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Development and Validation of an Immunohistochemical Method for Diagnosis of Bovine Tuberculosis in Formalin-Fixed, Paraffin-Embedded Tissues

[1]J. Martinez-Burnes, [1]O. Castillo-Martinez, [1]D. Solano-Terreros,
[1]N.I. de la Cruz-Hernandez, [2]J. Campuzano-Granados, [1]H. Barrios-Garcia,
[1]A. Snydelaar Hardwicke, [1]C. Almazan-Garcia, [1]J.A Guizarnotegui-Blanco and [3]J. Ramos-Vara
[1]Department of Graduate and Research, College of Veterinary Medicine,
Autonomous University of Tamaulipas, Tamaulipas, Mexico
[2]Department of Pathology, College of Veterinary Medicine,
National Autonomous University of Mexico, UNAM
[3]Animal Disease Diagnostic Laboratory, Department of Comparative Pathobiology,
College of Veterinary Medicine, Purdue University, West Lafayette, IN, USA

Abstract: The objective of the study was to develop an indirect Immunoperoxidase Method for bovine tuberculosis diagnosis in formalin fixed, paraffin embedded tissues and its validation compared to mycobacterial isolation and Ziehl Neelsen staining. About of 33 bovine lymph nodes with isolation of *Mycobacterium bovis* and 11 negative lymph nodes from tuberculosis free ranches were used. Sections of all lymph nodes examined were stained with Ziehl Neelsen and Immunohistochemistry (IHC). Rabbit anti-*M. bovis* polyclonal antibodies and horse anti-rabbit were used as primary and secondary antibodies. The immunologic reaction was detected with an immunoperoxidase DAB Method and counterstained with hematoxylin. Results show complete agreement between Immunohistochemistry and mycobacterial culture. From the 33 positive isolation cases, all of them (100%) were positive by IHC. From the 11 negative cases, all of them were negative to Mycobacterium by IHC. Regarding Ziehl Neelsen of the 33 positive isolation cases, 30 (90.9%) had acid-fast bacilli and from the 11 negative isolation cases none had acid fast bacilli. Results show that IHC represents a fast, sensitive and specific diagnostic tool for bovine tuberculosis in formalin fixed, paraffin embedded tissues allowing simultaneous observation of tissue lesions and antigens.

Key words: Bovine, formalin fixed, immunohistochemistry, tuberculosis, antibodies

INTRODUCTION

Tuberculosis is a chronic and infectious disease caused by Mycobacterium complex including *M. tuberculosis*, *M. bovis* and *M. africanum*. Granulomatous changes are characteristic in different tissues. This infection hinders bovine production and induces severe economic losses to livestock due to deaths, chronic disease and trade restrictions (Lopez, 2012). Tuberculosis is a relevant zoonosis and currently considered as a world emergency by the WHO. Because of the morbidity of human tuberculosis in Mexico the detection and intensive diagnosis of Mycobacterium was established as priority. It is also considered an important public health problem by the National Health Program and an important eradication program for bovine tuberculosis is in progress.

Diagnostic tests for bovine tuberculosis include tuberculin skin test in live animals using the caudal fold for preliminary screening with a 72-91% sensitivity and 78-96% specificity (Estrada-Chavez *et al*., 2004). Reactors are retested using the comparative cervical test (Estrada-Chavez *et al*., 2001). Postmortem examination is a practical method but only tuberculosis gross compatible lesions are detected. Definitive diagnosis of tuberculosis is based on the Gold Standard Method of bacterial isolation which requires an extended period for mycobacterium isolation, fresh tissues and biosafety regulations (Toledo-Ordonez *et al*., 1999). Histological identification of compatible lesions of tuberculosis and detection of acid fast positive bacilli by Ziehl Neelsen stain (ZN) is helpful but they have low sensitivity (Estrada-Chavez *et al*., 2004).

The Mexican Official Regulation National Campaign for Bovine Tuberculosis (*M. bovis*) establishes that for

Corresponding Author: J. Martinez-Burnes, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autonoma de Tamaulipas, km. 5 Carr. Victoria-Mante, CP 87000, Cd Victoria, Tamaulipas, Mexico

histological diagnosis of tuberculosis, tissue sections should be stained with Ziehl-Neelsen or using a Fluorescent Auramin test. However, rare acid fast bacilli presence is common in early lesions or even in severe lesions being frequently overlooked (false negative).

