EVIDENCE OF ANTIBODIES AGAINST DIFFERENT *Leptospira* spp. SEROGROUPS AND FACTORS ASSOCIATED WITH ITS PRESENCE IN SHEEP FROM VERACRUZ, MEXICO †

[EVIDENCIA DE ANTICUERPOS CONTRA DIFERENTES SEROGRUPOS DE *Leptospira* spp. Y FACTORES ASOCIADOS CON SU PRESENCIA EN OVEJAS DE VERACRUZ, MÉXICO]

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SUMMARY

Background. Leptospirosis is a bacterial zoonosis caused by pathogenic species of the genus *Leptospira* that affects mainly reproductive performance in sheep. **Objective**. To identify the seroprevalence of *Leptospira* spp., associated serogroups, and possible risk factors in the state of Veracruz, Mexico. **Methodology**. Blood samples were collected from 405 sheep in 55 farms located in 13 municipalities distributed along three ovine-producing regions in the state. Serological diagnosis was made by Microscopic Agglutination Technique (MAT). The prevalence and its confidence intervals (CI) were calculated using Vassarstats software, the Odds Ratio (OR) values were estimated with WinEpiscope. **Results.** An overall seroprevalence of 53.83% ($_{95\%}$CI:48.84-58.75), per municipality of 100% ($_{95\%}$CI :71.66-100) and per flock of 92.73% ($_{95\%}$CI: 81.58-97.65) were noted. The highest prevalence by region was 58.82% ($_{95\%}$CI$_{:\%}$:50.57-66.62) for Los Tuxtlas (p= 0.218) and per municipality 86.36% ($_{95\%}$CI:71.95-94.33) for Ángel R. Cabada. The predominant serogroup was Hardjo (45.93%; $_{95\%}$CI:41.02-50.92). By age, the highest values were 55.93% ($_{95\%}$CI:48.29-63.31) in the group of 19-36 months. The main recognized risk factors were region and municipality. Los Tuxtlas Region (OR =1.92; $_{95\%}$CI:1.08-3.43) and the municipalities of Ángel R. Cabada (OR=15.5; $_{95\%}$CI:5.1-47.2), Ayahualulco (OR=10.1; $_{95\%}$CI :3.6-28.7), and Coatzintla (OR=6.1; $_{95\%}$CI: 30.3 – 95.0). **Implications.** This study explores the presence of antibodies against *Leptospira* spp. in sheep, its associated serogroups and risk factors in the state of Veracruz, Mexico. This is this first report on the presence and relevance of this infection in sheep of this region. The role of sheep as a maintenance host for *Leptospira* spp. in endemic areas is stressed. **Conclusions.** The study concludes that *Leptospira* spp. in sheep has an overall seroprevalence of 53.83% in the state of Veracruz, Mexico and several serogroups present.

Keywords: *Leptospira* spp, microaglutination, sheep, cross-sectional study, zoonosis.

