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Histopathological and PCR *Mycobacterium bovis* Identification in Mexican Opossums (*Didelphis virginiana)* from Hidalgo, Mexico

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Abstract: *Mycobacterium bovis* is the causal agent of bovine tuberculosis, this bacillus affects domestic wild animals as well as human beings, thus considered a zoonosis. *Didelphis virginiana* is the Mexican marsupial in spite of being a wild animal, it has been adapted to live near to human and it can be included in the people's diet. The opossum could be susceptible to be infected by *M. bovis.* There were captured six adult animals in the Mexican state of Hidalgo; the animals were sacrificed using an overdose of sodic pentobarbital; samples were taken from lung, spleen, intestinal tract, liver, mediastinic, retrofaringeal, submaxilar lymph nodes; Necropsy findings and histophatological lesions are suggestive of chronic disease as it occurs in tuberculosis cases. Histopatological study showed calcification perfectly surrounded by dense irregular collagen tissue in which a detachment of capsule is found. Two of the specimens in the study showed positive in tuberculosis, using nested PCR. The present study shows the risk that could exist during the transmission of tuberculosis maybe due to *Didelphis virginiana* coexistence with infected bovine cattle and human.

Key words: *Didelphis virginiana*, *Mycobacterium bovis*, PCR, lung, disease, animals

INTRODUCTION

In Mexico, the bovine Tuberculosis (TB) is associated with both regionalization of the country, considering the levels of prevalence; in the state of Hidalgo, in the center of the country, the prevalence is >0.2% in bovines. *Mycobacterium bovis* is the causal agent of bovine tuberculosis, this bacillus affects domestic as well as human beings, thus considered a zoonosis (Romero *et al*., 2006). The bacterium infects other animal species like wildlife and this range of hosts complicate attempts to control or eradicate the disease (Rodriguez *et al*., 2010).

Mexican opossum (*Didelphis virginiana*) is the only marsupial that inhabits the American continent (Hernandez-Huerta *et al*., 2002; Barrios-Garcia *et al*., 2009) which could be susceptible to be infected by *M. bovis* (Fitzgerald *et al*., 2003) and could represent a risk for some areas of exploitation of bovine (Witmer *et al*., 2010) due to that Mexican opossum cohabits around agricultural fields; it is important to mention that in this region of the country, opossum can be included in the people's daily diet, thus included in the gastronomical annually fair taken place in April at the municipality of Santiago of Anaya. This fair has an attendance of 15000 people average and around 1100 dishes are presented (Bautista, 2008; Barrios-Garcia *et al*., 2009). The aim of the current study was to analyze Mexican opossums as risk factor and transmitter of *Mycobacterium bovis*, identified through PCR and histopathology.

MATERIALS AND METHODS

Mexican opossum capture was in a zone near to domestic animals, especially bovine and sheep cattle as well as at grain harvest time such as corn and sorghum (September to December). Three sampling were done in Santiago Tulantepec and a total of six animals were caught, four females and two males with an average age of 2.6 years and average weight of 1100 g. Captures were done using Tomahawk catch alive traps. Animals were sacrificed using an overdose of sodic pentobarbital

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(Anestesal® Pfizer). Pair samples were taken of each organ for histopathological and molecular analyses (lung, spleen, intestinal tract, liver, mediastinic, retrofaringeal, submaxilar lymph nodes), one was frozen at -20°C and the other fixed in diluted formalin at 10% (Prophet *et al*., 1995). For the histopathological study, the samples were processed by Paraffin-embedded method and 6 μm sections were staining with Hematoxyilin-Eosin (H-E) and Ziehl-Neelsen (ZN) methods.

