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Correlation Between Histophatological, Bacteriological and PCR Diagnosis of Bovine Tuberculosis

[1]H.R. Bermudez, [1]E.T. Renteria, [1]B.G. Medina,[1]S. Hori-Oshima, [1]De La Mora VA Valle,
[1]V.G. Lopez, [2]W.L. Yu, [1]G.R. Pio, [1]J.C. Herrera, [1]C. Pujol and [2]K. Nielsen,
[1]Instituto de Investigaciones en Ciencias Veterinarias Autonoma de Baja California,
Km. 3.5 Carr. San Felipe, Frac. Laguna Campestre s/n, cp 21386, Mexicali, Baja, Mexico
[2]Canadian Food Inspection Agency, Ottawa Laboratory (Fallowfield),
3851 Fallowfield Rd., K2H 8P9 Ottawa, Ontario, Canada

Abstract: About 553 Dairy cattle carcasses were inspected at a slaughter house in Baja California, out of 298 showed gross tuberculosis-like lesions. Microscopic examination of tissues using H and E and acid fast staining revealed 268 suspect cases. Of the 268, 104 were culture positive while 123 were PCR positive for *M. tuberculosis* complex. A total of 70 samples were culture and PCR positive and an additional 53 samples were PCR positives but culture negative. Thus PCR increases the rate of detection and will be a useful tool for control programs especially when considering the lower sensitivity of diagnosis by bacteriological culture, the occupational risk, technical difficulties with the protocol and the prolonged time of incubation.

Key words: *Mycobacterium bovis*, diagnosis, histopathology, PCR, incubation, bacteriological culture

INTRODUCTION

Mycobacterium bovis is the causal agent of bovine Tuberculosis (TB); it infects approximately 50 million animals all over the world causing economic losses of approximately 3 billion dollars per year (Steele, 1995). The disease is zoonotic, human populations may be infected by direct contact with diseased animals and by the consumption of non-pasteurized milk and its derivatives. In 2003, the World Health Organization (WHO) estimated that approximately 8.8 million persons developed tuberculosis and 1.7 million persons died of this disease (WHO, 2005). In Mexico, 28% of milk is marketed without pasteurization and is used for the preparation of cheese and other dairy derivatives (NOM, 2005) which implies a high risk to public health.

In Baja California, human pulmonary tuberculosis has the highest rate of human TB when compared to the other northern Border States with a rate of 58 cases/100,000 inhabitants (Epidemiological bulletin). In other countries it has been demonstrated that a reduction in bovine TB prevalence results in a lower risk exposure to human. The control method recommended in Mexico is the detection and slaughter of positive reactor animals (Epidemiological bulletin). One of the limitations in Mexico for TB eradication is that there is no economical compensation program that allows the reduction of economic loss due to the slaughter of tuberculoses animals (Corner *et al*., 1990; Corner, 1994).

The eradication TB program started in 1974 at Baja California State; however not as until 1995 was the program supported by histophatological and bacteriological diagnosis. Actually several countries relies the diagnosis in the traditional tuberculin test, histophatological and PCR from formalin fixed tissues and in some cases follow the bacteriological process which is expensive and complicated and there are not enough acilities to perform it and the identification of the Mycobacterial organism using PCR can be give us several benefits.

The aim of this study was to correlation histophatological, bacteriological and PCR diagnosis of bovine tuberculosis.

MATERIALS AND METHODS

Tissue samples were collected at the postmortem inspection in an abattoir in Tijuana, Baja California from June 2006-February 2007.

Sampling procedures: Inspection included the entire carcass with emphasis on the lung and liver and the retropharyngeal, mandibular, parotid, medastinal, tracheobronchial and hepatic lymphs nodes (NOM, 2005).

Corresponding Author: H.R. Bermudez, Instituto de Investigaciones en Ciencias Veterinarias Autónoma de Baja California, Km. 3.5 Carr. San Felipe, Frac. Laguna Campestre s/n, cp 21386, Mexicali, Baja, Mexico

Samples were removed from carcass, placed in sterile plastic bags and were sent to the laboratory for processing. Tissue samples were split in three parts, one for histophatological examination and the remainder two subsamples were used for bacteriology and fresh tissue PCR.

Histopathology: Examination, the samples were formalin fixed, embedded in paraffin and cut into 6 μm sections, slides were prepared using standard Hematoxylin-Eosin (HE) stain method (Bolin *et al*., 1997) and evaluated microscopically by a veterinary pathologist; histological lesions were recorded as suspect when granulomatous inflammation associated with central necrosis were present and the sample had no evidence of other non-mycobacterial etiologies. Acid-fast stain was applied to all positive suspect samples using the Ziehl-Neelsen staining procedure. If acid-fast bacilli were detected by these mean, the sample was considered positive (Fitzgerald *et al*., 2000).

Bacteriology: Lymph nodes were trimmed of excess fat, macerated in phenol red nutrient broth (Becton-Dickinson, Cockeysville, Maryland, USA) decontaminated in 2% NaOH for 10 min, centrifuged for 20 min and the supernatant fluid was decanted (Payeur *et al*., 1992). Stonebrick media were inoculated with the sediment. The inoculated media were incubated at 37°C for 12 weeks and examined for colony formation every week. The contaminated samples were discarded and inoculated once again. The growing colonies were classified to species using standard growth and were PCR confirmed (Liebana *et al*., 1995; Schmitt *et al*., 1997).