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RESUMEN

Antecedentes. La leptospirosis es una zoonosis bacteriana causada por especies patógenas del género *Leptospira* que afecta principalmente el desempeño reproductivo en ovinos. **Objetivo.** Identificar la seroprevalencia de *Leptospira* spp., serogrupos asociados y posibles factores de riesgo en el estado de Veracruz, México. **Metodología.** Se recolectaron muestras de sangre de 405 ovinos en 55 fincas ubicadas en 13 municipios distribuidos en tres regiones productoras de ovinos del estado. El diagnóstico serológico se realizó por la Técnica de Aglutinación Microscópica (MAT). La prevalencia y sus intervalos de confianza (IC) se calcularon con el software Vassarstats. las Razones de Momios (RM) fueron estimadas con WinEpiscope. **Resultados.** Se observó una seroprevalencia global de 53.83% ($IC_{95\%}$:48.84-58.75), por municipio del 100% ($IC_{95\%}$:71.66-100), y por rebaño de 92.73% ($IC_{95\%}$: 81.58-97.65). La mayor prevalencia por región fue 58.82% ($IC_{95\%}$: 50.57-66.62) para Los Tuxtlas (p=0.218) y por municipio 86.36% ($IC_{95\%}$ 71.95-94.33) para Ángel R. Cabada. El serogrupo predominante fue Hardjo (45.93%; $IC_{95\%}$:41.02-50.92). Por edad, los valores más altos fueron 55.93% ($IC_{95\%}$:48.29-63.31) en el grupo de 19-36 meses. Los principales factores de riesgo reconocidos fueron región y municipio. Región de Los Tuxtlas (OR=1.92; $IC_{95\%}$:1.08-3.43) y los municipios de Ángel R. Cabada (OR=15.5; $IC_{95\%}$:5.1-47.2), Ayahualulco (OR=10.1; $IC_{95\%}$:3.6- 28.7) y Coatzintla (OR=6.1; $IC_{95\%}$: 30.3 – 95.0). **Implicaciones:** Este estudio explora la presencia de anticuerpos contra *Leptospira* spp. en ovinos, sus serogrupos asociados y factores de riesgo en el estado de Veracruz, México. Este es el primer reporte sobre la presencia y relevancia de esta infección en ovinos de esta región. Se destaca el papel de las ovejas como hospedero de mantenimiento de *Leptospira* spp. en las zonas endémicas. **Conclusiones.** *Leptospira* spp. en ovinos tiene una seroprevalencia global de 53.83% en el estado de Veracruz, México y varios serogrupos presentes.

Palabras Clave: *Leptospira* spp, microaglutinación, ovinos, estudio transversal, zoonosis.

INTRODUCTION

Leptospirosis is an emerging disease that spreads at an alarming rate (Azhari *et al*., 2018). Leptospirosis is a zoonotic disease that affects humans, as well as wild and domestic animals. It is caused by a spirochete called *Leptospira* with more than 300 serotypes (Adler, 2015). In ovine, the disease generates productive and reproductive problems such as anorexia, depression, irritability, fever, polypnea, diarrhea, constipation, hemolytic disease, hemoglobinuria, anemia, jaundice, lameness or sudden drop in milk production, and epidemic abortions in acute states. Meanwhile, in subacute and chronic disease it can be observed infertility, increased number of services per conception, prolonged calving intervals, abortion, stillbirths, birth of weak animals and perinatal deaths (Consalter *et al*., 2019). All these effects translates into economic losses (Malone *et al*., 2010), being a serious problem, especially in the tropics, where seroprevalences of 54.9% have been found in Brazil (Eckstein *et al*., 2017), 44% in India (Balamurugan *et al*., 2021), 28.8 in Colombia (Parra *et al*., 2016), and 54.5% in Mexico (Arteaga-Troncoso *et al*., 2015).

Veracruz is the third state with the highest ovine production in Mexico. Only in 2019, 5,425 t of mutton were produced (SIAP, 2020). However, the condition in which ovine production develops is lacking adequate biosecurity measures, which in turn can determine the susceptibility of these animals to different serogroups of *Leptospira* spp. (Luna *et al*., 2018). Sheep can acquire *Leptospira* spp through the urine of infected rodents, by contact to infected animals or drinking water contaminated with the bacteria (Martins and Lilenbaum, 2017).

In particular, the serogroups Hardjo is the most frequent worldwide, although other serogroups such as Pomona, Ballum, Bratislava and Grippotyphosa have been found (Bautista *et al*., 2014). In Mexico, there are no official immunization protocols against *Leptospira* infection in sheep. Some of the factors that may determine a greater probability of presenting infection with *Leptospira* spp. in ovine are living with other animal species (Topazio *et al*., 2015), whether domestic or wild fauna, contact with aborted fetuses or placentas from infected animals, and even climatic conditions such as high temperature and humidity that favor the presence of the bacteria in the environment, among others (Arteaga-Troncoso *et al*., 2015). In Veracruz, the situation of ovine leptospirosis is unknown. Therefore, the objective of this study was to identify the seroprevalence of Leptospira spp., associated serogroups, and possible risk factors in the state of Veracruz, Mexico.

