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Presence of paratuberculosis in dairy cows culled in Tizayuca (Hidalgo, Mexico)


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Presencia de paratuberculosis en vacas lecheras de desecho en Tizayuca (Hidalgo, México)

Presença de paratuberculose em vacas leiteiras de descarte no Tizayuca (Hidalgo, México)

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Summary

Background: paratuberculosis (PTB) is clinically characterized by diarrhea and progressive weight loss in ruminants, and causes economic losses due to decreased milk production, premature animal disposal, and increased veterinary treatment costs. The presence of PTB has been increasing in milk producing bovine farms in Mexico, yet epidemiology data from specific farms remains scarce. Objective: to determine the proportion of cows positive for Mycobacterium avium subsp. paratuberculosis (Map) among culled cows. Methods: in the present study, 40 culled cows from 25 farms located at the complejo agropecuario industrial de Tizayuca (CAITSA) in Hidalgo (Mexico) were evaluated for PTB presence by histopathological and microbiological studies. Tissue and rectal scraping samples were taken from all cows, and studied for presence of histopathology PTB-compatible lesions. Samples were processed for Map isolation on selective agar culture media; typical Map colonies were confirmed by polymerase chain reaction (PCR). Results: potential PTB infection was found in 25% (10/40) of the animals evaluated by histopathology and acid-fast bacilli (AFB) staining, but...
only 7.5% (3/40) were positive for type 2 Map (C cattle strain) by PCR. **Conclusions:** the prevalence of PTB in cattle farms at CAITSA was confirmed, which may be valuable epidemiological information in Mexico.

**Keywords:** cull cattle, *Mycobacterium avium* subsp. *paratuberculosis*, *paratuberculosis* diagnosis, ruminant farms.

**Resumen**

**Antecedentes:** la paratuberculosis (PTB) se caracteriza clínicamente por diarrea y pérdida de peso progresiva en rumiantes y causa pérdidas económicas por la disminución de la producción de leche, eliminación prematura de animales y el aumento de los costos de tratamiento veterinario. La PTB se ha incrementado en las granjas productoras de leche de bovinos en México, pero se carece de información epidemiológica específica de granjas locales. **Objetivo:** determinar el porcentaje de infección por *Mycobacterium avium* subsp. *paratuberculosis* (Map) entre las vacas de descarte. **Métodos:** en este estudio fueron evaluadas 40 vacas de descarte de 25 granjas del complejo agropecuario industrial de Tizayuca (CAITSA), en el estado de Hidalgo (México), donde se evaluó la presencia de la PTB mediante estudios histopatológicos y microbiológicos. Se tomaron muestras de tejido y raspado rectal de todos los animales buscando lesiones compatibles con PTB. Las muestras fueron procesadas para el aislamiento de Map en medios de cultivo de agar selectivo, y las colonias identificadas como típicas fueron confirmadas por la prueba de reacción en cadena de la polimerasa (PCR). **Resultados:** en este estudio se observó positividad para PTB por histopatología y presencia de bacilos ácido alcohol resistentes (AFB) en el 25% de los animales (10/40), pero solo en el 7,5% (3/40) de las vacas de descarte se confirmó la cepa tipo 2 (cepa C) de Map mediante PCR. **Conclusiones:** se confirma la prevalencia de PTB en las vacas del CAITSA, lo cual podría tener valor como información epidemiológica en México.

**Palabras clave:** bovinos de descarte, diagnóstico de paratuberculosis, granjas de rumiantes, *Mycobacterium avium* subsp. *paratuberculosis*.

**Resumo**

**Antecedentes:** a paratuberculose (PTB) em vacas leiteiras caracteriza-se clinicamente pela presença de diarreia e perda de peso progressiva as quais provocam perdas econômicas pela redução da produção de leite, abate prematuro dos animais e aumento dos custos de tratamento veterinário. A PTB aumentou-se em fazendas leiteiras no México, mas ainda não há informações epidemiológicas específicas das fazendas locais. **Objetivo:** determinar a percentagem de infecção por *Mycobacterium avium* subsp. *paratuberculosis* (Map) em vacas de descarte. **Métodos:** se avaliaram por estudos histopatológicos e microbiológicos 40 vacas de descarte de 25 fazendas do complexo agropecuário industrial de Tizayuca (CAITSA), no estado de Hidalgo (México). Tomaram-se amostras de tecido e raspado anal de todos os animais procurando por lesões compatíveis com PTB. As amostras foram processadas para isolamento de Map em meios de ágar seletivos e as colônias classificadas como típicas foram confirmadas pelo teste de reação em cadeia da enzima polimerase (PCR). **Resultados:** observou-se positividade para PTB pelos estudos histopatológicos e microbiológicos e a presença de bacilos ácido álcool resistente (AFB) no 25% dos animais (10/40), mas só no 7,5% (3/40) confirmaram-se a cepa tipo II (cepa C) de Map por PCR. **Conclusões:** confirma-se a presença de PTB em vacas do complexo CAITSA, o qual poderia ter valor epidemiológico no México.

