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Identification of *Mycobacterium tuberculosis* Complex by Histopathology and PCR in White-Tailed Deer (*Odocoileus virginianus*) in Tamaulipas, Mexico

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Abstract: Bovine tuberculosis is caused by *Mycobacterium tuberculosis* complex which infects a wide hosts range including humans and therefore it is considered as zoonosis. White-tailed deer (*Odocoileus virginianus*) is considered as reservoir of bovine tuberculosis, playing an important role on the disease epidemiology. The aim of this study was to determine the presence of *M. tuberculosis* complex in free-ranging White-Tailed Deer (WTD) in Tamaulipas, Mexico. During the 2009-2010 hunting season, 44 mandibular lymph nodes and tonsils from WTD were collected, processed by routine histopathology, Ziehl Neelsen staining, mycobacterial isolation and PCR. From those, 4.5% had gross changes. Histopathology revealed morphological changes suggestive to tuberculosis such as macrophage aggregation, necrosis, giant cells, mineralization and Bacilli Acid-Alcohol Resistant (BAAR). Abscedative type changes were also observed. The presence of *M. tuberculosis* complex by amplification of DNA from a tissue sample by PCR was confirmed. To the researchers knowledge this is the first mycobacterial report on WTD in Mexico.

Key words: Tuberculosis, *Odocoileus virginianus*, PCR, histopathology, acid, Mexico

INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* complex. It has been reported worldwide in domestic animals and in wildlife and is considered as zoonosis (Quigley *et al*., 1997; Frolich *et al*., 2002; Michel, 2002; Payeur *et al*., 2002; Gortazar *et al*., 2003; Phillips *et al*., 2003; Vicente *et al*., 2007). Bovine Tuberculosis (bTB) caused by *Mycobacterium bovis*, becomes a very important disease because of the economic losses induced on bovine industry. It has been demonstrated that an important obstacle for TB eradication in cattle is the wildlife implication (Naranjo *et al*., 2008). In Mexico, the importance of units for wildlife management and conservation has increased due to the high income generated by the white-tailed deer (*Odocoileus virginianus*) hunting however, many of them still continues to operate the production of cattle sharing space with White-Tailed Deer (WTD) that is why TB could remain in the wild reservoirs (Whipple *et al*., 1997). Different studies have been conducted in several countries to identify the presence of *M. tuberculosis* complex in WTD. In 1994, a free-living WTD in Michigan was diagnosed with tuberculosis caused by *M. bovis* (Schmitt *et al*., 1997). Subsequently, conducted surveys identified an epidemic infection of *M. bovis* in free-living WTD in northeast Michigan (Schmitt *et al*., 1997; Brien *et al*., 2001). Those reports represented the first known reservoir of *M. bovis* in free-living wildlife in the USA and the first known epidemic of tuberculosis in WTD in the world (Schmitt *et al*., 1997). DNA analysis of isolates of *M. bovis* in WTD in Michigan, showed that majority of deer were infected with a common strain suggesting a single source of infection (Whipple *et al*., 1997).

Considering the lack of information in Mexico about the presence of tuberculosis in deer therefore, the demonstration of the presence of *Mycobacterium tuberculosis* complex in WTD becomes of high relevance which will reveal whether these animal species play an important role as a reservoir of TB in livestock diversified farms.

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MATERIALS AND METHODS

A total of 44 samples of lymphoid tissues including mandibular Lymph Nodes (LN) and tonsils from white-tailed deer were collected during the 2009-2010 Winter hunting season. The postmortem inspection was performed to identify gross lesions suggestive of tuberculosis. Samples were divided in two; one of them was laminated and fixed in 10% buffered formalin for histopathological analysis and the other one preserved in 6% sodium borate for bacteriology. Formalin fixed tissues were processed by routine histopathology methods, paraffin-embedded, cut at 6 μm thick sections and stained with Hematoxyilin-Eosin (H and E) and Ziehl-Neelsen (ZN) (Luna, 1968). Von Kossa staining was used to confirm or rule out the presence of calcium in some suggestive cases of calcification.

The presence and type of lesions compatible to tuberculosis were characterized by microscopic examination. The presence of bacilli acid-alcohol resistant was confirmed by microscopical examination of ZN stained slides. The method for bacteriological isolation was done by Petroff decontamination (Diaz-Otero *et al*., 2003). For primary isolation, the sediment was inoculated in Lowenstein-Jensen (LJ) and Stonebrink (ST) media, incubated for 10 weeks at 37°C with a 5% CO_2 pressure. The DNA extraction Paraffin-Embedded Tissue (PET) method to demonstrate the presence of the *M. tuberculosis* complex by PCR was done. Samples were dewaxed and homogenized in 400 μL of TE buffer with a polytron PT 1200E. The DNA extraction was done 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 min NaCl, Cetyl Trimethyl Ammonium Bromide (CTAB) solutions were incubated 10 min at 65°C. The DNA purification was done using organic extraction with chloroform/isoamilic alcohol (24:1).