MATERIALS AND METHODS

The study was carried out in the state of Veracruz, which is located on the coastal strip of the Gulf of Mexico between 17° 09´ and 22° 28´ of north latitude and 93° 36' and 98° 39' of west longitude. The 13 municipalities with the highest sheep inventory in the state were included in the study. All of them belonged to the Rural Development Districts (RDD) 002 Tuxpan, RDD 003 Martínez de la Torre, RDD 004 Coatepec and RDD 003 San Andrés Tuxtla (Figure 1).

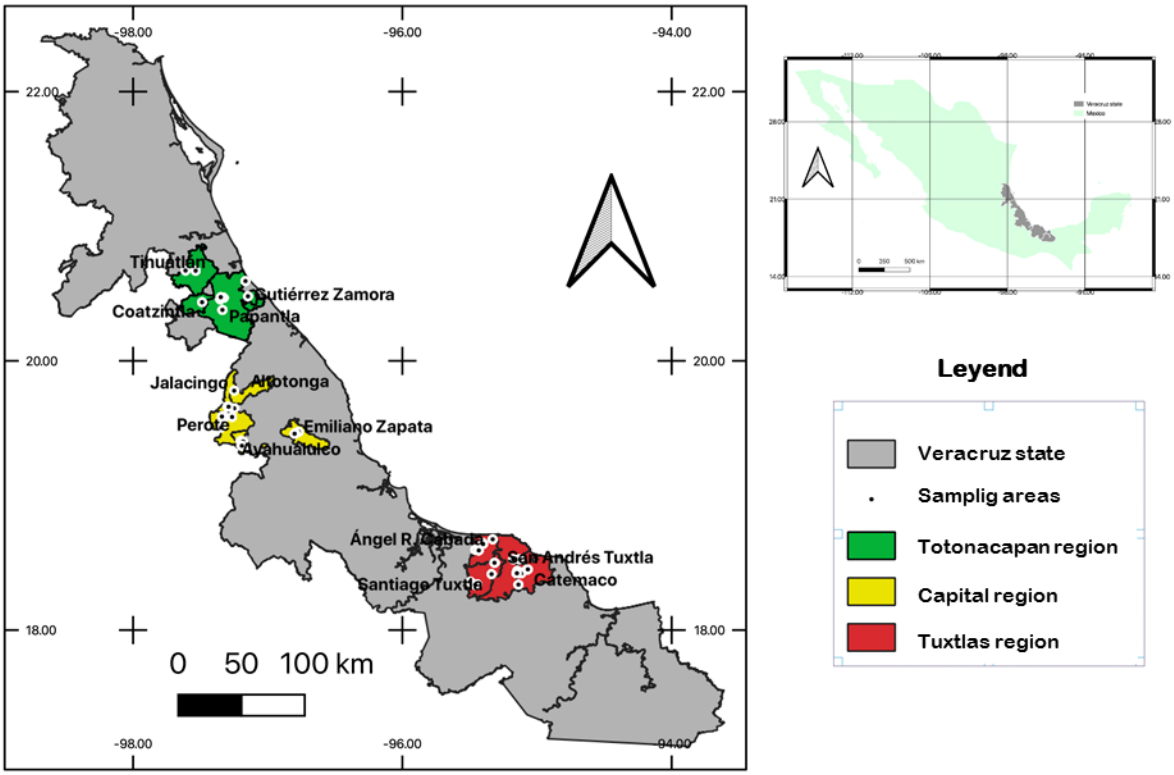

Figure 1. Geographic location of the study area in the state of Veracruz, Mexico.

