Comparison of Polyclonal Antibodies to Three Different Preparations of Mycobacterium Paratuberculosis in Immunohistochemical Diagnosis of Johne's Disease in Cattle

Judith R. Stabel, United States Department of Agriculture
Mark R. Ackermann, United States Department of Agriculture
Jesse P. Goff, United States Department of Agriculture

Available at: https://works.bepress.com/mark_ackermann/81/
Comparison of Polyclonal Antibodies to Three Different Preparations of Mycobacterium Paratuberculosis in Immunohistochemical Diagnosis of Johne's Disease in Cattle
Judith R. Stabel, Mark R. Ackermann and Jesse P. Goff

J VET Diagn Invest 1996 8: 469
DOI: 10.1177/104063879600800412

The online version of this article can be found at:
http://vdi.sagepub.com/content/8/4/469
Comparison of polyclonal antibodies to three different preparations of *Mycobacterium paratuberculosis* in immunohistochemical diagnosis of Johne’s disease in cattle

Judith R. Stabel, Mark R. Ackermann, Jesse P. Goff

**Abstract.** Polyclonal antisera were raised in rabbits against preparations of live and heat-killed *Mycobacterium paratuberculosis* and cell-wall proteins of *M. paratuberculosis* and were evaluated as diagnostic tools in immunohistochemical staining of bovine tissue. Live preparations of *M. paratuberculosis* (LMP) were inoculated intraperitoneally or intravenously at 10⁹/ml. Heat-killed *M. paratuberculosis* (HKMP) was prepared by treatment of bacteria at 85°C for 10 minutes. Cell-wall proteins were isolated from *M. paratuberculosis* and conjugated to keyhole limpet hemocyanin to improve antigenicity (KLH-CWPMp). The HKMP and KLH-CWPMp preparations were emulsified in incomplete Freund’s adjuvant before subcutaneous inoculation of rabbits. Antibody titers in the terminal blood sample were higher for HKMP and KLH-CWPMp than for LMP rabbits (1:1,024 vs. 1:64). The KLH-CWPMp antibody did not cross-react with *M. bovis*-infected tissues. Sensitivity and specificity of immunohistochemical detection of Johne’s disease (paratuberculosis) from bovine tissues was much higher for the KLH-CWPMp polyclonal antibody. Immunoreactivity of the antibody resulted in staining of bacteria in the cytoplasm of macrophages, mononuclear giant cells, and extracellular bacteria in both intestine and lymph node.

Johne’s disease (paratuberculosis) is a chronic enteritis in ruminants caused by the intracellular pathogen *Mycobacterium paratuberculosis*. Although diagnosis of *M. paratuberculosis* infection is difficult for a number of reasons, the primary hindrance is the paradoxical host immune response during various stages of the disease. During the initial phase of infection, animals are asymptomatic and shed low numbers of the organism. Subclinical infection is further characterized by a strong cell-mediated immune response and can be detected by such assays as lymphocyte proliferation to a T-cell-dependent mitogen and delayed-type hypersensitivity reactions or skin tests. A negligible humoral immune response during subclinical infection significantly reduces the usefulness of diagnostic tests measuring antibody production to *M. paratuberculosis*. In contrast, clinical disease is characterized by a strong humoral immune response and a weak cell-mediated response. Overt signs of clinical infection such as emaciation, anorexia, and profuse diarrhea are present, and high numbers of bacteria are shed in the feces. Because of this immunologic conundrum, the only definitive test for Johne’s disease at the present time is fecal culture. This test is both specific and sensitive; however, culture requires 8-16 weeks for positive identification of *M. paratuberculosis*.

Histopathologic analysis of tissues from infected animals is a rapid method of detecting paratuberculosis. Biopsy tissue or tissue samples taken at necropsy may be stained for acid-fastness to determine the presence of *M. paratuberculosis*. However, this method is nonspecific and does not distinguish among mycobacterial species. Immunofluorescent staining of tissues with fluorochromes such as auramine improves the level of detection but not the specificity and is used infrequently because of special equipment needed for detection, such as dark-field microscopes or flow cytometers. Immunohistochemical staining has been recommended as an alternative method of detection. Indirect immunoperoxidase staining has been used successfully for the diagnosis of paratuberculosis in tissue from a clinically infected cow. More recently, immunoperoxidase staining of bovine tissue was found to be better than acid-fast staining for detection of low numbers of mycobacteria. Immuno-peroxidase studies for detection of *M. paratuberculosis* in tissues have utilized either bovine antisera from cows clinically infected with paratuberculosis or commercial preparations of polyclonal antibodies to crude preparations of *M. paratuberculosis*. This is the first study to compare antigenicity of the whole bacterium with that of its cell-wall components in preparation of a polyclonal antibody. In the present study, we describe the production of 3 different polyclonal antisera in rabbits and demonstrate the efficacy of each of the antibodies in immunohistochemical detection of *M. paratuberculosis* in bovine tissue.
Materials and methods