**Palavras chave:** bovinos de descarte, diagnóstico de paratuberculose, granjas de ruminantes, *Mycobacterium avium* subsp. *paratuberculosis*.

**Introduction**

Paratuberculosis (PTB) or Johne’s disease is a deadly chronic intestinal infection that affects both domestic and wild ruminants. Paratuberculosis is caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), which is distributed worldwide. These mycobacteria infect macrophages and interfere with phagosome maturation, thereby evading the host’s first line of defense. The host immune system reacts to the infection by activating γδ-T cells, CD4+ T cells, and cytolytic CD8+ T cells. Map persists despite the immune system efforts. Release of cytokines and other cytotoxic mediators causes important damage to the intestinal epithelial cells (Coussens, 2001). PTB has an impact on dairy farms by decreasing milk production, inducing
animal miscarriage, stillbirth, and premature delivery (Tiwari et al., 2008). In Mexico, Map was first isolated in 1979 from bovines (Ramírez et al., 1979). Recent reports have demonstrated the presence of Map in cattle, sheep, and goats in several Mexican states (Estévez-Denaives et al., 2007). An immunological analysis was conducted in 2005 at complejo agropecuario industrial de Tizayuca (CAITSA) in Hidalgo (Mexico), to establish the prevalence of this disease among 28 dairy farms. Results showed that 25/28 (89.28%) of the tested farms had at least one bovine seropositive to Map infection, whereas seroprevalence among animal herds ranged between 1.82 and 24.07% (Miranda, 2005). To better understand the correlation between Map transmission and cow infection, our objective was to determine the relationship of culled cows with symptomatic Map and macroscopic and microscopic lesions compatible with PTB within the 28 CAITSA farms with a history of this disease.

**Materials and methods**

**Animals**

Animals used in the present study were 3.5-years-old *Bos taurus* (Holstein Friesian) females. To determine the amount of cows positive for Map, a pathologic examination was performed in 40 culled cows referred to the CAITSA necropsy room between October, 2004 and June, 2005. Animals originated from 25 CAITSA dairy farms previously identified as seropositive to PTB (range: 1.82% to 24.07%; Miranda, 2005). All animals evaluated from these 25 farms with positive prevalence for Map infection were discarded for various reasons (Table 1).

Following Mexican regulations, all bovines were necropsied at CAITSA veterinary medical services before leaving the facilities. After general inspection of each animal was conducted, samples from the terminal ileum, ileocecal valve, and mesenteric lymph nodes were taken and submitted to both histopathological and bacteriological analysis.

**Histopathological examination**

Some of the samples were fixed in 10% buffered formalin at pH 7.2 to 7.6, embedded in paraffin for subsequent 4-µm thick cuts, and stained with Ziehl-Neelsen for microscopic view of acid-fast bacilli (AFB), and hematoxylin and eosin staining (Gilardoni et al., 2012).

**Bacterial isolation**

Selected samples from small intestine and ileocecal valve tissues that showed lesions compatible with PTB by histopathology were then analyzed for Map identification. For this, samples were thawed for processing following the protocol described by Juste et al. (1991). In brief, each sample consisted of intestinal mucosal scraping using a sterile scalpel blade to obtain approximately 1 g of tissue, which was homogenized in 3 mL saline solution using a glass Tenbroek tissue grinder (Omni International, Kennesaw, GA). After this, samples were placed into 50 mL glass beaker and 30 mL 0.75% (v/v) hexadecylpyridinium chloride (HPC) was added and mixed in a stirring plate for one hour, and then let stand for 16 h at 25 °C. Next, 4 mL were taken from the intermediate phase and the supernatant was centrifuged at 1000 x g for 20 min. Then, the supernatant fluid was discarded. The pellet was suspended in 5 mL of an antibiotic-antimycotic solution (50 µg/mL vancomycin, amphotericin B, and nalidixic acid; Sigma-Aldrich, St. Louis, MO,