The present work was a multistage and stratified cross-sectional epidemiological study. The universe was that of the 2018 state ovine inventory corresponding to 698,520 heads (SIAP, 2021). Sample size was calculated according to the formula provided by Thrusfield (2005), for an estimated prevalence of 50% and 95% confidence: $n = \frac{z^2 pq}{B^2}$ where: n = sample size, z = 1.96 for 95% confidence, 2.56 for 99%, p = expected prevalence, q = 1- p, b = accuracy or admitted error.

In total, 405 blood samples were collected from sheep in 55 farms from 13 municipalities, located in three sheep-producing regions. The inclusion criteria were: females older than three months old, age and studs destined for service and absence of previous vaccination against leptospirosis as declared by owner. Meanwhile, the exclusion criteria were: lambs younger than three months old and males not destined for breeding rams.

A general questionnaire was applied for each farm, including variables such as: productive system (classified according to degree of confinement and/or grazing used as intensive, semi-intensive, and extensive), productive status (lambs, ewes, pregnant ewes, rams, lactating ewes), water sources (domestic service network, river, deep well) and contact with flood areas (yes / no). The concurrence on farm presence of domestic, wildlife, and noxious fauna was also considered. Additionally, an individual questionnaire collected data on each sampled sheep, on subjects such as breed, age, sex, productive status, body condition, health status, and presence of clinical signs suggestive of leptospirosis. This individual data was used to test for possible risk factor at the individual level.

A blood sample of 7 mL was taken by puncture of the jugular vein on each selected animal. The samples were identified and transported to the Microbiology Laboratory of the Faculty of Veterinary Medicine, University of Veracruz in Mexico where they were centrifuged at 1,000 X *g* for 15 minutes to obtain serum and aliquots of them were made and then stored at –20° C until processed by serological test.

The serological diagnosis was made in the *Leptospira* and Leptospirosis Laboratory of the Universidad Autónoma Metropolitana, Unidad Xochimilco, in Mexico City using the Microscopic Agglutination Technique (MAT), which has a sensitivity of 98.2% and a specificity of 96.4% (WHO, 2003). Serial double dilutions of each serum were made starting at 1:50, the antibody titer was determined at the highest serum dilution where 50% or more agglutination was

observed. Titers of 1:100 were considered as positives. Twelve serovars of *Leptospira* spp. were used as antigen, corresponding to three national isolates characterized by the US Department of Agriculture (Portland vere Sinaloa strain, Hardjo H-89, and Icterohaemorrhagiae strain Palo Alto, and nine international serotypes (Icterohaemorrhagiae, Pyrogenes, Grippotyphosa, Canicola, Pomona, Hardjo, Wolffi, Tarassovi, and Bratislava), from the Epidemiological Reference and Diagnosis Unit of WHO/FAO/OIE-Collaborating Center for Reference and Research on Leptospirosis, Australia and Western Pacific Region, Queensland, Australia.

Prevalences and their 95% confidence interval ($_{95\%}$CI) were obtained with the Vassarstats online program. Chi-square was used to test differences among several variables such as region, municipality, productive system, productive status, water source, and exposure to flood areas. Risk factors were estimated by Odds Ratio (OR) with WinEpi software.

RESULTS AND DISCUSSION

The general seroprevalence obtained from MAT was 53.83% ($_{95\%}$CI: 48.84–58.75). Out of the 55 farms analyzed, 92.73% ($_{95\%}$CI: 81.58-97.65) had at least one seropositive animal. Although sheep are generally considered to be resistant to *Leptospira* spp. infection and low prevalence rates have been found worldwide (Ellis, 2015), in this study, a seroprevalence higher than expected was recorded. Although, animals did not exhibit any clinical signs suggestive of leptospirosis, sheep could play a role as a maintenance host for this pathogen (Arteaga-Troncoso *et al*., 2015). It has been recognized that the circulation of individuals without clinical signs in endemic areas is important in the epidemiology of *Leptospira* spp. because they are not identified and act as transmitters for other animals (Almeida *et al*., 2019). In fact, in Santa Catarina, Brazil, Topazio *et al*. (2015) recognized the presence of ovine as a risk factor for the spread of *Leptospira* spp. to caprine.