Polymerase Chain Reaction (PCR) test for tissues and isolates: Frozen tissues were tested by PCR. Extraction of DNA from tissues was done using QIamp® Mini kit (Qiagen Inc) following the manufacturer's instructions. PCR procedures were applied as previously described using primers: IS41, 5' CCT GCG AGC GTA GGC GTC GG 3' and IS43, 5' TCA GCC GCG TCC ACG CCG CCA 3' complementary to sequences flanking the repetitive insertion element IS6110 specific of the Tuberculosis complex species (Liebana *et al*., 1995). Briefly the reaction consisted of 100 ng of DNA of each sample, 1X Taq buffer (invitrogen Inc.), 1.5 mM $MgCl_2$, 0.2 mM of each deoxy-nucleotide triphosphates, 2.5 U of Taq polymerase (Invitrogen Inc) and 25 pM of each primer in a total volume of 50 μL. DNA was amplified using a Hybaid Express thermocycler using 1 cycle at 94°C for 10 min, 30 cycles of the denaturing for 2 min at 94°C, alignment at 68°C for 2 min and an extension at 72°C for 2 min, finally 1 cycle at 72°C for 10 min. The expected amplified DNA band was 317 bp [15]; the AN5 strain was used as a positive control. BCG was used as positive control and water as negative control. PCR products were tested by electrophoresis in 1.5% agarose gels in 1X TAE buffer, stained with 5 μg mL^{-1} of ethidium bromide and observed by ultraviolet light (Transilluminator, 2000 Bio Rad Inc).

Statistical analysis: The statistical difference in the proportion ($p<0.5$) between the PCR and bacteriological results were estimated using Med Cal software. The sensitivity and specificity values were calculated with Epi info 6 (CDC, Atlanta, GA).

RESULTS AND DISCUSSION

A total of 553 dairy cattle were inspected at slaughter for presence of granulomatous lesions in lymphatic nodes at the abattoir in Tijuana, Mexico (Table 1). Of the 553 carcasses, 298 (54%) had tuberculosis like gross lesions in the head and thoracic lymph nodes. Of the 298 animals 268 (90%) were classified as TB suspect by histopathology examination using H and E stained tissue sections. The tissues from those 268 animals were stained with Ziehl-Neelsen method for the presence of fast-acid resistant organism and 217 (81%) were positive. Additionally, culture of those 268 animal tissues were processed for bacteriology, 104 (39%) were culture positive and were identified as *M. tuberculosis* complex by PCR means. Also, PCR was performed on 268 frozen tissues, 123 were positive (46%) of those, 70 (67.3%) were positives for both bacteriology and PCR and 53 (32.3%) were only positive for PCR (Table 1).

There was not statistical difference between PCR and bacteriological proportions. The sensitivity for PCR was 67.32% using culture as reference (Table 2). The results show that the PCR do not increased the rate of detection of *M. bovis* compared with the culture but this technique will be useful for detect *M. bovis* in control programs because its rapidity, lower price and lower risk. Post-mortem examination of tissue of 553 dairy cattle resulted in 298 showing gross TB-like lesions with a total

Table 1: Results on positive animals

Factors	Values
Animals	553
Number gross TB like lesions	298
Mycobacteriosis[a]	268
Culture positive[b]	104
PCR positive[c]	123

Confirmed by histopathology with H and E stain. [b]Colonies confirmed by PCR. [c]On frozen tissue samples

Table 2: Comparison of *Mycobacterium bovis* identification by culture and tissue PCR

	Bacteriology[a]		
PCR[b]	Positive (%)	Negative (%)	Total
Positive	70 (67.3)	53 (32.3)	123
Negative	34 (32.7)	111 (67.7)	145
Total	104.0	164.0	268

[a]Stonebrick medium; [b]Frozen tissue

of 268 cases showing microscopic lesions typical of bovine TB containing acid fast bacteria. A total of 39% (104) were positive by bacterial culture of which 34 were PCR negative. However, PCR identified an additional 53 culture negative samples as positive for the *M. tuberculosis* complex for a total of 123 (46%) of the tissue samples. Of the 268 samples, 111 were negative by culture and PVR.

The culture of *M. bovis* is the gold standard, however it is very time consuming requires special equipment and highly qualified personnel. This combined with low sensitivity as a result in some chronic cases with low number of viable microorganisms makes it a less than ideal diagnostic test (Corner *et al*., 1990; Kell and Young, 2000).

The diagnostic tests available have increased with the development of PCR techniques. Thus it is possible to identify *M. bovis* in any biological specimen without previous culture in both human and bovine samples (Cousins *et al*., 1992; Liebana *et al*., 1995). The use of PCR allows the identification of a specific sequence of the *M. tuberculosis* complex such the IS6110. Unlike other methods, PCR for IS6110 identifies the *M. tuberculosis* complex bacteria like *M. bovis*, *M. tuberculosis*, *Mycobacteria africanum*, *Mycobacteria canetti*, *Mycobacteria microti*, *Mycobacteria caprae* and *Mycobacteria pinnipedi* species, however the animal species and the geographic location of the animal provide a strong indication of which member of *M. tuberculosis* complex is involved in particular cases (Cousins and Florisson, 2005). The availability of numerous commercial kits for DNA extraction from tissues and body fluids has made sample preparation simple.

CONCLUSION

From this study, it is observed that combined with the lower cost of assay components and the widespread availability of multipurpose equipment, the PCR will be very useful in control programs of various infectious diseases.

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