DNA samples were precipitated with pure ethanol solution at -20°C. The mixture was then 17,000× g per minute for 10 min. After centrifugation, pure ethanol solution was removed and the DNA was provided with the application of 50 μL TE buffer. To determine the presence of *Mycobacterium tuberculosis* complex, PCR was used to type the IS 6110 which amplifies a fragment of 123 bp (Miller *et al*., 2002; Sharma *et al*., 2007) using amplificasa kit (BIOTECMOL®) doing an initial 25 μL PCR of final volume, of 15 mM of Tris-HCl (pH 8.05), 50 mM KCl of, 200 mM of dNTPs Each, 2.5 mM $MgCl_2$, 0.4 μM of the primers F 5 'CTCGTCCAG C GCCGCTTCGG 3' and R 5 'CCTGCGAGCGTAGGCGT CGG 3' (1 cycle of 10'94°C, 50 cycles 94°C 45 '72 2.15°C, 10'72°C) and 500 ng of DNA. A certified block to bTB donated by Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA) of Secretaria de Agricultura, Ganaderia, Desarrollo Rural, Pesca y Alimentacion (SAGARPA) and a second one donated by Universidad Autonoma de Tamaulipas as positive controls were used. The amplified products were analyzed in agar gel electrophoresis gel of 2% agarose with ethidium bromide.

RESULTS AND DISCUSSION

From the 44 samples collected from WTD, 39 were processed for histopathology, in 5 of them formalin fixed tissues were not available. Gross lesions in two samples were observed. Lesions consisted in diffuse areas of green to yellow purulent exudate. Autolytic changes in six processed tissue samples were observed and excluded. From the 33 examined samples, microscopic changes in 11 of them, some compatible to tuberculosis were detected. Changes were observed in mandibular LN and tonsils tissues. Microscopic changes were characterized and grouped in two types. The proliferative or granulomatous type included necrosis, aggregation of macrophages, giant cells, presence or absence of granulomas and calcification. The second was classified as abscedative type such as neutrophil aggregation, mixed aggregation of neutrophils and macrophages and necrosis. The proliferative or granulomatous type was observed in 5 cases, more frequently with aggregation of macrophages, followed by focal and diffuse necrosis, the presence of giant cell, some Langhans-type (Fig. 1) and calcification was confirmed in one case. Typical granulomas with fibroplasia were not observed. The abscedative type was observed in 6 cases with aggregation of neutrophils as

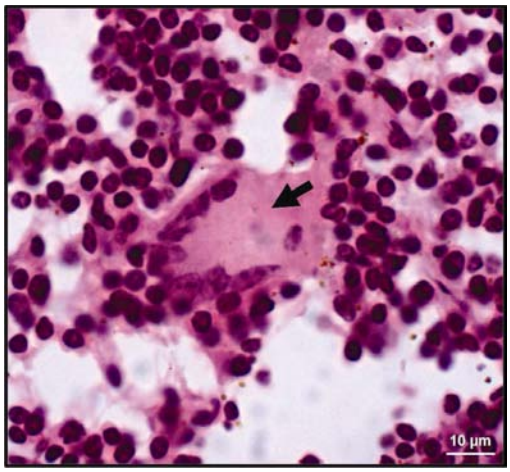

Fig. 1: Mandibular lymphoid section from a white-tailed deer with a Langhans-type multinucleated giant cell (arrow) (H and E)

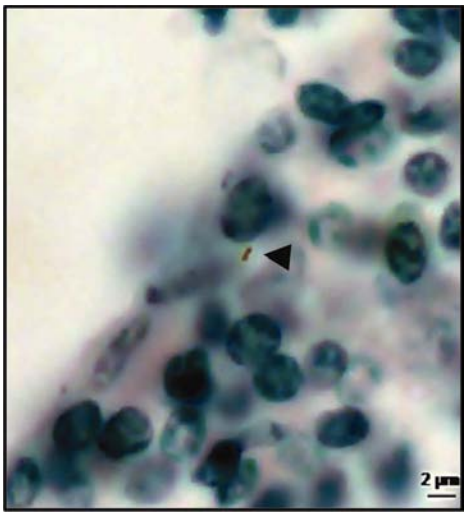

Fig. 2: Mandibular lymphoid section from a white-tailed deer showing a bacilli acid-alcohol resistant (arrowhead) (ZN)

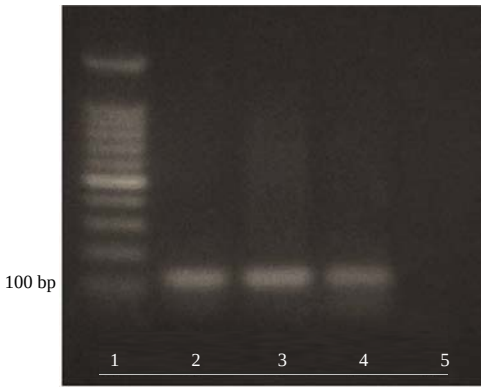

Fig. 3: Gel electrophoresis of IS 6110 amplification products, showing three bands at the locations expected for confirmation of *M. tuberculosis* complex. Lane 1: molecular 100 bp markers; lane 2: SENASICA control positive; lane 3: tissue positive from a cattle; lane 4: tissue positive from a white-tailed deer and 5: negative tissue control

