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Research paper

Relationship between the pathology of bovine intestinal tissue and current diagnostic tests for Johne's disease

Caitlin J. Jenvey[a], Jesse M. Hostetter[b], Adrienne L. Shircliff[a], Judith R. Stabel[a,*]

[a] USDA-Agricultural Research Service (ARS), National Animal Disease Center, Ames, IA, United States
[b] Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA, United States

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ABSTRACT

Johne's disease is an enteric disease caused by the intracellular pathogen *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Upon translocation from the lumen of the small intestine, mycobacteria have the ability to thwart innate defense mechanisms and persist within the macrophage in the lamina propria. In an effort to understand how the pathology of disease is reflected in current diagnostic tests, immunofluorescent (IFA) labeling was performed to quantitate macrophage and MAP numbers in the ileum of infected cattle and correlate results with common methods for diagnosis of MAP infection; including ELISA, IFN-γ assay, RT-PCR, culture of MAP, and histological classification of tissue sections. Predictive models for clinical and subclinical disease states, histopathology acid-fast (AF), MAP location, granulomatous inflammation and type classifications, as well as macrophage, MAP and macrophages with intracellular MAP IFA labeling were successfully developed. The combination of macrophage number and ELISA were the best predictors of clinical disease state, while macrophage number was the best and only significant predictor of subclinical disease state. Fecal culture and number of MAP were the best predictors of granulomatous inflammation, and of combined AF, MAP location and granuloma type, respectively. Additionally, fecal culture and tissue culture were the best predictors of numbers of macrophages and MAP, respectively, while both ELISA and tissue culture were the best predictors of number of macrophages with intracellular MAP.

1. Introduction

The gastrointestinal system contains the largest reservoir of tissue macrophages in the body. In response to bacterial infiltration, macrophages are recruited to the sub-epithelial lamina propria from the circulation *via* the bone marrow (Arsenault et al., 2014; Smith et al., 2011; Weber et al., 2009). *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is successfully phagocytized by intestinal macrophages, however, MAP are able to avoid degradation by preventing phagolysosome fusion and acidification (Arsenault et al., 2014; Benoit et al., 2008; Cheville et al., 2001; Hostetter et al., 2003), allowing it to persist within the macrophage throughout the course of the disease. Secretion of chemokines by infected macrophages results in recruitment of additional macrophages to the sub-epithelial lamina propria in an attempt to destroy the mycobacterium (Charavaryamath et al., 2011). Recently, we have observed increased numbers of macrophages in mid-ileal intestinal tissue of cows naturally infected with MAP, when compared to uninfected control tissue (Jenvey et al., 2017). It was of interest to us whether numbers of macrophages might be correlated with routine diagnostic tests used for the diagnosis of MAP infection, including MAP-specific antibodies *via* ELISA, MAP-specific interferon-gamma (IFN-γ) secretion, MAP RT-PCR of feces and tissue, and histological examination and classification of tissue sections. To date, there has been no research that has demonstrated if correlations exist between intestinal tissue macrophages *in vivo* and ante- and post-mortem methods used to diagnose MAP infection. The current study aimed to determine whether correlations were present between numbers of macrophages, numbers of MAP and numbers of macrophages with intracellular MAP, and common methods used for the diagnosis of MAP infection. Further, models to predict numbers of immunofluorescent-labeled (IF) macrophages, MAP or macrophages with intracellular MAP, or conversely, histopathology classification (AF, MAP location, granulomatous inflammation and type) or progression of disease status, were developed and applied.

* Corresponding author at: 1920 Dayton Rd, Ames, IA, 50010, United States.
E-mail address: judy.stabel@ars.usda.gov (J.R. Stabel).

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2. Materials and methods

2.1. Animals

Samples of mid-ileal tissue were collected at necropsy from a total of 20 Holstein dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and 8 uninfected control cows uninfected with MAP. Holstein dairy cows ranged in age from 4 to 9 years in this study, and were placed in three groups consisting of 8 non-infected healthy cows, 10 cows naturally infected with MAP but asymptomatic (*i.e.* subclinical), and 10 cows with the clinical form of disease. Prior to necropsy, infection was monitored bacteriologically for fecal shedding of MAP using fecal culture and PCR as previously described (Khalifeh et al., 2009), as well as serologic tests, such as ELISA and antigen-specific IFN-γ assay (Stabel and Whitlock, 2001). In addition to fecal shedding and serologic analysis, cows assigned to the clinical group also demonstrated weight loss and watery diarrhea. Prior to necropsy, animals categorized as clinical demonstrated serum ELISA antibody titers averaging 2.45 S/P ratio and fecal shedding averaging 2370 CFU of MAP/g of feces. Cows in the subclinical group were ELISA negative, and averaged < 17 CFU of MAP/g of feces. Infected animals had positive antigen-specific IFN-γ results ($Abs_{450nm}MPS - Abs_{450nm}NS$), averaging 0.23 ± 0.12 and 0.21 ± 0.07 for subclinical and clinical cows respectively. Because infection groups had similar mean values, IFN-γ results for all infected animals were collapsed into one group for analyses. All animal related procedures, such as euthanasia and necropsy, were approved by the IACUC (National Animal Disease Center, Ames, Iowa).

