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Assessment of the degree of concordance between the results of histopathological examination and bacterial culture in the diagnosis of bovine tuberculosis in Mexico

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Abstract

Bovine tuberculosis is a complex disease that is difficult to diagnose, control and eradicate and negatively impacts many farms. The objective of this study was to assess the degree of concordance between the results of histopathological examination and bacterial culture in the diagnosis of bovine tuberculosis lesions obtained in the laboratories certified by the Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (Ministry of Agriculture, Animal Production, Rural Development, Fisheries and Feeding) at the national level between January 2009 and December 2012 in Mexico. Tissue samples (10,818) from regular slaughter cattle that did not have tuberculin tests but had lesions suspected of having been caused by tuberculosis were sent to 10 authorized laboratories. Using Cohen's kappa to measure the reliability of the diagnosis, a general concordance was obtained between the histopathological examination and bacterial culture results with a kappa of 0.634 and a 95% (0.618 – 0.650) confidence interval (CI), which shows good concordance between the two techniques at the national level. The laboratory in Chihuahua had the highest kappa [k=0.784, 95% CI (0.754–0.814)] and the laboratory of the La Laguna Region in the state of Coahuila had a low value of global concordance [k= 0.334, 95% CI (0.257–0.412)]. The number of positive samples in bacterial culture was low in Tamaulipas (21 samples), Sonora (41 samples), and Yucatán (45 samples) because tuberculosis prevalence in those states is ≤ 0.04%. The reason for the disagreement between the two tests among some laboratories includes factors such as a lack of economic resources, infrastructure or personnel training. Correct sampling procedures, storage facilities, and shipping and sample processes are important in optimizing the bacteriological and histopathological results and obtaining correct diagnoses and differential diagnoses of bovine tuberculosis.

Keywords: Bovine tuberculosis; Concordance; Histopathology; Bacteriology; Cohen's kappa.

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Additional information and declarations can be found on page 10

Introduction

Bovine tuberculosis causes significant economic losses to farmers. Over 50 million cattle worldwide are estimated to be infected, generating an annual loss of 3 billion U.S. dollars due to costs associated with diagnostic tests, the slaughter of infected animals, the loss of commercial agreements, restrictions on movement, compensation payments, the maintenance of federal and regional programs of disease control, and research to develop improved control strategies (Waters *et al.*, 2012).

Mexico exports 1.2 million steers annually to the United States, which translates into a foreign exchange of more than 480 million dollars (SAGARPA, 2013). Bovine tuberculosis threatens to restrict this trade. Additionally, 28% of the milk produced nationally is either consumed unpasteurized or processed into dairy products without prior pasteurization, thereby presenting a substantial public health risk (CANATB, 2004; Hernandez, 1998).

Bovine tuberculosis is a complex, chronic disease that is difficult to diagnose, control and eradicate and has an adverse impact on the livestock industry. The causative agent is *Mycobacterium bovis* (*M. bovis*), which affects a wide range of mammals, including human beings (O'Reilly and Daborn, 1995; Acha, 2003).

As part of its efforts to control and eradicate this disease, Mexico has established the National Campaign Against Bovine Tuberculosis (*Mycobacterium bovis*) based on the Mexican Official Norm NOM-031-ZOO-1995, a national statutory guideline. These regulations state that lymph nodes and samples from lesions suggestive of tuberculosis from cattle that are reactive to the tuberculin test at routine slaughter are to be collected and sent to SAGARPA (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación)-authorized laboratories for the diagnosis of bovine tuberculosis via histopathological and bacteriological tests. After the isolation and identification of *M. bovis*, the Dirección General de Salud Animal del Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA) is notified. SENASICA then implements various strategies to locate the origin of the infection and control the disease.

Histopathological and bacteriological tests are the most reliable methods for detecting mycobacteria in animals with tuberculosis and other mycobacterioses. However, the sensitivity and specificity of these tests is affected by several factors, ranging from correct sampling, the medium used for transportation, the processing time of the sample, methods of sample disinfection for bacteriological culture, the amount of viable mycobacteria, and the inactivation of mycobacteria triggered by the host immune system, among others (Albernaz *et al.*, 2014; Pinto *et al.*, 2002).

