taken from the auricular and jugular veins in Vacutainer® tubes without anticoagulant and were transported as quickly as possible to the laboratory in a cooler at 4°C, and by means of spontaneous coagulation the sera were obtained and then were stored in Eppendorf® tubes at -20°C until serological analysis. The sera were examined by microscopic agglutination test (MAT). Antibody titers ≥1:100 were considered positive. The serovars of *Leptospira* spp. included in the diagnostic battery were Hardjo, Canicola, Icterohaemorrhagiae, Pomona and Grippotyphosa [20,32].

Statistical analysis

To describe the results of the serological and microbiological tests, percentages (%) were used. For analysis of the reproductive indicators, Statistix 8.0 software was used to calculate mean, standard deviation and range. Student's *t*-test was performed and the relative risk was calculated with the microbiological results. To determine the degree of relationship between serovars found and the altered reproductive indicators, Chi square test (X^2) was performed, and also analysis of variance (ANOVA) was used between the altered and not altered indicators positive to microbiological culture. Finally, the Pearson correlation coefficient was used to analyze the relationship between different variables such as clinical signs, diagnostic tests, reproductive indicators and data from the clinical examination of the sows.

RESULTS

Clinical examination found no increase in temperature or jaundice in any of the sows studied, while 2.5% (1/40) presented agalactia, 2.5% (1/40) anorexia and 5% (2 / 40) decay. In reviewing the records, the most common clinical sign was piglets born weak with 72.5% (29/40), followed by 40% (16/40) of birth of mummified piglets, 22.5% (9/40) of the sows had submitted abortion, 15% (6/40) puerperal endometritis, 10% (4/40) repeating zeal, 2.5% (1/40) low total born and 2.5% (1/40) hemoglobinuria.

When comparing the average of the reproductive indicators with the goals set by the ACP, neither of the sows overshoot the weaning-service interval, 20% (8/40) had a high mortality in the first 24 h, 40% (16/40) had high pre-weaning mortality, 52.5% (21/40) had mummified piglets per litter, 70% (28/40) was above the limit of stillborn piglets per litter and 72.5% (29/40) had low total piglets born per litter.

In both farms, factors associated with disease occurrence included: direct contact with other sows, the presence of rodents, the service method, the presence of other domestic and wild species on the farms, reproductive failures in recent years, the presence of other diseases, type of farm (open) and that the source of drinking water for the animals came from a natural birth.

In 32.5% (13/40) of the urine samples *Leptospira* spp. was isolated, 20% (8/40) of the farm 1 and 12.5% (5/40) of the farm 2.

The 77.5% (31/40) of the sera tested positive, and two or more serovars were found in 45.1% (14/31) of the MAT titers. The most common serovar of *Leptospira* spp. in the study population was Grippotyphosa with 67.5% (Table 1). Furthermore, 31 of the positive sera corresponded to the titer 1≥100, 17 to 1≥200, 7 to 1≥400 and 1 to 1≥800.

The results obtained by ANOVA show that there is no statistically significant difference ($P > 0.05$) between the altered reproductive indicators and the not altered reproductive indicators that were positive to *Leptospira* spp.; however, with the presence of *Leptospira* there is increased risk of alteration in the indicators M24h (1.27), TPB (1.08), SB (1.15) and MUM (1.27).

According to the results of X^2 test between serovars and reproductive indicators, there is only significant relationship between the SB average with the serovar Pomona ($P = 0.001$); and likewise, when calculating the Pearson correlation coefficient was found that to more number of positive samples to the serovar Pomona was higher the presentation of weak piglets ($P = 0.0382$).

DISCUSSION

The most common findings in this study included the presentation of clinical signs such as the birth of weak piglets and the alteration of indicators such as SB. These are of the major alterations caused by the bacteria, in accordance with a study in which disease was confirmed in animals with this type of reproductive failures and these were the most affected [8]. These failures occur because *Leptospira* can be located in the uterus; thus generating, in the case of breeding females who become infected during pregnancy, reproductive manifestations as a result of intrauterine infections that occur in the last period of gestation. This can also by transplacental infection occurring during the period of leptospiremia [16].

Table 1. Positive samples with antibody titres to *Leptospira* spp. serovars by microscopic agglutination test (MAT) from sows in Cundinamarca, Colombia.