Antigen preparation and polyclonal antibody production. *Mycobacterium paratuberculosis* used in polyclonal antibody production was an ileal isolate from a cow with clinical Johne’s disease (strain Kay). Bacteria were grown in Middlebrook 7H9 medium and harvested at 0.3 *A*<sub>400nm</sub>. Bacteria were washed 3 times in phosphate-buffered saline (PBS, pH 7.4) for 30 min at 7,500 rpm in a refrigerated centrifuge (4 C). After washing, the bacterial pellet was resuspended in 3 times the wet weight in distilled water. The diluted bacteria were treated 3 times in a French pressure cell at 18,000 lb/in². The suspension was diluted 3 times with distilled water and centrifuged 3 times for 15 min at 800 g in a refrigerated centrifuge to remove unbroken cells. The supernatant from this final wash was centrifuged at 27,000 x g for 50 min. The supernatant from this centrifugation was decanted, and the final pellet contained the cell walls of the original *M. paratuberculosis* preparation. This pellet was washed after resuspension 3 times with PBS and 3 times with distilled water by centrifuging at 27,000 x g for 50 min. The pellet was resuspended in PBS, and the sample was lyophilized. The sample was reconstituted in 0.1 M borate buffer (pH 10.0) to a final concentration of 5 mg of cell wall protein (CWP) per milliliter. Twenty-five milligrams of solubilized keyhole limpet hemocyanin (KLH) was added to 5 mg CWP. The KLH conjugation was performed to enhance the antigenicity of the CWP, particularly small proteins, and peptides in the preparation that might not be highly antigenic on a singular basis. One milliliter of 0.3% glutaraldehyde was added to this mixture and stirred at room temperature for 2 hr. Glycine (1 M) was added, and the preparation was stirred at room temperature for 30 min. The suspension was dialyzed against borate buffer (0.1 M, pH 8.5) overnight at 4 C. The KLH-CWP conjugate was stored at 4 C until used.

Two additional preparations of *M. paratuberculosis* (strain Kay) were utilized in this study. The bacteria were washed 3 times with PBS and resuspended to a concentration of 1 × 10<sup>8</sup>/ml. One preparation (HKMp) was killed by heat treatment at 85 C for 10 min and the other preparation was untreated and consisted of live *M. paratuberculosis* (LMp). Polyclonal antibodies to the 3 *M. paratuberculosis* preparations were raised in New Zealand White rabbits. Prior to inoculation of rabbits, KLH-CWP and HKMp preparations were emulsified in incomplete Freund’s adjuvant. Rabbits were anesthetized with a xylazine/ketamine cocktail prior to blood withdrawal and inoculation. Preinoculation blood samples were obtained from the central artery of the ear. Rabbits were randomly assigned to treatment groups, with 3 animals in the KLH-CWP and HKMp groups and 2 animals in each of the LMp treatments. Rabbits were injected subcutaneously with either KLH-CWP (1 mg protein) or HKMp (1 x 10<sup>8</sup>/ml) at 4 different sites (0.25 ml each). The remaining rabbits were injected intraperitoneally with 1 ml LMp (1 x 10<sup>8</sup>/ml) in 1 site or injected intravenously in the ear with 0.1 ml of LMp. Blood samples were collected and injections were repeated on days 14 and 28, and antibody titers were measured by the complement fixation assay (CFA). A second round of blood collection and injections was performed on days 49, 63, 77, and 91. Rabbits were anesthetized and blood collected via cardiac puncture on day 101. Serum samples were harvested and frozen at –20 C. Antibody titers of serum samples from the terminal blood collection were determined by CFA.

Tissue sections. We conducted a retrospective analysis of tissues from 5 animals with *paratuberculosis*. Tissue samples from all 5 animals had granulomatous lesions typical of *paratuberculosis*. Samples from the ileum and mesenteric lymph nodes of all cows were fixed in buffered zinc-formalin (Z-fix), processed routinely, and embedded in paraffin wax. Sections were cut at 6 µm and stained with hematoxylin and eosin (HE) and Ziehl-Neelsen (ZN) by conventional methods. Replicate unstained sections were made for immunohistochemistry. Ileal tissues from all 5 animals had acid-fast rods within the cytoplasm of macrophages. The macrophages were present in the lamina propria and submucosa.

