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Technical note

Prevalence of various *Leptospira interrogans* serovars in unvaccinated cows in the states of Puebla, Tabasco and Veracruz, Mexico

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Abstract:

The objective was to compare the prevalences of antibodies against different serovars of *Leptospira interrogans* among the states of Puebla, Tabasco and Veracruz, as well as among some of their municipalities, and to determine if the health status of the cows influences their

fertility. Blood samples were taken from 423 cows (*Bos taurus* x *Bos indicus* and *Bos indicus*) from 24 ranches in 11 municipalities in the aforementioned states. The prevalences of the Hardjo and Inifap serovars were higher ($P<0.05$) in the state of Veracruz than in the state of Puebla, but the prevalence of the Wolffi serovar was higher ($P<0.05$) in the state of Puebla than in the state of Veracruz. The prevalences of the Hardjo and Palo Alto serovars were higher ($P<0.05$) in the state of Tabasco than in the state of Puebla, but there were no differences between these two states in the prevalences of the Inifap and Wolffi serovars ($P>0.05$). The number of serovars in the state of Veracruz was higher ($P<0.05$) than in the state of Puebla, but the number of serovars in Tabasco was intermediate; in addition, there was an important variation ($P<0.05$) between municipalities and between ranches in the prevalence of the different serovars. Overall, the serovar with the highest frequency was Inifap, while the serovar with the lowest frequency was Tarassovi. The health status of the cows did not influence the pregnancy rate ($P>0.05$); however, vaccination of cattle against *Leptospira interrogans* is recommended in order to decrease the risks associated with this bacterium in cattle and humans.

Key words: Prevalence, *Leptospira interrogans*, Hardjo, Wolffi, Tarassovi, Cows, Pregnancy rate.

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Leptospirosis is an infectious disease of bacterial origin classified as a globally distributed zoonosis, affecting numerous species of domestic and wild animals[(1)]. It is a disease of great social and economic impact on livestock, especially in cattle, due to the losses caused by abortions, perinatal mortality, birth of weak calves, infertility and decreased milk production[(2)]. There are two species: *Leptospira interrogans*, which is pathogenic, and *Leptospira biflexa*, which is saprophytic and is found on the surface of soil and water. *Leptospira interrogans* is pathogenic for humans and animals, with more than 250 serovars identified, while *Leptospira biflexa* has 60 serovars[(3)]. Nationally and globally, it is known that bovine leptospirosis is mainly caused by the Hardjo serovar, whose maintenance host is the bovine; however, in different studies it has been shown that the Hardjo, Wolffi and Tarassovi serovars are the most frequent in Mexico[(3)], although Leptospiras *santarosai* and *kirschneri* have been detected with potential impact on cattle[(4)]. Knowledge of the prevalences in a locality, the determination of its maintenance hosts and the monitoring of the emergence of new *Leptospira* serovars are essential to understand the epidemiology of leptospirosis in a region and to focus control strategies[(5)]. Based on the above, the objective was to compare the prevalences of antibodies against different serovars of *Leptospira*

interrogans among the states of Puebla, Tabasco and Veracruz, as well as among some of their municipalities, and to determine if the health status of cows influences their fertility.

The present study was carried out in 24 ranches dedicated to bovine production. Six ranches were located in three municipalities in the state of Tabasco; eleven were located in five municipalities in the state of Puebla; and seven were located in three municipalities in the state of Veracruz. Eleven of the sampled ranches produced milk and calves (dual-purpose system), while thirteen ranches only produced calves (cow-calf system).

The ranches were selected based on a non-probabilistic convenience sampling, according to the interest of the ranchers to participate in the present study. On the other hand, the sample size depended on the budget of the study, therefore, not all the cows from each ranch were sampled; however, at least 12 cows were selected in each. Within each ranch, cows were selected by simple random selection.