Los Tuxtlas Region had the highest seroprevalence (58.8%, OR=1.9). In this region, Ángel R. Cabada municipality had the highest prevalence among all the municipalities (86.4%, OR=1.9), but there were others with a prevalence over the average (Catemaco, 57%; San Andrés Tuxtla; 56.3%) (Table 1). Sheep in the municipalities of Ángel R. Cabada, Ayahualulco, and Coatzintla had over six times more chances of resulting seropositive against to *Leptospira* spp. than sheep from other tested municipalities in the state of Veracruz. The climate in this region is warm-humid-regular with an average temperature of 25°C. Agricultural activities occupy more than 84% of its surface and only 5.4% is jungle, while water bodies represent 2.8% of the total area in this region. Papaloapan, one of the 10 main rivers in the state of Veracruz, crosses the region, which also exhibits several lakes and lagoons. The tropism of *Leptospira* spp. in tropical and subtropical areas for tributaries of natural or artificial water (Ansdell, 2017), could explain the high prevalence found in sheep of this region and municipalities. Martins and Lilenbaum (2014) noted that tropical conditions are an ideal scenario for the maintenance and spreading of leptospires, as a result of the concurrence of abundant rainfall and the diversity of wild and domestic reservoirs that may contribute to disseminate the agent. For other domestic ruminants residing in tropical areas, the presence of rodents triplicates the chances of the occurrence of seroreactive animals (Higino *et al*., 2013). The environmental conditions that *Leptospira* spp. requires to persist relies on multiple variables such as soil and water pH, temperature, and even environmental microbial communities (Barragan *et al*., 2017).

Serovar Hardjo had the highest prevalence with 45.9% ($_{95\%}$CI: 41.02-50.92), while serovar Grippotyphosa had the lowest one with 0.2% ($_{95\%}$CI: 0.01-1.59) (Table 2). Machado *et al*. (2016) argued that sheep show evidence of being positive to a small number of serogroups. Twelve serogroups were used for this study but, except for serovar Pomona, seropositive animals were found to all the used serogroups. Indeed, the prevalence for the serovar Harjo is high, but for the other ten serovares was mostly below 4%. No previous study on the presence of leptospirosis in sheep has been done in the state of Veracruz, but one research that was conducted in goats of 14 municipalities of central Veracruz showed an overall seroprevalence of 23.40% and seroreaction against seven serovars were found (Peña-Ramírez *et al*., 2011).

Infection in domestic species, such as ovine, with more than one serovar may be due to grazing and intra-species transmission of the agent within the flock (Garba, 2017). Larger herd size was the common risk factor for *Leptospira* spp. seroprevalence when several livestock species were grazing together (beef cattle, sheep, and deer) in New Zealand (Subharat *et al*., 2012; Dreyfus *et al*., 2018). In northeastern Brazil, Campos *et al*. (2017) suggested the possibility of interspecies *Leptospira* transmission among cattle, sheep, and goats when consorted rearing occurred, so they concluded that the presence of dogs, rodents, and wild animals (reservoirs), and the raising of different species are considered important factors in the epidemiology of *Leptospira* spp. Wild animals that inhabit large areas

have contact with flooded sites and feed on rodents that may be infected with *Leptospira* spp. (Viera *et al*., 2017). Moreover, although the water sources supplying ovine farms and sheep access to flooded areas were not relevant in this study, taking biosecurity measures should not be neglected because these conditions are ideal for *Leptospira* spp. transmission.