Immunohistochemistry (IHC) is a highly sensitive and specific diagnostic method in tissues with minimal laboratory biosecurity requirements and faster than mycobacterial isolation (Sumi *et al*., 2001). One main advantage of IHC for the pathologist is the simultaneous detection of characteristic tuberculosis lesions and mycobacterium antigens (Haines and Clark, 1991). Despite of IHC advantages it is not routinely used in Mexico for tuberculosis diagnosis. The main purpose of the current study was to develop and validate a fast indirect immunohistochemical assay for the detection of mycobacterium in formalin fixed, paraffin embedded bovine tissue sections.

MATERIALS AND METHODS

For the IHC assay, a polyclonal antiserum against *Mycobacterium* sp. was produced in rabbits using a previously described protocol (Espitia *et al*., 1991) at the Biomedical Research Institute, UNAM. Briefly, three, 2 months old rabbits were immunized by IM and SC injections of *M. tuberculosis* H37/Rv strain heat killed and 3 mg of filtered proteins from culture suspended on 1 mL of Al (OH) during the 1st 2 weeks. Later, rabbits were weekly inoculated with bacilli and suspended proteins on PBS. Immunoelectrophoresis was used to detect the serum antibody levels and rabbit antiserum was obtained and stored at -70°C until used.

Forty four lymph nodes were used including 11 lymph nodes collected at slaughter houses from cattle from certified tuberculosis free ranches and negative to mycobacterial culture (negative cases). Thirty three bovine lymph nodes with gross changes suggestive to tuberculosis and confirmed mycobacterial isolation were considered as positive cases. Samples were fixed in 10% buffered formalin for histopathological analysis. Formalin fixed tissues were processed by routine histopathology methods, paraffin embedded cut at 4 μm thick sections and stained with Hematoxyilin-Eosin and Ziehl-Neelsen (Luna, 1968).

The presence and type of lesions compatible to tuberculosis such as granulomatous inflammation with giant cells and caseous necrosis were characterized by microscopic examination. The presence of bacilli acid alcohol resistant was confirmed by microscopical examination of Ziehl-Neelsen stained slides. Paraffin-embedded tissues from all cases were processed by immunohistiochemistry.

An indirect Immunohistochemistry was developed and optimized as follows. Formalin fixed tissues processed routinely embedded in paraffin were serially sectioned at 4 μm thickness floated on a gelatin free warmer bath and mounted on silane treated slides. Tissue sections were then heated at 60°C for 10 min, deparaffinized twice in xylene for 5 min each time. Then, immersed in graded ethanol xylene (50/50%) twice for 5 min each time and ethanol for 5 min. Rehydrated into TBS (0.05M Tris-HCl, 0.03 M NaCl, 0.1% Tween-20) pH 7.6 twice for 5 min each time.

Endogenous peroxidase and alkaline phosphatase activities were blocked by immersing the slides in Dual Endogenous Enzyme Block (DakoCytomation, Inc., Carpinteria, CA) at room temperature for 5 min. After rinsing with PBS Tween-20 the sections were subjected to either antigen retrieval with steam heat while slides were immersed in preheated at 90-95°C citrate buffer solution (0.01 M, pH 6.0) for 20 min or no antigen retrieval.

After washing slides with PBS Tween-20, nonspecific binding sites were blocked by incubating the sections with horse serum (Vector Laboratories, Burlingame, CA) or with Protein block, serum free (DakoCytomation, Inc., Carpinteria, CA) for 5 min at room temperature.

The blocking serum was removed and sections were covered with the rabbit anti-Mycobacterium polyclonal antibody (primary antibody) at dilution of 1:7500 and incubated in a humidified chamber for 30 min at room temperature.

Normal rabbit serum at dilution of 1:3000 was used as negative control. The slides were incubated with biotinylated horse anti-IgG rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min at 37°C. The slides were then washed 2 times in PBST for 5 min each time before being immersed in avidin-biotin conjugate (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) and incubated for 30 min at 37°C. Sections were washed in PBS and then stained with 3, 3 iminobenzidine tetrahydrochloride for 2 min.

Finally sections were rinsed with tridistilled water, lightly counterstained with hematoxylin, dehydrated through graded ethanol to xylene and mounted. Light microscopy was used to detect mycobacterium antigen presence.

Caprine lymph nodes and intestine sections positives to *M. paratuberculosis* and positive lymph nodes to *M. bovis* by culture were used as positive controls. Rabbit serum was used as negative control replacing the primary antibody.