The females used in this study had one or more calvings, most of them being *Bos taurus* x *Bos indicus*, although some pure *Bos indicus* cows of the Brahman breed were also used (N= 403 and 20, respectively). The females showed no clinical signs of any disease at the time the samplings were performed. The present research only included adult cows, since there was not enough economic resource to sample calves (both sexes), steers and heifers. However, because cows stay longer in the herd, they are more likely to become infected and, consequently, more likely to have antibodies against all kinds of diseases.

The cows had no history of leptospirosis vaccination prior to the study, so it is valid to assume that the presence of antibodies in the females was due solely to natural exposure to the bacterium. The cows were evaluated reproductively by means of ultrasonography (rectal route) of the uterus and ovaries, in order to determine their reproductive status (pregnant or non-pregnant); however, in the herds from the state of Tabasco, it was not possible to determine such status.

Blood samples were obtained by puncture of the coccygeal vein. To obtain the serum from each of the blood samples, these were centrifuged at 3,000 xg for 10 min. Serum samples were stored at -20 °C. The serological diagnosis for the detection of antibodies against Leptospira was made using the microagglutination (MAT) technique[6,7]. For this purpose, five strains were included, three of international reference (Hardjo, Wolffi and Tarassovi) and two national isolates (Inifap and Palo Alto; Hardjo and Icterohaemorrhagiae serovars, respectively) obtained at INIFAP. This technique was performed in 96-well microplates; 50 µl of each dilution of the serum were used, from 1:50 to the last double dilution where 50 % agglutination was observed in the field, in phosphate buffer solution (PBS). As antigen, 50 µl of each strain of Leptospira cultured in EMJH medium for 8 days with a titer of $2X10^8$/ ml were added. After 1 h of incubation at ambient temperature, the reactions were observed

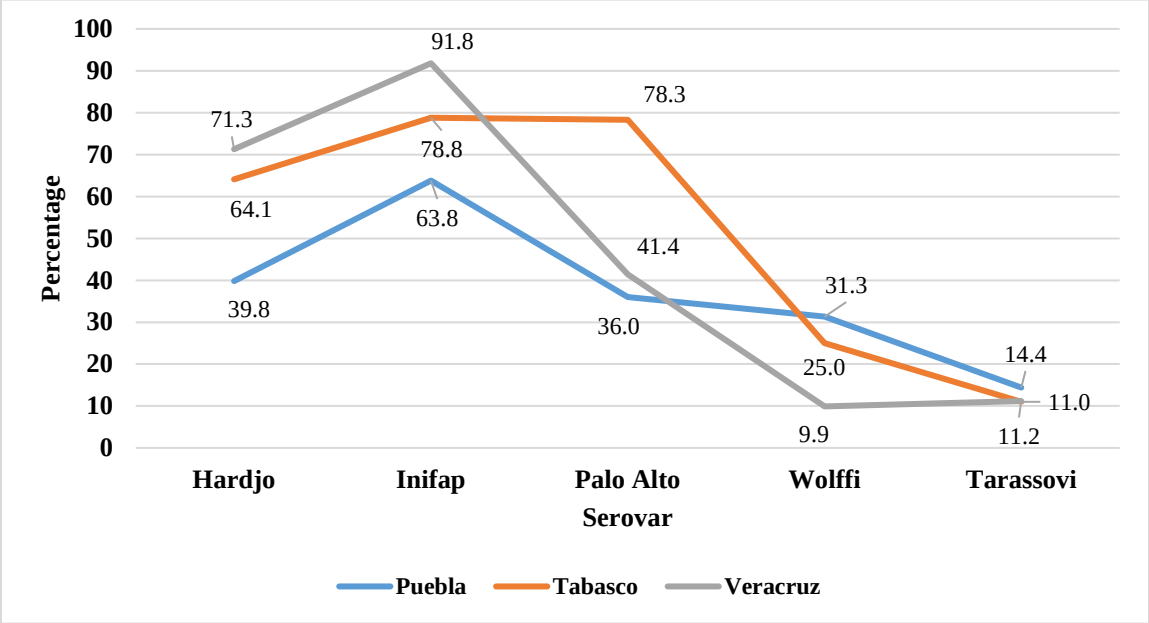
under a dark-field microscope. All sera that showed 50 % or more agglutination at a dilution of 1:100 or more were considered positive. When a serum reacted to two or more antigens, the highest serum titer was taken for the analysis of the data.