The seroprevalence by sex was similar between males and females, 53.2% ($_{95\%}$CI: 41.6-64.6; OR=1.0) and 54.0% ($_{95\%}$CI; 48.4-59.4; OR=1.0), respectively (Table 3). By age, for analysis purpose a range of three age groups was established, the 19-36 months group had a seroprevalence of 55.93% ($_{95\%}$CI: 48.29-63.31; OR=1.3), the older than 36 months group with 54.33% ($_{95\%}$CI: 45.28-63.31; OR=1.2), and the 4-18 months group with 49.5% ($_{95\%}$CI: 39.47-59.57). Other variables that were analyzed in this study were the productive system, the productive status, the source of water, and the exposure to flooding zones. The highest prevalence of antibodies against *Leptospira* spp. were obtained in the semi-intensive system, 56.5% ($_{95\%}$CI: 47.3-65.2); in the lamb stage, 66.7% ($_{95\%}$CI: 31.0-91.0); from drinking tap water, 56.3% ($_{95\%}$CI: 47.5–56.7), and in individuals who did not have contact with flooding zones, 55.6 ($_{95\%}$CI: 49.1 - 61.9).

Table 1. Seroprevalence against *Leptospira spp*. by region and municipality in sheep from Veracruz, Mexico.

Variable	Levels	Positives	Sampled	Prevalence (%)	95% C. I.	Odds Ratio	Odds Ratio 95% C.I.
Region	Los Tuxtlas	90	153	58.8	50.5 – 66.2	1.9	1.0 - 3.4
	Capital	99	184	53.8	46.3 – 61.1	1.5	0.8 - 2.7
	Totonacapan	29	68	42.6	30.9 – 55.2	1.0	
Municipality	Ángel R. Cabada	38	44	86.4	72.0 - 94.3	15.5	5.1 - 47.2
	Ayahualulco	33	41	80.5	64.6 - 90.3	10.1	3.6 - 28.7
	Coatzintla	5	7	71.4	30.3 - 95.0	6.1	1.0 – 36.5
	Gutiérrez Zamora	4	7	57.1	20.2 - 88.1	3.3	3.6 - 28.7
	Catemaco	21	37	57.0	40.0 - 72.5	3.2	1.2 – 8.4
	San Andrés Tuxtla	18	32	56.3	38.0 - 73.2	3.2	1.2 – 8.5
	Tihuatlán	9	16	56.2	30.6 - 79.2	3.2	0.9 - 10.6
	Jalacingo	19	36	53.0	36.0 - 69.2	2.7	1.0 - 7.1
	Altotonga	18	32	48.7	32.2 - 65.3	2.3	0.9 - 6.0
	Emiliano Zapata	15	32	46.9	29.5 - 65.0	2.1	0.8 - 5.8
	Perote	14	38	36.8	22.3 - 54.0	1.4	0.5 - 3.7
	Santiago Tuxtla	13	40	32.5	19.0 - 49.2	1.1	0.5 - 3.0
	Papantla	11	38	29.0	16.0 - 46.0	1.0	

Region: Chi-square= 4.957, p=0.175; Municipality: Chi-square=53.163, p=0.000, C.I. =Confidence interval.

Table 2. Seroprevalence (%) for *Leptospira* serovares in sheep from Veracruz, Mexico.

Serovares	Positive (no.)	Seroprevalence (%)	95% Confidence Interval
Hardjo	186	45.9	41.02-50.92
Portland vere Sinaloa strain	32	7.9	5.55-11.08
Wolffi	19	4.6	2.93-7.36
Hardjo H-89	19	4.6	2.93-7.36
Palo Alto strain	17	4.2	2.54-6.77
Tarassovi	16	3.9	2.35-6.47
Pyrogenes	10	2.4	1.26-4.64
Bratislava	4	1.0	0.32-2.69
Canicola	3	0.7	0.19-2.33
Icterohaemorrhagiae	2	0.4	0.08-19.7
Grippotyphosa	1	0.2	0.01-1.59
Pomona	0	0	0-1.17

Table 3. Seroprevalence for *Leptospira* serovars by production system, productive status, water source, and exposure to flood areas in the study sites from Veracruz, Mexico.