The confirmation of the disease via histopathology and bacteriological culture is invaluable to the Epidemiological Surveillance System of Bovine Tuberculosis of the general cattle population (Kaneenea *et al.*, 2006; Frankena *et al.*, 2007; Shittu *et al.*, 2013). In this context, the degree of concordance between histopathological and bacteriological tests is of the utmost importance when the tests are used in the diagnosis of a notifiable disease, as in the case of bovine tuberculosis (*Office International des Epizooties* list of diseases, OIE, http://www.oie.int/en/).

Cohen's kappa coefficient is used to measure the agreement between tests and indicates the association between categorical and binary variables. It is an estimate of agreement beyond chance. This statistical test has been used in the medical field to measure the reliability and validity of a diagnosis and to estimate the risk factor between tests (Kraemer *et al.*, 2002).

The objective of this study was two-fold: 1) to assess the degree of concordance between the results of histopathological examination and bacterial culture of lesions suggestive of tuberculosis sampled by inspectors at slaughtering facilities and sent to SAGARPA-authorized laboratories between January 2009 and December 2012, and 2) to determine whether the degree of concordance remained constant over the course of those years.

Material and methods

Data

The Dirección de Campañas Zoosanitarias of SENASICA collected information on histopathological and bacteriological results provided by SAGARPA-authorized laboratories from the following locations: Chiapas, Chihuahua, La Laguna Region, Jalisco, Monterrey, Sonora, Tabasco, Tamaulipas, Veracruz, and Yucatán. These laboratories are authorized to make the formal diagnosis of bovine tuberculosis and handle the largest quantities of samples.

The dataset analyzed consisted of the results of tissue samples (10,818) from beef cattle, dairy cattle or crossbred animals with lesions suggestive of tuberculosis found at routine slaughter and collected between January 2009 and December 2012.

Tissue samples

Sampling for histopathological and bacteriological studies of organs showing lesions or secretions compatible with or suggestive of tuberculosis is conducted according to NOM-031-ZOO-1995 and the 2015 epidemiological guidelines (SENASICA, 2015) as follows:

a) Lymph nodes: head, prescapular, anterior and posterior mediastinal, and right and left bronchial lymph nodes are sampled; mesenteric lymph nodes are sampled for miliary tuberculosis.
b) Lungs: caseous, calcified lesions are sampled. Samples must be taken to include 2 cm of tissue around the lesion.
c) Uterus: organ and exudate (yellow pus having the appearance of curdled milk) samples must be taken.
d) Other tissues: spleen, liver, kidney, bone marrow, ovaries, testes, and mammary gland.

Histopathological diagnosis

Non-laminated tissue samples measuring 2 cm^3 and with the fat removed were set in formalin and processed via embedded paraffin to obtain the histological cuts, which were stained with hematoxylin-eosin and observed through an optical microscope. The results were:

1. Negative for tuberculosis: no typical granulomatous lesions or mycobacteria were observed.

2. Suggestive of tuberculosis: typical granulomas were observed, but no acid-alcohol-resistant bacteria were detected.

A histopathological diagnosis aids in the detection of tuberculosis when macroscopic or microscopic lesions are observed in a tissue sample.

Bacteriological diagnosis

Non-laminated tissue samples measuring 2 cm^3 and with the fat removed were submerged in a saturated solution of sodium borate and analyzed. The samples were examined via microscopy and bacterial culture. The tissues were homogenized, decontaminated and inoculated in Stonebrink medium and Lowenstein-Jensen medium. The inoculated tubes were incubated at 37 °C for 9 weeks, and weekly records were kept for the time of bacterial growth and pigment formation. Generally, atypical bacteria grew in 7-21 days, whereas *Mycobacterium bovis* grew after the fourth week of incubation.

Both tests, histopathology and bacterial culture, were performed in parallel.

Statistical analyses

Cohen's kappa statistic was used to assess the concordance between the results of the histopathological examination and bacteriological culture. However, because the value of kappa can be affected by the prevalence of the feature under study, minimum and maximum values were also calculated, as suggested by Lantz *et al.* (1996). The Jackknife technique was used to calculate the confidence interval for kappa (Efron, Tibshirani, 1993). No assumption was made on which of the two diagnostic tests was better.