Seven response variables were analyzed: prevalence of antibodies against the serovars Hardjo, Inifap, Palo Alto, Wolffi and Tarassovi; number of serovars per cow; and pregnancy rate. Antibody positivity was recorded as 1 when a cow had antibodies against a particular *Leptospira interrogans* serovar (Hardjo, Inifap, Palo Alto, Wolffi or Tarassovi), while antibody negativity was recorded as 0. Like each of the five antibody prevalences, the pregnancy rate was also recorded as a binary variable. The pregnancy rate was coded as 1 when a cow was pregnant at the diagnosis by rectal ultrasonography; otherwise (not pregnant), this reproductive variable was coded as 0.

The study was conducted under a completely randomized design. In order to determine the effect of the factors state of the Mexican Republic, municipality nested within state of the Mexican Republic, and ranch nested within state of the Mexican Republic x municipality on the prevalences of antibodies against the serovars of *Leptospira interrogans*, a logistic regression model was used. The analyses were performed with the GENMOD procedure (PROC GENMOD) of the SAS program[8], considering a binomial distribution and applying a logit link function. The number of serovars per cow was analyzed in a very similar way to the prevalences, with the only difference that a Poisson distribution was considered, since this variable is of the counting type. The pregnancy rate was also analyzed by logistic regression, with the GENMOD procedure of SAS[8], considering a binomial distribution and applying a logit link function. For the analysis of this reproductive variable, the statistical model included health status of the cow, state of the Mexican Republic, and municipality nested within state of the Mexican Republic as fixed effects. Cow's health status was defined as the presence/absence of antibodies against any of the five serovars of *Leptospira interrogans*. When a cow presented antibodies against at least one of the five serovars, the health status was recorded as seropositive; when a cow did not present antibodies against any of the serovars of *Leptospira interrogans*, the animal health status was recorded as seronegative. The convergence criterion was 10^{-8} in the seven statistical analyses.

The prevalence of the Hardjo serovar in the states of Tabasco and Veracruz was higher ($P<0.05$) than in the state of Puebla (64.1 and 71.3 % *vs* 39.8 %; Figure 1). The prevalence of the Inifap serovar was higher ($P<0.05$) in the state of Veracruz than in the states of Puebla and Tabasco, with values of 91.8, 63.8 and 78.8 %, respectively. These values are considerably higher than those reported in the scientific literature for the Hardjo serovar identified in cattle from the states of Campeche[9], Oaxaca[10,11], Yucatán[12-14], Veracruz[15,16], Tamaulipas[17] and the State of México[18].

Figure 1: Prevalences of *Leptospira interrogans* serovars, by state



The prevalence of the Palo Alto serovar was higher ($P<0.05$) in the state of Tabasco than in the states of Puebla and Veracruz (78.3 *vs* 36.0 and 41.4 %). The prevalence of the Palo Alto serovar for the state of Veracruz reported in the present study is two times higher than that found (19.8 %) in a previous study conducted in the south of this same state[16]. The average prevalence of the Palo Alto serovar (51.9 %) obtained in the present study is similar to that reported by Ramos *et al*[10] for the state of Oaxaca (57.1 %), but much higher than those reported (8.8, 8.0, 3.1, 1.6, 1.0 and 0.0 %) by other authors for other states of the Mexican Republic[13,14,19,20,21].