In order to check the presence of the *M. tuberculosis* complex by PCR, DNA extraction was done; the frozen samples were homogenized in 400 μL of TE buffer with a polytron PT 1200E. The DNA extracting was made with lysozyme (20 mg mL^{-1}) (1 h at 37°C), 10% Sodium Dodecyl Sulphate (SDS) and proteinase K (20 mg mL^{-1}) (10 min at 65°C); 5 M NaCl, CTAB solutions were incubated 10 min at 65°C. The DNA purification was made using organic extraction with chloroform/isoamilic alcohol (24:1). DNA samples were precipitated with pure ethanol solution at -20°C for precipitation. The mixture was then centrifuged at maximum rotations per minute for 10 min. After centrifugation pure ethanol solution removed and DNA was provided with the application of 50 μL TE buffer. The purity and concentration of the DNA was quantified using the Nanodrop spectometer (Nanodrop technologies) (Estrada *et al*., 2004). The DNA integrity was determinated by electrophoresis observations. The amplification of the 372 pb fragment of the *MP70* gen of the *M. tuberculosis* complex were used for the tuberculosis diagnostic in the specimens (Romero *et al*., 2006). The nested PCR was made doing an initial PCR 25 μL of final volume and of 15 mM of Tris-HCL (pH 8.05); 50 mM of KCl; 200 mM of each dNTPs; 2.5 mM $MgCl_2$, 0.4 μM of the primers TB1F 5' GAA CAA TCC GGA GTT GAC AA 3' y TB1R 5' AGC ACG CTG TCA ATC ATG TA 3' (30 cycle of 12' to 96°C, 30" 94°C, 30" 55°C, 1' 72°C) and 500 ng of DNA. A posterior reamplified of 1 μL previous product with oligonucleotide M22-3, 5' GCT GAC GCG TGC ACT GTC GGG C 3' and M22-4, 5' CGT TGG CCG GGC TGG TTT GGC C 3' (94°C/5', 35 cycle of 94°C/1', 65°C/45", 72°C/42", 72°C/10'). The amplified products were analyzed in Agar gel electrophoresis of 2% agarose gel with ethidium bromide.

RESULTS AND DISCUSSION

Animal physical conditions were good in general. The macroscopic lesions found at necropsy and the histopathological changes are shown in the Table 1. In one of the animals studied (Table 1), near 5 mm diameter whitish dots were observed in the dorsal surface of both lungs. At the mediastinal lymph nodes were observed granulomatous lesions mainly at cortical zone.

Table 1: Results by PCR and histopathology description of lesions in the necropsy of tuberculosis diagnostic in opossums

			Histopathological diagnostic	
Opossums identification	Necropsy observations	PCR	HE staining	ZN
1	NVL	-	*	*
2	NVL	-	*	*
3	NVL	+	Cronical interstitial pneumonia	-
4	NVL	-	*	*
5	Whitish dots in lungs, supraclavicular lymph nodes enlarged	+	Encapsulated focal classification	-
6	NVL	-	-	-

The obtained results in the histopatological diagnostic in the opossum 3 years 5 are indication of TB. No Visible Lesion (NVL) were not made histopathology diagnostic because in the necropsy were no founded lesions that indicate TB (*)

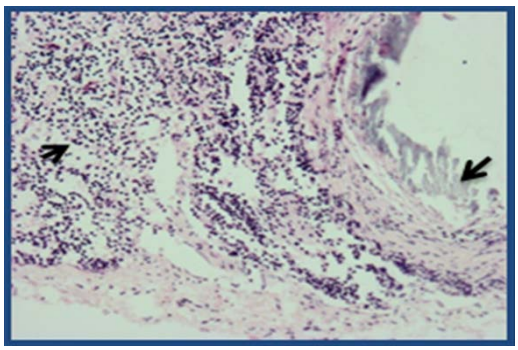

Fig. 1: Opossum five, Mediastinal lymph nodes showed encapsulated focal calcification surrounded by dense collagen tissue perfectly irregular and infiltration of macrophages, lymphocytes (left). H and E staining 100X

At histopatological study with hematoxilin and eosin this animal showed calcification perfectly surrounded by dense irregular collagen tissue as consequence of swollen process in which a detachment of capsule is found. Those injuries could be a suggestion of chronic swollen process as in the tuberculosis case (Fig. 1, opossum 5), although when the Ziehl Neelsen staining no acid alcohol resistant bacillus were found.

Two of the specimens in the study showed positive in tuberculosis, using nested PCR, it was seen in it an amplification product of 208 pb (Fig. 2). In Table 1, the obtained results by PCR, histopathology and the injury description to the necropsy to diagnose tuberculosis in opossums are shown. Meanwhile the rest of the studied specimens do not show amplifications to *MP70* gen. The summary of results is shown in Table 1.