the predominant finding followed by mixed aggregation of neutrophils and macrophages and necrosis. Calcification was not observed. The presence of Bacilli Acid-Alcohol Resistant (BAAR) by Ziehl Neelsen (ZN) was only observed in one case (Fig. 2), associated to a sample with microscopic lesions suggestive of tuberculosis. From the 42 samples processed for bacteriological isolation, there was only one bacterial growth in Stonebrink media, the colony had characteristics of mycobacteria to the 2nd week of being inoculated. A bacterial growth smear was stained with Ziehl Neelsen and confirmed the presence of BAAR. However, because of fungal contamination, the sample could not be identified by biochemical tests. From the embedded paraffin tissue of the case in which mycobacterial growth occurred, the PCR was performed and tested positive to tuberculosis, amplification product of 123 bp was detected (Fig. 3). The PCR positive sample was associated to microscopic changes suggestive of tuberculosis as focal necrosis, aggregation of macrophages, Langhans-type multinucleated giant cells (Fig. 1) and the presence of BAAR in ZN (Fig. 2).

The gross lesions observed in 4.5% of LN of the head of WTD in this study, results higher than in similar studies in Michigan, United States where 3.5% (Schmitt *et al*., 1997) or 0.6% (Palmer *et al*., 2002) of gross lesions of the head were described in WTD. The percentage of gross lesions is also higher than in another similar study in Ontario, Canada where only 0.2% of WTD had gross lesions in the pleura (Belli, 1962). The high proportion of gross lesions of this study could be associated to a small number of samples tested, compared with more extensive sampling and populations of more than 10,000 samples tested in Michigan, United States (Palmer *et al*., 2002) or compared with the 354 samples processed also in Michigan (Schmitt *et al*., 1997) or the 440 cases studied in Ontario, Canada (Belli, 1962).

An important factor to consider in the diagnosis of TB in WTD is the presence of not visible lesions of tuberculosis, a term that refers to the existence of the disease but without presenting macroscopic lesions. Previous studies have revealed that the bacteriological isolation in animals showed no detectable gross lesions (Gavier-Widen *et al*., 2009). The results of this study revealed the presence of higher number of cases of WTD with microscopic changes compared to those with gross lesions. Regarding to the types of microscopic changes suggestive of TB in WTD of this study in which the aggregation of macrophages and necrosis were more frequent are consistent with early lesions described in red deer (*Cervus elaphus*) which consists only in aggregations of macrophages in tonsils and LN with early granuloma lesions or aggregation of macrophages. Early lesions described in WTD range from necrosis to caseation but only found in tonsils (Gavier-Widen *et al*., 2009). It is important to mention that in this study typical granulomas were not found. Characteristic lesions of TB detected in this study such as Langhans-type multinucleated giant cells in one WTD, match with microscopic lesions-detected on WTD in United States (Schmitt *et al*., 1997) and red deer in Spain (Gortazar *et al*., 2008). An important finding was the presence of diffuse

calcification in a tonsil which matches with a study that found partial mineralization LN in WTD (Schmitt *et al*., 1997). Microscopic lesions in tonsils and LN of WTD, consisting of focal or multifocal lesions and granulomas caseonecrotic, some with a calcified necrotic core have also been reported (Palmer *et al*., 2002).

Important changes were also found on the abscedative type which was accompanied by focal necrosis and the presence of neutrophils, this is consistent with what was reported in a study by Palmer which indicate the presence of neutrophils in compatible microscopic lesions of TB in tonsils and LN (Palmer *et al*., 2002).

In European deer, there is also evidence of the presence of an abscess capsule in LN of the head (Martin-Hernando *et al*., 2010). It is possible that variations in the characteristics of the microscopic lesions may be associated to factors such as the host species, stage of development of the lesions and the host immunity (Gavier-Widen *et al*., 2009).

Unfortunately, bacterial growth obtained in this study could not be identified because of fungal contamination problems. The negative results of bacteriological culture of the sampled animals could be associated to the small number of bacilli in the tissue collected and the destruction of the bacilli during the decontamination process (Schmitt *et al*., 1997). The alternative was taken to detect the presence of the *M. tuberculosis* complex using PCR as it has been reported by other researchers (Sharma *et al*., 2007; Sangster *et al*., 2007; Gavier-Widen *et al*., 2009).

CONCLUSION

The presence of gross lesions as well as the frequency and type of microscopic changes suggestive of TB in lymphoid tissues of WTD were detected in the present study and results similar to other studies of WTD and red deer. Microscopic changes such as necrosis, aggregation of macrophages, giant cells, some of the Langhans-type and calcification as well as the presence of neutrophils in some cases, correspond to what has been described in cases of TB in the WTD and red deer. The presence of BAAR and a mycobacterial colony growth was observed 2 weeks after being sown. The presence of *M. tuberculosis* by PCR from paraffin embedded tissue was also confirmed associated to microscopic changes suggestive of TB and the presence of BAAR in Ziehl Neelsen. Now-a-days, there are not reports on WTD infected with TB, the present study is the first case reported in Mexico.

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