The prevalence of the Wolffi serovar in the state of Puebla was higher ($P<0.05$) than in the state of Veracruz (31.3 *vs* 9.9 %); the prevalence of the Wolffi serovar in the state of Tabasco (25.0 %) was similar to the prevalences found in the states of Puebla and Veracruz. The prevalence of the Wolffi serovar obtained in the present study for the state of Veracruz is similar to the corresponding average prevalence (11.0 %) found in a previous study conducted in four municipalities in the south of this state[16]. In several studies carried out in other states of the Mexican Republic (Yucatán, Oaxaca, Estado de México), low prevalences (less than 10 %) for the Wolffi serovar have also been found[11,12,14,18]. On the contrary, the prevalences of the Wolffi serovar (77.7 and 66.0 %) reported by Córdova *et al*[9] and Luna *et al*[19] are at least two times higher than the Wolffi serovar prevalences reported in the present study for the states of Puebla and Tabasco.

The prevalences of the Tarassovi serovar found in the states of Puebla, Tabasco and Veracruz were similar to each other (P<0.01), with values of 14.4, 11.0 and 11.2 %, respectively. These prevalences are similar to those previously reported for the states of Veracruz[16] and Tamaulipas[17], but much lower than those reported by Cárdenas-Marrufo *et al*[12], Segura-Correa *et al*[13] and Luna *et al*[19], who reported prevalences for the Tarassovi serovar with values of 66.6, 53.6 and 53.3 %, respectively. On the contrary, other authors have reported prevalences for this same serovar less than 8.0 %[9,10,11,18,21].

The average prevalences of the Hardjo, Inifap, Palo Alto, Wolffi and Tarassovi serovars were 58.4, 78.1, 51.9, 22.1 and 12.2 %, respectively, indicating that the Inifap serovar was the most frequent, while the Wolffi and Tarassovi serovars were the least frequent. This order of magnitude of the prevalences is similar to that found in Oaxaca[11] and Campeche[9]. On the contrary, in Tamaulipas, Veracruz and Estado de México, Wolffi, Tarassovi and Hardjo serovars were found to have similar prevalences[16-18]. In the state of Yucatán, a higher prevalence was determined for the Tarassovi serovar (53.6 %) than for the Hardjo (31.6 %) and Wolffi (9.4 %) serovars[12], a result that also differs from that obtained in the present study.

The number of *Leptospira interrogans* serovars present in the cows from the state of Veracruz was greater (P<0.05) than the number of serovars present in the cows from the state of Puebla (2.23 *vs* 1.68); the number of serovars of *Leptospira interrogans* present in the cows from the state of Tabasco was intermediate (2.05). In general, it was expected to find a higher prevalence of each of the serovars studied and a higher number of serovars per cow in the states of Veracruz and Tabasco than in the state of Puebla, since there is greater rainfall and environmental temperature in the states of Veracruz and Tabasco than in the state of Puebla; however, the Hardjo serovar was the only serovar with higher prevalence in Veracruz and Tabasco in relation to Puebla (Figure 1). In a study comparing the prevalence of leptospirosis from the different ecological regions of Mexico, it was found that the prevalence was higher in the dry tropical and humid tropical regions than in the arid/semi-arid and temperate regions[19]; however, this study lacked a formal statistical analysis. Vinetz[22] reported that Leptospirosis occurs most often in countries with tropical climate, where rainfall and environmental temperature are higher.

In the state of Puebla, the municipalities of Ayotoxco, Hueytamalco and San José Acateno had higher prevalences of the Hardjo serovar (P<0.05) than the municipality of Nauzontla. In the state of Tabasco, the prevalences of the Hardjo serovar in the municipalities of Cunduacán, Huimanguillo and Ranchería El Puente were similar (P>0.05; Table 1). In the state of Veracruz, the municipalities of Cotaxtla, Medellín and San Rafael had similar prevalences of the Hardjo serovar.