<table>
<thead>
<tr>
<th>Culling cause (1 cow each)</th>
<th>Map type “C” cattle strain confirmation by PCR</th>
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<tr>
<td>Reproduction problems (infertility)</td>
<td>-</td>
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<tr>
<td>Acute abomasal impaction</td>
<td>+</td>
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<tr>
<td>Clostridiosis</td>
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<tr>
<td>Paratuberculosis</td>
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<td>Undetermined</td>
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<td>Myocarditis</td>
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<td>Reproduction problems (pelvic ring fracture)</td>
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<tr>
<td>Acute ruminal acidosis</td>
<td>-</td>
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<tr>
<td>Traumatic reticulitis</td>
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Table 1. Culling causes and Map type “C” cattle strain confirmation by PCR in cows showing lesions compatible with PTB.
USA) and incubated for 16 h at 25 °C. After this, 8 drops from the intermediate layer were inoculated in three Petri dishes with Löwenstein-Jensen (LJ) medium; only two of the dishes were supplemented with mycobactin J (2 mg/L; Allied Monitor (Fayette, MO, USA). For Map identification, inoculated dishes were incubated at 37 °C and microscopically screened from week 10 to week 14, recording growth rate, colony morphology, bacillary morphology, and staining affinity.

**PCR confirmation of Map isolates**

Confirmation of Map among developed colonies was performed using molecular testing by PCR analysis. Briefly, to proceed to DNA extraction, colonies from LJ culture with mycobactin J were collected with sterile loops and suspended in 250 µL sterile PBS in 2-mL conic tubes. DNA extraction included boiling each colony sample for 20 min. Definitive identification of the developed colony was performed by PCR testing using the following primers: sense 5'-CGCTGCTGGAGTTGATTG IS 900 -3' and antisense 5'-TTTCCTTCGGTGCGTTTTC IS 900 -3', which amplify an expected 973 bp product (Whittington et al., 2000). Each PCR reaction contained 10 µL of 1x buffer, 3 µM of MgSO₄, 0.6 µL of dNTPs (10 mM), 1 nM of each primer, 5 U of Taq polymerase (Life Technologies Corporation, CA, USA), 2 ng of DNA and 50 L of molecular biology grade H₂O to a 50 µL final volume. The amplification protocol consisted of an initial denaturing cycle at 95 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension cycle 72 °C for 5 min. Visualization of the amplification products was performed by gel electrophoresis in 1% w/v agarose gel with TAE 1X, and stained in 1% ethidium bromide solution.

**Results**

From all 28 selected farms (based on previous seropositive PTB results), only 25 farms discarded bovines during the evaluation period. From these farms, all with positive prevalence for the disease, a total of 40 cows were discarded and evaluated for the anatomopathological study. Those cows had various health disorders and only one cow was culled due to PTB (Table 1). According to histopathology, only 10 cows (25%) showed lesions compatible with PTB. AFB and leukocyte infiltration were also observed in such lesions (Figure 1).

Colony development of expected Map growth on solid culture after 10 and 14 weeks of incubation was observed among samples from histopathological lesions compatible with PTB (taken from 10 cows). Macroscopically, colonies were white and smooth, and were of 1-3 mm in diameter. Smears taken from these colonies were stained with ZN staining to confirm the Map bacillary morphology and AFB staining (Gilardoni et al., 2012). PCR analysis was performed for Map molecular characterization of these colonies. Identification was confirmed in all the tested colonies, where the expected 973bp product was amplified after using primers from IS900 (Figure 2). The specific PCR test confirmed Map identification and also that culture from the three colonies belonged to type C Map cattle strain within 3 out of the 10 PTB compatible samples from the 40 initially analyzed cows (3/49; 7.25%).

**PCR strain typing of Map isolates**

DNA extracted from bacterial colonies was used as template for PCR specific strain type test following the methodology described by Collins et al. (2002). Strain identification was performed by PCR testing, using the following primers: sense 5’-CACTTCTTACTCGGACTTC DMC531-3’ and antisense 5’-TGCCCTCGAGCCGAGGAGGAGTA DMC531-3’, which amplify an expected 310 bp product for type C Map (cattle) strain; and sense 5’-GCTTGACAACGTATTGAGAA DMC136-3’ and antisense 5’-GCTTGAGTGATGCCCTCAATGACG DMC136-3’, to amplify an expected 162 bp product for type S (sheep) strain (Collins et al., 2002). Each PCR reaction consisted of 1x reaction solution enhancer (Life Technologies Corporation), 1x buffer, 2 µM of MgSO₄, 0.2 L of dNTPs, 1-pM of each primer, 5 U of Taq polymerase (Life Technologies Corporation, CA, USA), 2 ng of DNA and 50 L of molecular biology grade H₂O to a 50 µL final volume. The amplification protocol consisted of an initial denaturing cycle at 95 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension cycle 72 °C for 5 min. Visualization of the amplification products was performed by gel electrophoresis in 1% w/v agarose gel with TAE 1X, and stained in 1% ethidium bromide solution.
Paratuberculosis in culled cows in Tizayuca (Mexico)