Immunohistochemical procedures. Sections were stained by a previously described procedure with slight modifications. Sections were deparaffinized with xylene and rehydrated through a series of ethanol baths (100% for 2 min, 95% for 1 min, 70% for 1 min) at room temperature. Slides were washed in distilled water for 5 min and rinsed in 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 50 mM Tris HCl, 150 mM NaCl; pH 7.6) for an additional 5 min. Slides were incubated for 4 min in hot HCl (2 N) to aid in antigen retrieval. Samples were incubated with 0.1% trypsin/CaCl<sub>2</sub> solution for 20 min at 37 C and then washed in TBS for 5 min. Blocking was performed in 10% normal goat serum for 30 min at room temperature. The sections were then incubated with rabbit anti-*M. paratuberculosis* polyclonal antibodies diluted in TBS for 1.5 hr at 37 C and washed for 5 min in TBS. Goat anti-rabbit biotinylated link antibody was incubated on sections for 30 min at room temperature. Sections were rinsed for 5 min followed by incubation with streptavidin-alkaline phosphatase solution for 30 min at room temperature. After washing, samples were incubated with chromagen, Histomark red, for 30 min at room temperature and counterstained with Harris hematoxylin after a final wash. A double staining procedure was followed as above with an anti-macrophage monoclonal antibody (anti-CD68, clone EBM11) diluted 1:20, goat anti-rabbit biotinylated link antibody, streptavidin-horseradish peroxidase conjugate, and metal diaminobenzidine as the chromagen in the first reaction. After rinsing in TBS-BSA for 15 min, slides were blocked in goat serum and then incubated with anti-*M. paratuberculosis* polyclonal antibody to CWP, goat anti-rabbit biotinylated link antibody, streptavidin-alkaline phosphatase, and Histomark red. Control sections either lacked primary antibody or the primary antibody was replaced with rabbit serum from a nonvaccinated rabbit. Positive control sections were comprised of cell pellets infected or noninfected with *M. paratuberculosis*. Bovine macrophages were grown in tissue culture flasks; half of the flasks were infected and others remained noninfected. After 18 hr of incubation, cells were released from the flasks, fixed, pelleted, and processed.

Results

Serum antibodies to *M. paratuberculosis* were measured on day 28, 14 days after the secondary boost. Titers to *M. paratuberculosis* were negligible for rabbits.
inoculated with LMP regardless of inoculation route. Positive antibody titers were detected in sera from rabbits inoculated with the HKM prep., ranging from 1:16 to 1:64. Titers were immeasurable at this stage for animals inoculated with the KLH–CWP preparation. After the last set of injections, titers had increased significantly in all treatment groups. One rabbit given LMP intraperitoneally had a 1:16 titer, whereas 1 rabbit given an intravenous inoculation of LMP reached a 1:64 titer. The other rabbits in this group had no demonstrable titer to *M. paratuberculosis* (< 1:8). Two of the rabbits given the HKM prep. had titers of 1:1,024, and the third rabbit had a 1:128 titer. Similarly, 2 of the 3 rabbits given the KLH–CWP prep. had titers reaching 1:1,024, and the other had a 1:512 titer.

The 3 different polyclonal antibodies to *M. paratuberculosis* were utilized in immunohistochemical staining of ileum and lymph node from cows infected with paratuberculosis. Similar staining intensities were present in both tissues of all animals. A serial titration of the polyclonal antisera demonstrated that the KLH–CWP antibody had a higher binding affinity to *M. paratuberculosis* than did the other 2 antibodies. The KLH–CWP antiserum was diluted to an end point of 1:60,000 with positive staining. In contrast, the HKM prep. and the LMP antibodies reached earlier end point dilutions of 1:30,000 and 1:10,000, respectively. Reactivity of the 3 polyclonal antisera at a 1:30,000 dilution with bovine ileal tissue from cows infected with *M. paratuberculosis* is depicted in Figs. 1-3. No positive staining was observed for the LMP antibody at this dilution (Fig. 1). However, very low intensity staining was observed for the HKM prep. antibody and staining was further enhanced with the 1:30,000 dilution of the KLH–CWP antibody (Figs. 2, 3). Immunoreactivity was present in the cytoplasm of macrophages and giant cells and extracellularly. No staining was apparent in other cell types present. Further verification that *M. paratuberculosis* was associated with macrophages was obtained by double-label immunohistochemical staining (Fig. 4).
cells were immunoreactive for both *M. paratuberculosis* and CD68.