Serovar	Antibody titres				Total (%)
	1:100	1:200	1:400	1:800	
Hardjo	5	2	0	0	7 (17.5)
Canicola	8	1	0	0	9 (22.5)
Pomona	4	1	0	0	5 (12.5)
Icterohaemorrhagiae	7	1	0	0	8 (20)
Grippotyphosa	7	12	7	1	27 (67.5)

At this stage the microorganism invades the placenta and infects the fetuses, which often die in the uterus. Generally the most common manifestation of the infection in the herd is abortion, but stillbirths, partially mummified fetuses and birth of weak piglets can be observed. These patterns are presented depending on gestation stage the fetuses die, because they can autolyzed in the uterus and mummified, generating minor injuries in the uterine lining and so the disease does not manifest itself with abortion [45].

Positive cultures, 32.5% (13/40), indicate a high percentage of positive animals in the population. In the few studies on *Leptospira* spp. isolation, the number of isolates is generally low even in large population samples [24]. This is possibly because of the difficulty of isolation that is due to different causes such as: the long incubation period needed by the bacteria, the presence of contaminating microorganisms such as *Bacillus* spp. that multiplies rapidly and inhibits the growth of *Leptospira*, and the acid pH of urine that inactivates and lyses the bacteria in less than three hours [19].

The total of positive culture results reveal that humans, pigs and other animal species from the farms and surrounding areas, are at risk of exposure to the bacteria, because these positive sows are eliminating the microorganism through urine to the environment [6]. This is why it is important to perform the identification of bacteria in urine. It establishes whether the animal is a carrier, although the non-detection of the microorganism in the urine does not rule out that this is a chronic renal carrier because it may indicate that at the moment of the test the animal was not excreting detectable amounts of the bacteria. To be considered *Leptospira* free, individuals must show negative urine samples from three consecutive weeks [49].

The total of positive sera (77.5%) indicates a high seropositivity of swine leptospirosis in the population, similar to a report in breeding sows in Vietnam where found a seroprevalence of 73% [9], and differs with other report of 16.1% in the state of Alagoas in Brazil [47]. These results reflect the possibility of increased economic losses for pig farmers and for the economy of countries, taking into account that the prevalence of leptospirosis in pigs in Central America is between 17% and 75% [44].

Regarding serovars of *Leptospira* spp. identified, Grippotyphosa has the largest presentation (67.5%). This shows that there is an incidental infection in the population, as has been reported in different countries such as Thailand, where conducted a study with breeding sows that with different titers reached 55% of positive samples for this serovar [31]; therefore, as pigs are not maintenance hosts of this serovar, the results of this study suggest that synanthropic rodents that are found on farms may be transmitting the bacteria to pigs [36].

The production of titers against two or more serovars which in this case was 45.1% of the positive samples is a common finding in these studies, similar to the obtained in Argentina where 46% of the sera revealed titers against two serovars and 45% on three or more [37]. The production of titers against several serovars may be in some cases the result of cross reactions between these [48], and also could be that the animals are in the acute phase of infection, in which occurs the induction of antibodies against common antigens of *Leptospira* [46].

In this study some sera showed varied titers against each serovar, and these results could be explained because some serovars produce higher MAT titers cause the immunogenicity differs depending on the serovar, for example titers ≥512 for the serovar Pomona and titers ≥32 for other serovars in a swine population studied in Australia [12].

Researchers in Brazil detected antibodies in sows after being inoculated with *Leptospira interrogans* but they did not showed clinical signs, showing the subclinical infection that can be present commonly in pigs [4]. These findings may explain why in the present study some sows had serological evidence without clinical manifestations, which is important from an epidemiological point of view, because the persistence of *Leptospira* spp. in these populations is generated from these apparently healthy cases, highlighting the importance of identifying animals with subclinical infection to control the transmission of the bacterium in the human-animal-environment interface.

The obtention of samples that were positive to MAT but negative to culture (52.5%) can be attributed to that isolation of *Leptospira* spp. is long and complex, that the period of leptospiruria varies and is not constant during the day, and because animals can be in the acute phase of the disease and have not started to eliminate the microorganism [19], and although serological tests are the most used, it is necessary the combination with direct detection tests like the isolation through culture or molecular techniques to provide a definitive and individual diagnosis and understand the distribution of the microorganism in the population [40]. Culture-positive results but negative to MAT, 7.5% (3/40) obtained in this study, can be explained because when the infection is endemic in farms can be found sows with titers <100; also, should be aware that the MAT test has limitations and there is not always correlation between the found serogroups and the results in the isolation and subsequent identification [16,27,49].