Discussion

Tizayuca is a municipality of Hidalgo state located in central-eastern Mexico, where dairy farming is part of the local economy. Unfortunately, PTB represents a great threat among producers because of its high prevalence levels, strong economic impact, and public health relevance due to a possible association with Crohn’s disease (Gilardoni et al., 2012).

Only 25 of 200 cow farms in CAITSA were evaluated in this study (Miranda, 2005), since authorizations to evaluate the rest of the farms were not granted. Twenty-five out of 28 dairy farms were found seropositive to Map in a previous study, where cows with lesions compatible with PTB were detected (Miranda, 2005). We decided to evaluate cows from these farms to confirm if this illness was a factor to select cows for culling. Pathology reports suggest that 80% of the causes to cull a cow from a productive farm are due to low milk production, weight loss, or illness related to decreased fertility. Nevertheless, in some cases it is recommended to perform histopathology and bacterial isolation to obtain a definitive diagnosis of the illness. Only 2.5% (1/40) of the culled cows presented macroscopic lesions compatible with PTB in the present study; histopathology resulted in diagnosing this disease in 10% of cows, which

Figure 1. Lesions suggesting PTB. A) Lymphoid tissue in the small intestine pointing granulomatous lesions associated to paratuberculosis (H-E, 20X). B) Presence of abundant AFB (arrows) in ileocecal valve tissue (ZN, 40X).

Figure 2. PAGE stained with ethidium bromide. Lane 1 = molecular weight markers; lane 2, positive control to Map S strain (sheep); lanes 3 to 5, amplification products; lane 6, negative control; and lane 7, positive control to Map C strain (cattle).
was confirmed by positive bacterial isolation and molecular identification by PCR.

Only one out of the 40 culled cows was taken to the necropsy room with presumptive PTB diagnosis. This cow presented emaciation and diarrhea, and acid-fast bacilli were observed in samples obtained from smears of rectal scraping. This procedure should be considered insufficient since PTB diagnosis in this area is considered positive by associating with bacillary morphology of the clinical microscopic aggregates. However, this procedure did not detect PTB in all the infected cows, since mycobacterial excretion in feces is intermittent and AFB determination is not specific for Map. Presence of additional damage to the rectum only occurred in advanced stages; therefore it is recommended to use more sensitive and specific tests, such as ELISA and PCR (Clarke, 1997; Oble, 1980).

The frequency of positive PTB cases found in this study was greater than that reported in previous CAITSA studies (Oble, 1980). Sometimes, infected cows with Map only develop tissue lesions; thus, diagnosis after necropsy underestimates the true proportion of PTB cases (González et al., 2005). In the present study, three colonies were cultured using intestinal samples from histological lesions compatible with PTB; however, we did not find reports of Map isolates from samples without apparent tissue lesions (González et al., 2005).

We determined through specific PCR type test that the three presumptive Map isolates of developed colonies from positive histological samples corresponded to type C strain, which has already been described as the most common strain in goats and other ruminants in Mexico (Estévez-Denaives et al., 2007). As mentioned before, a large proportion of culled cows from CAITSA farms are sold to other dairy farms or local butchers and may be sub-clinically infected and undiagnosed, representing a potentially greater number of culled animals that may be infected with Map. Additionally, it is likely that Map is disseminated to other farms through this practice. Further testing is needed to determine PTB prevalence in Mexico and eventually establish control strategies. Currently there are no campaigns to limit mobilization of PTB ruminants, which allows this mycobacteriosisis to silently spread across the country (Möbius et al., 2008). In addition to feces, handling of cows might increase PTB prevalence in farms where infected cows do not present evident PTB lesions (Crossley et al., 2005). The use of sensitive tests, such as histopathology and PCR, are highly recommended to better understand the epidemiology of PTB.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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Miranda BMV. Evaluación del impacto económico de la paratuberculosis en ganado bovino lechero (Sistema intensivo),