Table 1: Least squares means and standard errors for prevalence (%) and number of *Leptospira interrogans* serovars, by municipality

Municipality	Serovar					Number
	Hardjo	Inifap	Palo Alto	Wolffi	Tarassovi	
Ayotoxco	65.0 ± 10.7^{ab}	90.0 ± 6.7^{ab}	60.0 ± 11.0^{ab}	80.0 ± 8.9^{a}	35.0 ± 10.7^{a}	3.30 ± 0.41^{a}
Hueytamalco	49.7 ± 5.0^{ab}	68.9 ± 4.7^{b}	21.4 ± 4.6^{c}	6.9 ± 4.1^{c}	NE	1.36 ± 0.12^{e}
Nauzontla	7.7 ± 7.4^{c}	15.4 ± 10.0^{c}	46.2 ± 13.8^{b}	38.5 ± 13.5^{b}	NE	1.08 ± 0.29^{e}
San José Acateno	67.5 ± 7.3^{ab}	77.9 ± 5.9^{b}	17.8 ± 5.6^{c}	20.9 ± 5.7^{bc}	5.0 ± 3.4^{c}	1.77 ± 0.18^{d}
Xochitlán	28.6 ± 17.1^{bc}	57.1 ± 18.7^{bc}	42.9 ± 18.7^{b}	28.6 ± 17.1^{bc}	NE	1.57 ± 0.47^{d}
Cunduacán	41.7 ± 14.2^{abc}	58.3 ± 14.2^{b}	41.7 ± 14.2^{b}	NE	NE	1.42 ± 0.34^{de}
Huimanguillo	63.8 ± 6.8^{ab}	78.4 ± 7.6^{b}	86.8 ± 5.1^{a}	25.0 ± 10.8^{bc}	13.2 ± 5.5^{abc}	2.24 ± 0.22^{bcd}
El Puente	81.8 ± 11.6^{a}	90.9 ± 8.7^{ab}	90.9 ± 8.7^{a}	NE	9.1 ± 8.7^{bc}	2.73 ± 0.50^{a}
Cotaxtla	67.1 ± 9.9^{ab}	89.4 ± 5.5^{ab}	70.1 ± 7.6^{a}	NE	27.7 ± 11.5^{ab}	2.56 ± 0.26^{ab}
Medellín	81.7 ± 5.8^{a}	95.4 ± 3.2^{a}	40.6 ± 8.0^{b}	6.5 ± 3.8^{c}	9.1 ± 4.4^{bc}	2.34 ± 0.23^{bc}
San Rafael	62.6 ± 6.6^{ab}	88.3 ± 4.6^{ab}	18.0 ± 5.3^{c}	14.8 ± 4.9^{bc}	5.0 ± 3.4^{c}	1.86 ± 0.18^{cd}

^{a,b,c,d,e} Means with different literal within columns are different (P<0.05).
NE= not estimable.

In the state of Puebla, the municipalities of Ayotoxco, Hueytamalco and San José Acateno had higher (P<0.05) prevalences of the Inifap serovar than the municipality of Nauzontla. Cunduacán, Huimanguillo and Ranchería El Puente, municipalities in the state of Tabasco, had similar (P>0.05) prevalences of the Inifap serovar. Similarly, the municipalities of Cotaxtla, Medellín and San Rafael showed no difference (P>0.05) in the prevalence of the Inifap serovar (Table 1).

The municipalities of Ayotoxco, Nauzontla and Xochitlán, in the state of Puebla, had higher (P<0.05) prevalences of the Palo Alto serovar than the municipalities of Hueytamalco and San José Acateno. In Tabasco, the prevalences of the Palo Alto serovar in the municipalities of Huimanguillo and Ranchería El Puente were higher (P<0.05) than in the municipality of

Cunduacán. In the state of Veracruz, the municipality of Cotaxtla showed a higher (P<0.05) prevalence of the Palo Alto serovar than the municipalities of Medellín and San Rafael (Table 1). A higher (P<0.05) prevalence of the Wolffi serovar was found in the municipality of Ayotoxco than in the other municipalities of the state of Puebla. The prevalences of the Wolffi serovar found in the three municipalities of the state of Veracruz were similar (P>0.05; Table 1).