A comparison of kappa coefficients between the years studied was also carried out using the homogeneity score test of kappa to determine whether the kappa coefficients varied over the years ($\alpha = 0.05$). The kappa statistic has a Chi-Square distribution and the comparison between Cohen's kappa coefficients has a null hypothesis (H_0) that states that all kappa coefficients that are compared are equal. The Epidat Analysis software version 4.0 was used (Organización Panamericana da Saúde, 2014).

Results and discussion

Table 1 shows the total number of samples sent to SAGARPA-authorized laboratories for the diagnosis of *M. bovis*. The results for both diagnostic tests from the 10 certified laboratories appear in Table 2. The highest frequencies of sample submission were for the states of Jalisco, Chihuahua, and Veracruz (Table 1) for the simple reason that these states, along with Chiapas, have the highest population of cattle in the country. However, the state of Veracruz, with a population close to 3.7 million cattle, received less samples than the state of Jalisco (Table 1), with a population of 3 million cattle (Servicio de Información Agroalimentaria y Pesquera –SIAP– http://www.siap.gob.mx/poblacion-ganadera/). This is probably related to the fact that the

Table 1. Total samples from routine slaughter received at certified laboratories from 2009 to 2012.

Laboratory	2009	2010	2011	2012	Total
Chiapas	215	187	270	193	865
Chihuahua	553	325	625	562	2065
Jalisco	557	746	844	413	2560
Laguna Region	93	91	61	84	329
Monterrey	29	235	364	326	954
Sonora	251	262	92	194	799
Tabasco	188	150	324	156	818
Tamaulipas	91	74	39	131	335
Veracruz	364	236	519	419	1538
Yucatán	87	91	217	160	555
Total	2,428	2,397	3,355	2,638	10,818

state of Jalisco, where 10.6% of the country's total dairy cattle population is found, has a higher prevalence of bovine tuberculosis (0.067-0.25%) than the state of Veracruz (.003-.046%), which has only 1.5% of the country's total dairy cattle population (SAGARPA http://www.senasica.gob.mx/?id=6246).

Using the data from Tables 1 and 2, the percentages of positive results to histopathology, bacterial culture and to both tests were calculated (Table 3). For every 100 samples received, 39 were positive to both histopathology and bacterial culture at the laboratory in the state of Jalisco and only 22 at the laboratory in the state of Veracruz (Table 3). Also, two states with approximately the same amount of cattle (1.5 million), Tabasco and Tamaulipas, showed very different results. The laboratory in the state of Tabasco received more than double the amount of samples the laboratory in the state of Tamaulipas did; 12% of samples were positive to tuberculosis in the former and 3% in the latter (Table 3). The state of Tabasco has a tuberculosis prevalence of 0.05-0.36% and the state of Tamaulipas, 0.038% (SAGARPA http://www.senasica.gob.mx/?id=6246).

M. bovis was identified in 2,499 (206 + 2,293; Table 2) out of 10,818 suspicious samples (23.1%, Table 3) via bacterial culture. This result is in accordance with the findings in Brazil (Araújo *et al.*, 2005), where out of a total of 72 samples, 23.6% were positive according to bacterial culture. In Ecuador, 36.3% of samples suggestive of tuberculosis were positive after culturing (Pérez, 2011).

The laboratories with the highest positive results to bacterial culture were those from the state of Jalisco (41.2%, Table 3) and the La Laguna Region (29.5%, Table 3). The states of Jalisco and Coahuila (where La Laguna Region is located) have 329,189 and 438,048 head of dairy cattle, respectively (SIAP http://www.siap.gob.mx/poblacion-ganadera/), the highest population in the country, and the prevalence of tuberculosis is 0.03-0.46% for Jalisco and 0.07- > 4% for La Laguna Region (SAGARPA http://www.senasica.gob.mx/?id=6246). Pérez *et al.* (2008) have indicated that the prevalence of tuberculosis in dairy cattle can reach 16%.

On the other hand, the laboratories with the lowest positive results were those from the states of Sonora (5.1%, Table 3) and Tamaulipas (6.3%, Table 3). These states are important for their beef cattle production and the prevalence of tuberculosis is 0.04% (SAGARPA http://www.senasica.gob.mx/?id=6246).