As for the relationship of the reproductive indicators, a greater number of MAT-positive animals was obtained when the indicators were altered; it is important to note that although the MAT test is the "gold standard" for leptospirosis, also has flaws and is not always good because of the variations in the immune response, and also it indicates that there was exposure to the bacteria while the culture confirms the diagnosis and the presence of the bacteria in the time when the urine sample is taken to the animal [21,22,39].

In a study on a swine herd in Brazil, compared the alteration of the reproductive indicators with serology, finding only significant difference in the number of weak piglets between seropositive and seronegative, while the alteration of other indicators such as return to estrus, stillborn, mummified piglets, nonproductive days, total born per litter and live births per litter had no significant difference [18]; similar to a report in Denmark [30], where only found association with the number of stillbirths, which was significant ($P = 0.02$). These reports support the results of the present study in the analyzed sows that show a greater number of MAT-positive animals when the reproductive indicators were altered.

The reproductive indicators related to the *Leptospira* serovars found in this study, by X^2 test, demonstrated significant association between the average of SB and the serovar Pomona; these results are in accordance with the literature since this serovar has the swine species as reservoir, its related with the presence of the disease and has been found associated with the production of piglets born dead [41].

In contrast, in southern Vietnam reported significant relationship in the case of positive sows to the Grippotyphosa serovar who had a prolonged weaning to service interval, and in positive sows to the serovar Tarassovi that had higher number of stillborn piglets, but in the case of the serovars Icterohaemorrhagiae, Bratislava, Autumnalis and Pomona there was not specific associations [10]. With the results obtained in the present investigation only was found association between the serovar Pomona and the birth of dead piglets.

Through the Pearson correlation coefficient it was found that the greater the number of positive samples to serovar Pomona there is a greater presentation of weak piglets; these results differ from a study in Japan [23], where the serovar Icterohaemorrhagiae represents a greater risk to the presentation of weak piglets. This difference is explained because the different serovars produce various types of infection, which in turn depends on environmental conditions, on the distribution of these in different geographical areas, on the presence of reservoirs as synanthropic rodents and various species of wild animals, and specifically on factors of each animal as susceptibility [15].

Not finding statistically significant relationship between indicators and the presence of *Leptospira* spp., can be explained by geographical factors, because the infection is endemic in farms and not marked clinical signs occur and because the mode of presentation of leptospirosis depends on the farm being studied [5,35]. However, according to the relative risk calculated in this study, animals with *Leptospira* spp. have an incre-

ased risk of presenting alteration of the reproductive indicators as the M24h, the average of TPB, of SB and of MUM. These results are relate to previous reports that identify in the pig population the *Leptospira* serovars found in the present study associated with the type of reproductive failures mentioned above [47].

CONCLUSIONS

In conclusion, the principal serovars of *Leptospira* spp. identified by MAT in the population of pigs were: Grippotyphosa with 67.5% (27/40), followed by Canicola with 22.5% (9/40), Icterohaemorrhagiae with 20% (8/40), Hardjo with 17.5% (7/40) and finally Pomona with 12.5% (5/40). *Leptospira* spp. was isolated in 32.5% (13/40) of urine samples, indicating that there is a risk of transmission of the bacteria for both the rest of the animals, and workers and veterinarians of the farms, representing a problem for public health. Similarly, was demonstrated that animals with *Leptospira* spp. have a higher risk to present alterations of the M24h, the average of TPB, SB and MUM.

These results are the second report published in Colombia and the sixth worldwide about the isolation of *Leptospira* spp. in swine, and they reflect the impact of this pathogen in public health and livestock production. Also, indicate the need to know in each region the true role of the isolated serovars in the pathophysiology of swine leptospirosis which affects animal, human and environmental health and has implications for epidemiology and veterinary economics.

MANUFACTURER

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Ethical approval. This study was approved and performed under the guidelines of the Ethics Committee of the Faculty of Agricultural Sciences of the Universidad de La Salle.

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