These antisera were evaluated for cross-reactivity with *M. bovis* antigens by immunohistochemical staining of tissues from infected animals. Prefemoral and cervical lymph nodes and liver samples obtained from pigs intravenously infected with *M. bovis* (10^4 organisms/inoculum) were stained with the *M. paratuberculosis* polyclonal antibodies. Tissues were devoid of positive reactivity regardless of which antiserum was used when evaluated at the same dilutions that demonstrated positivity in *M. paratuberculosis*-infected tissues. Polyclonal antibodies at 1:1,000 dilution were still unable to detect *M. bovis* organisms; however, acid-fast staining revealed numerous *M. bovis* organisms in these tissues. Sections of lymph nodes from a cow naturally infected with *M. bovis* were also stained with various dilutions (1:1,000, 1:5,000, 1:10,000, 1:20,000) of the 3 polyclonal antibodies, with no detectable cross-reactivity.

**Discussion**

*Mycobacterium paratuberculosis* was detected in intestinal tissue and associated lymph nodes of infected cattle by all 3 polyclonal antibodies tested. However, the polyclonal antibody raised against the CWP preparation was unquestionably the most sensitive antibody of the 3. We originally thought that a live preparation of *M. paratuberculosis* would be highly antigenic and would induce the greatest level of antibody. However, the heat-killed *M. paratuberculosis* and the *M. paratuberculosis* KLH-CWP preparations had substantially higher end point titers in the resulting polyclonal antibodies than did the live *M. paratuberculosis* inoculum. The LMP inoculum was designed to simulate a natural infection state, with all the cellular components of the bacterium intact. In contrast, using heat to kill the organism may have denatured some of the proteins on the cell surface, resulting in an increase in antigenicity. Alternatively, treatment of *M. paratuberculosis* with heat may also have resulted in the exposure of more antigens by altering the waxy layer of the cell wall. Isolation of the mycobacterial cell wall from other cell components for the CWP preparation similarly enhanced the antigenicity of the inocula. The sensitivity of the polyclonal antibody to the CWP preparation in the immunohistochemical staining of tissue from infected animals suggests that some portion of the cell wall is highly antigenic and capable of stimulating a high degree of humoral immune response to *M. paratuberculosis*. Although little is known about specific antigenic components of *M. paratuberculosis* compared with other mycobacterial species, a surface glycopeptidolipid has been recognized as being highly immunoreactive. The presence of mineral oil (incomplete Freund’s adjuvant) in the HKMp and CWP inoculum preparations may also have contributed to the high titers. The respective antigen/oil emulsions formed depot sites at the sites of injection, allowing for a more protracted release of antigen and thereby increasing the time of exposure to the immune system, with resultant increases in titer.

An indirect immunoperoxidase assay for diagnosis of paratuberculosis in cattle has been previously described. That method was proposed as a screening test for cattle with paratuberculosis, using sera in the immunohistochemical detection of the organism in formalin-fixed ileocecal tissue obtained from a clinically infected animal. Positive detection of the organism would indicate the presence of antibodies to *M. paratuberculosis* in the serum, which could be used as a criterion to determine infectivity in that animal. Although this method has potential as a serologic diagnostic tool, the sensitivity achieved appeared to be relatively low. Comparison of this technique with other serologic diagnostic tests was not conducted, so true efficacy of immunohistochemistry as a serodiagnostic tool could not be determined.

Immunohistochemical methodology is more often utilized to determine presence of antigens in tissues. Three immunohistochemical methods were compared for detection of *M. paratuberculosis* in bovine tissues, the peroxidase-antiperoxidase, streptavidin-biotin, and avidin-biotin complex techniques. There was no difference in sensitivity among the 3 methods; however, the immunoperoxidase techniques were superior to ZN (acid-fast) staining for detection of low numbers of *M. paratuberculosis* in tissue sections. Comparative evaluation of immunohistochemistry, acid-fast staining, and culture for detection of *M. paratuberculosis* in tissues of infected goats demonstrated a higher percentage of positives obtained via immunohistochemical analysis than via the other 2 methods.

The simplicity and the speed of the immunohistochemical technique define its value as a diagnostic tool. Although acid-fast staining of smears from intestinal material or tissue sections is relatively easy, it is non-specific. In addition to mycobacteria, other organisms such as *Nocardia* and *Corynebacteria* also stain acid-fast, which makes a definitive diagnosis impossible. In the present study, production of a polyclonal antibody to cell wall proteins of *M. paratuberculosis* resulted in a highly sensitive, species-specific tool for the detection of paratuberculosis in tissue sections.

**Acknowledgements**

We thank Kim Driftmeyer, Trudy Bosworth, and Janis Hansen for their excellent technical assistance.

**Sources and manufacturers**

a. National Animal Disease Center, Ames, IA.

b. Pierce, Rockford, IL.
References