The presence of mycobacterium antigens was evaluated by IHC in lymph nodes sections from cases with positive mycobacterial isolation (positive cases) and lymph nodes from cattle from certified tuberculosis free ranches and negative to mycobacterium culture (negative cases). IHC results were compared with mycobacterium culture and presence of acid fast bacilli in sections stained by Ziehl-Neelsen. Kappa statistics were used to

diagnostic tests (Fleiss, 1981). The following measure statistical agreement among the three standards or levels for the strength of agreement as described by Fleiss were used: >0.81 = Excellent, 0.61-0.80 = Good, 0.41-0.60 = Moderate, <0.40 = Poor (Fleiss, 1981).

Sensitivity, specificity and predictive value were determined for the IHC and ZN results compared to mycobacterium culture and isolation as reference test using Receiving Operating Characteristics Curve analysis (ROC Curve) (Griner *et al*., 1981; Zweig and Campbell, 1993).

RESULTS

From the thirty three positive cases by mycobacterium culture, 30 of them (90.91%) had acid fast bacilli by ZN. Three cases were negative (9.09%). All of the negative cases (n = 11) to mycobacterium culture did not have acid-fast bacilli.

All of the positive cases to mycobacterium culture were confirmed to be positive to mycobacterium antigens by IHC. Negative cases to mycobacterium culture were also negatives by IHC. A comparison of results between mycobacterium culture, Ziehl-Neelsen and IHC are shown in Table 1.

Under the laboratory conditions of the current study the comparative results from IHC and positive cases by culture, the kapp a statistics was $\kappa = 1$ indicating excellent agreement between IHC assay and culture.

In contrast, comparison between Ziehl-Neelsen staining and mycobacterium culture the kappa statistics was $\kappa = 0.83$ indicating also a high agreement however, disagreement between Ziehl-Neelsen and culture isolation as the reference test was found in 9. 09% cases. Three of 33 positives cases by culture did not have acid fast bacilli.

RO curve analysis showed a 100% sensitivity of IHC to detect mycobacterium antigens in paraffin-embedded tissues. Sensitivity of ZN was 90.9% because failed to detect 9.01% of positive culture cases (false negatives). Specificity of IHC and ZN compared to mycobacterium culture was 100%. False positive cases were not detected.

Mycobacterium antigens were easily detected in the cytoplasm of macrophages and giant cells as brown gold staining and presence of granules or spheres and no the characteristic bacilli shape was frequently observed (Fig. 1). Positive staining was mainly observed surrounding the necrotic core of the granulomatous inflammation.

Table 1: Comparison of Ziehl Neelsen and immunohistochemical staining for mycobacterium and isolation by culture

	Ziehl Neelsen		Immunohistochemistry	
Isolation	Positive	Negative	Positive	Negative
Positive (33)	30	3	33	0
Negative (11)	0	11	0	11

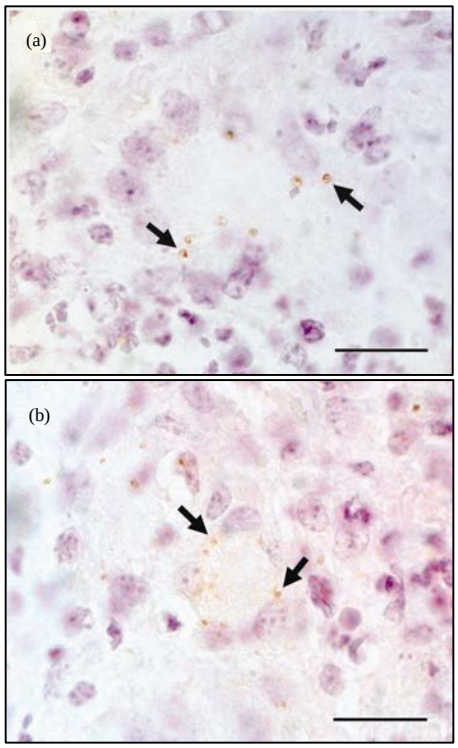

Fig. 1: Lymph node of bovine. Immunohistochemical staining of mycobacterial antigens with a rabbit anti-*M. bovis* polyclonal antibody. a) Multiple granular aggregates within the cytoplasm of a giant cell (arrows) and b) revealing gold-brown staining (arrows) within a giant cell. Avidin-Biotin-Peroxidase Complex Method counterstained with hematoxylin. Bar = 20 μm

DISCUSSION

During the optimization of IHC protocol and to minimize nonspecific staining tissue sections were pretreated with dual endogenous enzyme block to eliminate false positive staining caused by endogenous peroxidase and alkaline-phosphatase activities (Ramos-Vara, 2005). The nonspecific binding sites were initially blocked using horse serum, from the species in which the second antibody was prepared for 5 min at room temperature however, better results were obtained using protein block, serum free to saturate nonspecific immunoglobuling sites.