Table 2. Results from histopathological (H) and bacteriological (B) tests provided by certified laboratories from 2009 to 2012.

	2009				2010				2011				2012				Total			
	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B	H/B
Laboratory	-/-	-/+	+/-	+/+	-/-	-/+	+/-	+/+	-/-	-/+	+/-	+/+	-/-	-/+	+/-	+/+	-/-	-/+	+/-	+/+
Chiapas	135	0	39	41	100	0	35	52	108	7	74	81	109	12	23	49	452	19	171	223
Chihuahua	379	6	29	139	235	0	25	65	426	3	68	128	409	5	43	105	1,449	14	165	437
Jalisco	188	5	112	252	292	30	136	288	318	7	182	337	163	2	115	133	961	44	545	1,010
Laguna Region	4	3	61	25	33	2	36	20	25	1	10	25	57	1	6	20	119	7	113	90
Monterrey	0	3	19	7	221	12	2	0	325	7	20	12	299	3	10	14	845	25	51	33
Sonora	227	4	8	12	254	5	2	1	83	6	0	3	179	8	5	2	743	23	15	18
Tabasco	84	6	60	38	89	17	12	32	269	17	12	26	142	3	9	2	584	43	93	98
Tamaulipas	90	0	1	0	61	1	9	3	31	4	3	1	106	6	13	6	288	11	26	10
Veracruz	206	1	40	117	117	1	45	73	348	3	68	100	316	4	49	50	987	9	202	340
Yucatán	70	1	14	2	77	1	10	3	161	1	33	22	138	8	7	7	446	11	64	34
Nationwide	1,383	29	383	633	1,479	69	312	537	2,094	56	470	735	1,918	52	280	388	6,874	206	1,445	2,293

Test result: (-) negative, (+) positive.

Table 3. Percentage of positive samples to histopathology, bacterial culture and to both tests from 2009 to 2012.

Laboratory	Samples received	Samples positive to histopathology	%	Samples positive to bacterial culture	%	Samples positive to both tests	%
Chiapas	865	394	45.5	242	28.0	223	25.8
Chihuahua	2,065	602	29.1	451	21.8	437	21.2
Jalisco	2,560	1,555	60.7	1,054	41.2	1,010	39.4
Laguna Region	329	203	61.7	97	29.5	90	27.4
Monterrey	954	84	8.8	58	6.1	33	3.5
Sonora	799	33	4.1	41	5.1	18	2.2
Tabasco	818	191	23.3	141	17.2	98	12.0
Tamaulipas	335	36	10.7	21	6.3	10	3.0
Veracruz	1,538	542	35.2	349	22.7	340	22.1
Yucatán	555	98	17.7	45	8.1	34	6.1
Total	10,818	3,738	34.5	2,499	23.1	2,293	21.2

Upon histopathological examination, 34.5% of the samples (Table 3) were positive for tuberculosis; the minimum value was 4.1% from the laboratory in Sonora and the maximum value was 61.7% from the laboratory in La Laguna Region (Table 3). In Argentina, Latini *et al.* (1997) found 81.8% of 248 samples to be positive for tuberculosis upon histopathological examination. However, Pinto *et al.* (2002) reported a low sensitivity for the histopathology test (38.7% positive results). Latini *et al.* (1997) indicated that the sensitivity of histopathology can be affected by the technique used, the number of slides examined, the manner in which the sample is collected, and how it is shipped and preserved.

The quantification of the degree of concordance between the histological and bacteriological tests enables an overview of the degree of agreement between these two tests. Low values of kappa indicate discrepancy probably due to detection of infection through different mechanisms that could be interpreted as false positive and false negative results, whereas high values indicate accuracy in the diagnosis. Estrada-Chávez *et al.* (2004) stated that false positive results generate economic and social problems because a misleading result can contribute to the dissemination of the disease.