Necropsy findings and histophatological lesions are suggestive of chronic disease as it occurs in tuberculosis cases. It is important to consider the bovine tuberculosis in the state of Hidalgo in its control stage. A factor that is affecting the bovine tuberculosis control programs

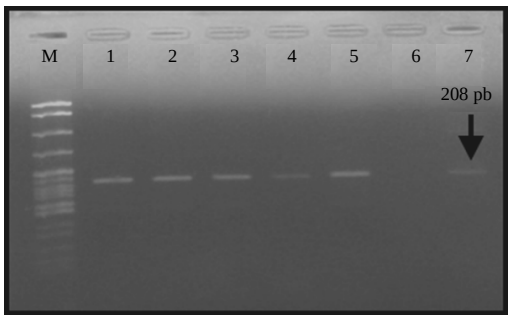

Fig. 2: Results of nested PCR shown positive specimens. M: Molecular weight marker. In the line 1: correspond to opossum number 3: Lung; line 2: lymph node, opossum 5: line 3: Lung; line 4: Mediastinal lymph node; line 5: Retropharyngeal node; line 6: negative control (Water MILI-Q) that corroborated specificity of the test; line 7: Positive control (stock AN5 DNA)

could be the wildlife presence, since they are not subject to governmental control programs as cattle (Woodroffe *et al*., 2006; Witmer *et al*., 2010). Thus, it could be a source of the disease spreading to healthy heads because they are usually feed close to farm animals feeders which makes them liable to get infected of bovine tuberculosis and remain as reservoir of the disease and then to be able to transmit it to humans, since these wildlife are part of human diet. On the other hand, Corner (2006) reported that *M. bovis* detected in cattle and deer was found in wildlife animals (Opossums, deers, pigs) and they concluded that genomic track of isolated *M. bovis* was the same as in bovine cattle. It has been reported that there are two types of susceptible animals to the infection by *M. bovis*, those that act as reservoir host or those which maintain or spread the disease called spillover animals could get infected but occurrence of disease is low among the population of these animals may be due to that the tuberculosis is slow, progressive and that individuals may possess resistance to the disease and only persists inside the population when there is a reservoir host in the bovine tuberculosis ecosystem (Thoen *et al*., 2006). Diegel *et al*. (2002) has been reported in previously studies that animals could develop tuberculosis. There are two very important factors to be considered: Transmission track and inoculated dose necessary to develop the disease but clinically animals may not have symptoms or during necropsy granulomatous injuries could be found which are characteristic of the disease or absence of it. This researcher in experimental studies has observed that the opossum is possible to get infected by *M. bovis*, a dose of 1×10^{7} cfu^{-1} using nasal track. Other studies proved oral and intramuscular track with a dose of 1×10^{5} cfu mL^{-1} of the bacillus. In the case of inoculation VO of 4 inoculated, only one gave positive to culture (Intestine and lymph nodes) while those inoculated IM, 3 of them gave positive to culture (Lymph nodes, liver and kidney); only one animal gave negative to culture in spite of having the characteristic injuries of tuberculosis. This suggests that opossums (*D. virginiana*) were sensible to infection by *M. bovis* by different tracks which occur in a natural way. Nevertheless, their susceptibility is relative; its importance is in acting as spreading species spillover (Fitzgerald *et al*., 2003).

PCR technique has been used now a days as a rapid diagnostic technique and highly sensible compared with the culture techniques. Nested PCR detects even fentograms (Estrada *et al*., 2004), besides its high specificity and that only detects to *M. tuberculosis* species including *M. bovis*; this characteristic makes this technique ideal for tuberculosis diagnosis. It is probable that due to this high sensibility a larger number of positive animals have been identified compared to the histopathological technique (Table 1). From these results, the researchers concluded that opossums are getting infected with the bacillus as shown by PCR tests, may be due to coexistence with infected bovine cattle, it is possible that infective dose needed to cause macroscopic and microscopic lesions observable in these animals could not be reached. In the case of the only animal with such suggesting lesions they could be due to constant reinfections by domestic animals; this coincides with reports where in spite of having identified the opossum as a species susceptible to get infected by *M. bovis* such susceptibility is low compared with host reservoirs, thus this will be catalogued as spreading species that only get infected when tuberculosis incidence when its habitat is high, it can return infected by this way and may transmit infection to other animals of its same species as well as to cattle and human beings (Thoen *et al*., 2006).

It would be interesting to study the state of infection in Mexican opossum in regions with a higher incidence of bovine tuberculosis to be able to evaluate if there is a higher number of infected animals and if they could really be a risk to public health of inhabitants of the state of Hidalgo who consume such animals in their diet.

CONCLUSION

With this study, the researchers have observed that a third of the studied animals were found infected by tuberculosis, even though it has have been reported that infective dose do not reach the necessary level to spread the disease amongst other animals, thus the researchers can not consider as disease reservoirs for the regional

bovines, even though we can not rule out that they represent a public health issue, since the researchers know that they are part of local diet in some communities in this state.

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