The non-antigen retrieval treatment demonstrated better results than Heat Steam Antigen Retrieval Method. It is well known that a number of aldehyde containing fixatives can produce cross links between proteins during tissue preservation that limits access of antibodies to antigenic sites resulting in false negative results (Ramos-Vara, 2005). However, studies of optimization of immunohistochemical methods comparing antigen retrieval methods for the detection of antigens informalin fixed, paraffin embedded tissues have also demonstrated that non-antigen retrieval methods are required for some polyclonal antibodies such as rabbit anti *Mycobacterium bovis* polyclonal antibody (Ramos-Vara and Beissenherz, 2000). Therefore, for standardization of an IHC protocol and for each antibody a systematic approach for antigen retrieval should be followed to obtain optimal results.

Optimization of the IHC protocol under the conditions of the present study allowed the reduction of the initial primary incubation time from overnight to only 30 min.

The non-specific staining initially observed in the present study could be also associated to polyclonal antibodies. It is well known that polyclonal antibodies have a high affinity but also may have mmunological promiscuity caused by multiple antigen detection through the variety of epitopes recognized. Therefore, the higher number of different anti-bodies to the target protein the greater the cross-reactivity with similar epitopes in other proteins, hence the likelihood of false positives (Ramos-Vara, 2005). Conversely, monoclonal antibodies reduce or inhibit background or non specific staining because specific antibodies are used to detect single proteins increasing affinity and specificity (Ramos-Vara, 2005). Unfortunately, monoclonal antibodies for mycobacterium assays are not commercially available.

Identification of *Mycobacterium bacilli* by Ziehl-Neelsen and antigens by IHC was mainly observed at the periphery of the necrosis in the cytoplasm of macrophages and giant cells which was consistent with previous reports (Cancela and Marin, 1993).

Rare single acid fast bacilli or as small aggregates were not easily detected in majority of positive cases. Previous reports have also described the presence of very few bacilli in tissue slides by Ziehl-Neelsen the reduced number of acid fast bacilli have been associated to immunocomplex with antibodies excess and few bacilli in the granulomatous inflammation (Cancela and Marin, 1993). In addition, severe and extensive necrotic areas in granulomas in the present study difficulted bacilli detection. Furthermore, detection of mycobacterium by Ziehl Neelsen in tissue slides is time consuming.

In contrast, mycobacterium antigens were easily detected by IHC as brown or gold granules or spheres. The granular shape of mycobacterium antigens stained by IHC and not the characteristic bacilli forms have been described earlier (Orrell *et al*., 1991; Cancela and Marin, 1993).

One advantage of IHC is the detection of mycobacterium antigens from entire bacilli but also from its fragments caused by the granulomatous inflammation or also from multiple isoforms (epitopes) of the target protein. In contrast, recognition of a mycobacterium by ZN Method it is limited to complete stained bacilli, making difficult its identification.

Therefore, sensitivity of IHC is higher than ZN. Additionally, magnification of the antigen-antibody reaction revealed by the colored reaction in IHC, substantially decreased the observation time by microscopists to detect mycobacteria when compared to ZN staining. Furthermore the simultaneous detection and characterization of granulomatous lesions and mycobacterium antigens using IHC it is an important advantage for pathologists and also recognized by others (Haines and Clark, 1991).

Results of the present study revealed 100% sensitivity for IHC to detect mycobacterium antigens. Sensitivity of ZN staining was 90.9%. Specificity of IHC and ZN was 100% false positive were not detected. The high sensitivity of IHC to detect mycobacterium antigens in animal tissues has been also reported earlier (Thoresen *et al*., 1994). The higher sensitivity of IHC compared to ZN staining to detect mycobacterium antigens has also been described in caprine and bovine tissues (Cancela and Marin, 1993). Researchers associated the few bacilli stained by ZN to the immunoresponse generated in the granulomatous inflammation. To the researchers knowledge, in spite of IHC advantages, this technique it is not used for routine diagnosis of bovine tuberculosis.

CONCLUSION

In this study, the IHC Method developed in the present study was found to be highly sensitive and specific in detecting mycobacterium antigens in formalin fixed, paraffin embedded tissues. It is concluded that IHC is an important diagnosis tool for mycobacterium in tissues. IHC facilitates mycobacterium detection, even in cases with rare bacilli or its fragments in tissues. These advantages are not found in ZN staining. The researchers conclude that IHC is particularly useful for veterinary diagnostic laboratories because of the reduced time for diagnosis compared to mycobacterium culture and

isolation, minimal biosafety requirements the simultaneous observation of lesions and antigens and its application to paraffin embedded tissues allowing retrospective studies.

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