The concordance results are usually classified according to the following scale (Gilchrist, 2009):

a) -1 to 0.00, entirely inadequate concordance
b) 0.01 to 0.39, insufficient concordance
c) 0.40 to 0.75, good concordance
d) 0.76 to 0.99, excellent concordance
e) 1, perfect concordance

The average Cohen's kappa statistic for the national concordance between the histopathological examination and bacterial culture of lesions suggestive of tuberculosis was k = 0.634 (good concordance) with a 95% confidence interval of 0.618–0.650 (Table 4). This value was greater than that obtained in Ecuador (k = 0.49, Pérez *et al*., 2011) and Argentina (k = 0.48, Latini *et al*., 1997).

However, states varied in their Cohen's kappa statistic. Chihuahua's laboratory showed excellent concordance (k = 0.784), whereas concordance was insufficient in two laboratories, La Laguna Region (k = 0.334) and Tamaulipas (k = 0.295). The rest of the laboratories showed good concordance.

The differences between laboratories could be due to several factors, such as general handling of the samples at shipping, the duration between sampling and laboratory processing, a low number of viable mycobacteria, a lack of economic resources, inadequate infrastructure and deficiencies in the training of laboratory personnel. However, as mentioned above, the histopathology test may not be reliable [as Pinto *et al.* (2002) reported (38.7% positive results)].

Additionally, the low prevalence of tuberculosis (<0.1 to <0.01%) in 25 regions could also explain the low concordance results obtained in some laboratories. If prevalence is low then the number of true "negative" results will be high, as will be the probability that both histopathology and bacterial culture classify the samples as "negative" (United States Department of Agriculture [USDA], 2009). Because the kappa statistic eliminates the influence of chance, low kappa values (0.115) will

Table 4. General kappa, 95% confidence interval, and minimum and maximum kappa.

	95% confidence interval				
Laboratory	General kappa	Lower limit	Upper limit	Minimum kappa	Maximum kappa
Chiapas	0.543	0.489	0.596	-0.123	0.581
Chihuahua	0.784	0.754	0.814	-0.045	0.828
Jalisco	0.556	0.527	0.585	-0.128	0.563
Laguna Region	0.334	0.257	0.412	-0.213	0.356
Monterrey	0.423	0.317	0.530	-0.042	0.842
Sonora	0.462	0.317	0.606	-0.024	0.905
Tabasco	0.489	0.416	0.562	-0.091	0.676
Tamaulipas	0.295	0.129	0.461	-0.059	0.782
Veracruz	0.672	0.633	0.712	-0.074	0.731
Yucatán	0.410	0.305	0.515	-0.073	0.735
Nationwide	0.634	0.618	0.650	-0.083	0.702

be obtained. Thus the inclusion in Table 4 of the minimum and maximum values of kappa should aid in the analysis of the data (Lantz *et al.*,1996).

Differential diagnoses should also be considered. For example, Valencia *et al.* (1997) tested 505 samples, 49% of which were positive for mycobacteria; the rest were diagnosed as actinobacillosis, actinomycosis, eosinophilic granuloma, and other diseases.

Chakravorty *et al.* (2005) stated that the diagnosis of tuberculosis changes for several reasons, such as (1) inadequate sample quantity and volume, (2) the use of the sample in several diagnostic tests (histology/cytology, biochemical analysis, microbiology and PCR) may result in a non-homogenous distribution of microorganisms, (3) the paucibacillary nature of the samples, (4) the presence of inhibitors that weaken the performance of techniques based on nucleic acid amplification, and (5) inefficient sample processing techniques worldwide. Shitaye *et al.* (2006) stated that false negative results may be associated with low numbers of mycobacteria that are not evenly distributed throughout the body, in particular in samples infected with *M. bovis.*

Table 5 shows the concordance coefficients for the years 2009 to 2012 for each laboratory. There is sufficient statistical evidence to infer that the national average kappa coefficients have remained constant over many years, as have the average kappa coefficients for the laboratories in Chiapas, Jalisco and Tamaulipas ($P > 0.05$). This is not so for the other laboratories. The laboratories in Chihuahua, Jalisco and Veracruz had more concordance in 2009. The laboratory in Sonora was very inconsistent. 2010 and 2011 were better years for the laboratories in Tabasco and Yucatán. La Laguna Region and Monterrey showed very interesting results. The worst year for the laboratory in La Laguna Region was 2009; 2010 was the worst year for the laboratory in Monterrey. However, they improved markedly in 2011 and 2012. Major decisions and adjustments must have occurred. This type of analysis is thus very useful as a management tool and for quality control.