2.2. ELISA and IFN-γ assay

A commercial kit was used to measure serum antibodies to MAP (HerdChek, IDEXX, Wetbrook, ME) with ≥0.55 S/P ratio considered as positive cut-off values as described by the manufacturer. Antigen-specific IFN-γ secretion was measured by ELISA (Bovigam, Thermo Fisher Scientific, Carlsbad, CA) after stimulation of whole blood with a whole cell sonicate of MAP for 18 h as previously described by Stabel and Whitlock (2001). Results were expressed as $MPS_{Abs450nm} - NS_{Abs450nm}$ with values ≥0.1 considered positive.

2.3. Fecal and tissue culture

At necropsy, up to 22 tissues (2–10 g) were collected, including proximal, mid-, and distal ileum, ileo-cecal valve, proximal, mid-, and distal jejunum, duodenum, along with corresponding lymph nodes of each region, as well as liver and spleen. Tissues were rinsed with 0.15 M PBS and cut into multiple cross-sections for culture of MAP and PCR to assess infection status. Cross-sections immediately adjacent were processed for histopathology and IF. Tissues (2 g) were homogenized in 0.9% hexadecylpyridinium chloride (HPC) prior to incubation for 3 h, then centrifuged at 1700 × *g* for 20 min. Similarly, fecal samples (2 g) obtained just prior to necropsy were decontaminated overnight at 39 °C in 0.9% HPC, followed by centrifugation at 1700 × *g* for 20 min the following day. Fecal and tissue sample pellets were resuspended in 1 ml of an antibiotic solution containing 100 μg/ml naladixic acid (Sigma Chemical Co., St. Louis, MO), 100 μg/ml vancomycin (Sigma) and 50 μg/ml amphotericin B (Sigma) (Stabel, 1997). After overnight incubation at 39 °C, decontaminated samples (200 μl) were inoculated onto slants of Herrold's Egg Yolk Medium (HEYM; BBLTM Herrold's Egg Yolk Agar Slants with mycobactin J, amphotericin, nalidixic acid, and vancomycin; Becton Dickinson and Co., Sparks, MD) in replicates of 4 and incubated for 12 weeks at 39 °C. Tubes were examined and colony counts enumerated every 4 weeks during the 12-week period. At 12 weeks, a final read was taken and colony count averaged across all 4 slants for each cow.

2.4. Real time-polymerase chain reaction

Commercial DNA extraction kits were used to extract MAP DNA from fecal (MagMax Total Nucleic Acid Isolation Kit, Applied Biosystems, Foster City, CA) and tissue (Ultraclean Tissues and Cells DNA Isolation Kit, MoBio Labs, Carlsbad, CA) samples as per manufacturer's instructions. The RT-PCR was performed as previously described for detection of the IS900 target gene (Stabel et al., 2014). Each PCR plate contained a standard curve that was generated using MAP strain K-10 genomic DNA (10 fg/μL to 1 ng/μL), no template negative controls, and a positive control consisting of MAP strain 19,698 genomic DNA. Results were expressed as cycle threshold (Ct).

2.5. Tissue snap-freezing protocol

At necropsy, the entire section of ileum extending from the ileo-cecal valve through the distal flange was excised and then cut equally into proximal, mid- and distal sections. A dry ice bath was prepared by combining 95% ethanol with dry ice and mixed until a slurry consistency was achieved. Isopentane (Sigma-Aldrich, St. Louis, MO) was added to a tin cup and the cup was placed into the dry ice bath. The mid-ileal intestinal samples were immediately washed with PBS, pH 7.4, and a cross-section (2 gm) was positioned luminal side down on a section of liver covered with Tissue-Tek Optimum Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA), in order to protect the villi during the freezing process and to ascertain tissue orientation post-freezing. The intestine-liver sample was wrapped in foil and placed in the isopentane for at least 5 min. The snap-frozen sample was transferred to dry ice for transport to storage at −80 °C, where it remained until tissue sectioning could be performed.

2.6. Frozen sections

The mid-ileal intestinal samples were removed from −80 °C and placed in a cryostat at −20 °C for at least 30 min prior to sectioning. Tissue samples were embedded in O.C.T., cut in 6 μm sections and adhered to ColorFrost Plus microscope slides (ThermoFisher Scientific, Carlsbad, CA). Tissue sections were allowed to air-dry overnight at room temperature before fixing for 5 min at −20 °C. Tissue sections were stored at −80 °C until immunofluorescence staining could be performed.

2.7. Histochemistry

Serial sections of frozen mid-ileal tissue were stained with Harris-Hematoxylin and Eosin (H and E) and Ziehl-Neelsen (ZN) for staining of acid-fast (AF) bacteria, following fixation in 1:1 acetone methanol for 5 min at −20 °C.

2.7.1. H and E staining

H and E staining was performed using a Leica autostainer. Briefly, frozen slides were equilibrated to room temperature and re-hydrated in deionized water for 5 min. Slides were then placed in Harris-Hematoxylin for 5 min, rinsed in a deionized water bath for 5 min, dipped in acid alcohol, followed by another rinse in a deionized water bath for 5 min. Sections were then placed in 70% alcohol for 1 min and then placed in Eosin solution for 10–15 seconds to stain the cytoplasm. Slides were then dehydrated in 95% alcohol, twice for 1 min each, followed by 100% alcohol three times for 1 min each. Sections were then cleared using Propar three times for 5 min each and coverslipped.

2.7.2. ZN staining

Frozen slides were equilibrated to room temperature and re-hydrated in deionized water for 5 min. Slides were placed in carbol fuchsin solution for 60 min at room temperature. Slides were de-colorized in stock acid alcohol until sections appeared pale pink