In the state of Puebla, the municipality of Ayotoxco had a prevalence of the Tarassovi serovar similar (P>0.05) to that of San José Acateno. The prevalences of the Tarassovi serovar found in the state of Tabasco were not different from each other (P>0.05). In the state of Veracruz, the prevalence of the Tarassovi serovar in the municipality of Cotaxtla was higher (P<0.05) than in the municipality of San Rafael (Table 1). Apparently, this is the first time that the prevalence of these five serovars has been reported for these eleven municipalities in the states of Puebla, Tabasco and Veracruz, since no scientific publications on the subject were found in the literature.

The number of *Leptospira interrogans* serovars found in the municipality of Ayotoxco was greater (P>0.05) than the number of serovars found in the other municipalities of the state of Puebla. A higher (P<0.05) number of serovars was found in the municipality of Ranchería El Puente than in the municipalities of Cunduacán and Huimanguillo. The number of serovars found in the municipality of Cotaxtla was greater (P<0.05) than the number of serovars found in the municipality of San Rafael (Table 1).

Cows with antibodies against *Leptospira interrogans* had similar (P>0.05) pregnancy rate to cows without antibodies (55.5 *vs* 51.5 %); there was also no difference (P>0.05) in the pregnancy rate between states, or between municipalities (Table 2). In accordance with this result, it has been reported that *Leptospira interrogans* is not related to the generation of follicular cysts, which negatively affect the fertility of cows[23].

Table 2: Pregnancy rates (%) and their respective standard errors and 95 % confidence intervals (95 % CI) by animal health status of the cow, state of the Mexican Republic and municipality

Effect/level	Pregnancy rate	95 % CI
Animal health status		
Seronegative[a]	51.5 ± 8.5[c]	35.2 – 67.4
Seropositive[b]	55.5 ± 3.7[c]	48.1 – 62.6
State of the Mexican Republic		
Puebla	51.4 ± 6.4[c]	38.9 – 63.7
Veracruz	55.5 ± 5.8[c]	44.0 – 66.5
Municipality		
Ayotoxco de Guerrero	48.2 ± 11.8[c]	26.9 – 70.2
Hueytamalco	51.2 ± 6.0[c]	39.7 – 62.7
Nauzontla	38.0 ± 13.5[c]	16.7 – 65.3
San José Acateno	48.8 ± 7.0[c]	35.6 – 62.2
Xochitlán	69.8 ± 18.0[c]	30.2 – 92.5
Cotaxtla	49.4 ± 9.0[c]	32.5 – 66.4
Medellín de Bravo	68.0 ± 8.1[c]	50.6 – 81.6
San Rafael	48.4 ± 7.3[c]	34.6 – 62.5

[a]Seronegative= absence of antibodies against *Leptospira interrogans*.
[b]Seropositive= presence of antibodies against *Leptospira interrogans*.
[c]Pregnancy rates are not different ($P>0.05$).

In conclusion, the prevalences of the Hardjo and Inifap serovars were higher in the state of Veracruz than in the state of Puebla, but the prevalence of the Wolffi serovar was higher in the state of Puebla than in the state of Veracruz. The prevalences of the Hardjo and Palo Alto serovars were higher in the state of Tabasco than in the state of Puebla, but there were no differences between these two states in the prevalences of the Inifap and Wolffi serovars. The number of serovars of *Leptospira interrogans* in the state of Veracruz was greater than in the state of Puebla, but the number of serovars in the state of Tabasco was intermediate; in addition, there was important variation in the prevalence of the different serovars of *Leptospira interrogans* between municipalities and between ranches. Overall, the serovar with the highest frequency was Inifap, while the serovar with the lowest frequency was Tarassovi. The health status of the cows did not influence their fertility; however, farmers in the municipalities evaluated should vaccinate against *Leptospira interrogans*, as a preventive measure to reduce the risks associated with this bacterium in cattle and humans.

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