While conducting a study in the State of Mexico, a high-prevalence area for bovine tuberculosis, Estrada-Chávez *et al.* (2004) obtained a concordance index of

Table 5. Kappa ± standard error (SE) calculated for the years 2009 to 2012.

	2009	2010	2011	2012	
Laboratory	**Kappa ± SE**	**Kappa ± SE**	**Kappa ± SE**	**Kappa ± SE**	***P-value***
Chiapas	0.569 ± 0.05	0.614 ± 0.05	0.559 ± 0.05	0.600 ± 0.06	0.8795
Chihuahua	0.844 ± 0.02	0.790 ± 0.03	0.710 ± 0.03	0.760 ± 0.03	0.0072*
Jalisco	0.590 ± 0.03	0.564 ± 0.02	0.570 ± 0.02	0.470± 0.03	0.0629
Laguna Region	-0.029 ± 0.00	0.254 ± 0.00	0.647 ± 0.09	0.794 ± 0.07	0*
Monterrey	-0.218 ± 0.12	-0.015 ± 0.00	0.433 ± 0.09	0.662 ± 0.08	0*
Sonora	0.641 ± 0.09	0.210 ± 0.18	0.474 ± 0.17	0.201 ± 0.14	0.0322*
Tabasco	0.313 ± 0.05	0.549 ± 0.07	0.591 ± 0.06	0.215 ± 0.14	0.0025*
Tamaulipas	†	0.320 ± 0.15	0.122 ± 0.19	0.310 ± 0.11	0.4302
Veracruz	0.763 ± 0.03	0.610 ± 0.04	0.653 ± 0.03	0.548 ± 0.04	0.0063*
Yucatán	0.162 ± 0.11	0.306 ± 0.14	0.487 ± 0.07	0.162 ± 0.11	0.0272*
Nationwide	0.633 ± 0.01	0.629 ± 0.01	0.643 ± 0.01	0.625 ± 0.01	0.8607

† The laboratory did not obtain any positive bacteriological results for that particular year.

* *P-value* ≤ 0.05: sufficient statistical evidence to reject the null hypothesis of equality.

k = 0.52 between bacterial culture and histopathology for suggestive lesions. This result points to the utility of the concordance index for the definitive diagnosis of tuberculosis in high-prevalence herds, as well as for evaluating other diagnostic tests.

Discrepancies in the degree of concordance between laboratories could be due to several factors. *M. bovis* culture is the most widely used test, but is very time-consuming and requires a particular laboratory infrastructure, as well as special equipment and highly qualified personnel (Bermudez *et al.*, 2010). Thus, the differences in kappa indexes among laboratories could have been due to differences in the reliability of both methods in each laboratory at each time period.

In a study conducted in the state of Baja California, Mexico, 553 samples from suspected cows were analyzed, 268 of which were suggestive of tuberculosis via histopathology. These 268 samples were subjected to (a) a bacteriological test resulting in 104 positive samples and (b) a PCR analysis resulting in 123 positive samples (70 samples were positive via PCR and culture; 53 were positive via PCR). These results indicate the complexity of *M. bovis* diagnosis and the utility of reviewing the official methods of laboratory tuberculosis confirmation in order to include PCR.

Conclusion

This study demonstrated a good average concordance coefficient at the national level between the histopathological and bacteriological tests in the diagnosis of bovine tuberculosis. It also showed that the number of positive samples according to bacterial culture was related to the prevalence of bovine tuberculosis reported by national zoosanitary agencies. However, there is a need for improvement in the procedures associated with the diagnosis of this disease in some laboratories certified by SAGARPA in Mexico. This information, then, may be useful as a decision-making tool for government officials.

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Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

Jacobo Carrisoza Urbina: Collected the data, interpreted the results, and wrote the manuscript.

Estela Flores Velásquez and José A. Gutiérrez Reyes: Collected the data and interpreted the results.

Noé O. Juárez López: Designed the study, performed the statistical analyses, interpreted the results and wrote the manuscript.

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