Variable	Levels	Samples analyzed	Positive samples	Prevalence (%)	95% C.I.	Odds Ratio	Odds Ratio 95% C.I.
Productive system	Semi-intensive	124	70	56.5	47.3 - 65.2	1.5	0.7 - 3.1
	Extensive	242	130	53.7	47.2 - 60.1	1.4	0.7 -2.8
	Intensive	39	18	46.1	30.4 - 62.6	1.0	
Productive status	Lambs	9	6	66.7	31.0 - 91.0	2.7	0.5 - 13.4
	Ewes	184	103	55.9	48.5 - 63.2	1.7	0.8 - 4.0
	Pregnant ewes	108	57	52.8	43.0 - 62.4	1.5	0.6 - 3.6
	Rams	78	41	52.6	41.0 - 63.9	1.5	0.6 - 3.7
	Lactating ewes	26	11	42.3	24.0 - 62.9	1.0	
Water source	Tap water	135	76	56.3	47.5 – 56.7	1.3	0.6 - 2.6
	River	118	65	55.1	45.7 - 64.2	1.2	0.6 - 2.6
	Deep Well	107	55	51.4	41.6 - 61.1	1.1	0.6 - 2.6
	Other	45	22	49.0	33.9 - 64.0	1.0	
Exposure to flood areas	No	243	135	55.6	49.1 - 61.9	1.2	0.8 - 1.8
	Yes	162	83	51.2	43.3 - 61.9	1.0	

Productive system: Chi-square= 1.27, p=0.529; Productive status: Chi-square= 2.437, p=0.657; Water source: Chi-square= 1.1, p=0.777; Exposure to flood areas: Chi-square= 0.01, p=0.92. C.I.= confidence interval

Recent studies consider that there is a higher risk for host-adapted strains, while flocks with fewer technical resources generally have incidental infections (Martins & Lilenbaum, 2017). In this study, a higher frequency of antibodies was demonstrated for sheep in a semi-intensive system; that is to say, those animals that combine pen feeding and grazing. In this regard, it has been pointed out that the probability of ovine becoming infected is higher in semi-intensive systems, which they determined as a risk factor (Machado *et al.*, 2016). Whereas in intensive flocks, clinical infections are more often produced by serovar Hardjo (Ellis, 2015). Therefore, the transmission of *Leptospira* spp. depends on the coexistence with other species such as cattle, as well as on external environmental factors, such as rain and the accumulation of surface water (Correia *et al.*, 2017). In general, the state of Veracruz complies with the environmental and geographical conditions necessary for spreading *Leptospira* spp.

CONCLUSIONS

Leptospira spp. has a high seroprevalence in sheep and is widely distributed on farms in the state of Veracruz, Mexico. The most frequent serovar is Hardjo, but other serovars could be also found. The high *Leptospira* spp. seroprevalence in sheep found prevented the identification of associated risk factors.

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Conflict of interests. The authors hereby declare that they have no conflict of interest.

Compliance with ethical standards. This study comply with Mexican and institutional (Universidad Veracruzana) regulations on the care and use of animals as stated by Approval No. 008/21 issued by the Bioethics and Animal Welfare Commission, Faculty of Veterinary Medicine and Animal Science, University of Veracruz, Mexico.

Data availability. Data are available with the corresponding author (dmartinez@uv.mx) upon reasonable request.

Author contribution statement (CRediT). B. L. Gabriel-Vejar- Methodology, Resources, Investigation, Data curation, Writing – original draft. **D. Martínez-Herrera** - Conceptualization, Methodology, Resources, Supervision, Funding acquisition, Project administration. **D. Vázquez-Luna** - Conceptualization, Methodology, Resources, Investigation, Supervision. **J. I. Torres-Barranca** and **Patricia Meléndez-Valadez** - Data curation Methodology, Resources, Investigation, Validation. **J. A. Villagómez-Cortés** ‾ Data curation, Formal Analysis, Writing – review & editing. **O.R. Leyva-Ovalle** – Data curation, Formal Analysis. All authors critically reviewed the manuscript and approved the final version of the manuscript.

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