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Short Communication

Sample handling substantially affects Johne's ELISA

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ABSTRACT

Detection methods for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are imperfect, yet crucial for diagnosis of Johne's disease. Our purpose was to test for significant and biologically relevant changes in Johne's ELISA results associated with how field-collected blood samples were transported to the laboratory, prepared and stored prior to testing, while removing potential confounding by test kit and laboratory variables. Blood samples were collected from 21 cows that previously had MAP ELISA scores ranging from negative to highly positive. Samples for immediate laboratory processing were subjected to different transportation temperatures (on ice, 26 °C) and preparation methods (serum separated, hemolyzed and serum separated, clotted whole blood), but were tested using the same ELISA kit in the same laboratory. Samples for laboratory processing after one week of storage were subjected to different storage temperatures (4 °C, −20 °C) and preparation methods (serum separated, hemolyzed and serum separated, clotted whole blood), and again were tested using the same ELISA kit in the same laboratory. Finally, samples were evaluated by time to processing (one day, one week) and storage temperature (4 °C, −20 °C). Data were checked for normality and analyzed with repeated measures ANOVAs. Significantly ($P = 0.027$) higher MAP ELISA scores were recorded for whole blood and hemolyzed samples transported at 26 °C than serum separated samples. Sample storage for one week at −20 °C resulted in significantly ($P < 0.001$) lower MAP ELISA scores, regardless of handling method, compared to samples stored at 4 °C for one week. Method of sample preparation, as well as transportation temperature and medium-term storage temperature, affects MAP ELISA results. Such discrepancies will inevitably result in improper classification of MAP-infected cattle, impeding both biosecurity measures on uninfected farms and MAP control programs.

1. Introduction

Johne's disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), and in cattle is characterized by chronic diarrhea and weight loss. Detection methods for MAP infection are imperfect; therefore, reported sensitivity and specificity of ELISA and culture can be variable. Specificity of MAP serum ELISA is typically reported to be 98–99%; sensitivity, however, has a broader range of values: for example, estimates of 43%, 59% and 37% have been reported (Sockett et al., 1992; Whitlock et al., 2000; Eamens et al., 2000, respectively). Furthermore, as more test-positive animals are removed from a herd, the sensitivity of a serum ELISA reportedly decreases, making the test less useful (Whitlock et al., 2000). There can be substantial differences (28% coefficient of variation) between ELISA test kits and laboratories, even when using the same test method (Jacobson et al., 1999). Differences between test kits and laboratories have been attributed to kit lot number, random error, inter-laboratory variation and an interaction of kit lot number by laboratory (Dargatz et al., 2004). In 2006, to resolve confusion over different

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reported values, field experts defined "consensus-based" sensitivity and specificity to be 60 ± 5% and 99.9 ± 0.1%, respectively for culture and 30 ± 5% and 99.0 ± 1.0%, respectively, for serum ELISA (Collins et al., 2006).

The agreement between MAP ELISA results and faecal culture results may be poor (Jacobson et al., 1999). The ELISA is most likely to agree with faecal culture or necropsy results when tested animals are in the very late stages of Johne's disease (Nielsen et al., 2002; Whitlock et al., 2000). Despite variable results and unpredictable agreement with faecal culture, the ELISA is one of the tests recommended by the United States National Voluntary Bovine Johne's Disease Control Program (www.aphis.usda.gov/vs/nahps/johnes). The program, developed under the guidance of the United States Department of Agriculture and the United States Animal Health Association, recommends use of screening tests (ELISA) and confirmatory tests (faecal culture) for cattle herds based on MAP prevalence, herd size and herd composition. Specifically, if the Johne's disease status of a herd is unknown, it is recommended to screen cattle for the presence of MAP antibodies with the ELISA test. If cattle are found to be ELISA-test positive, the program recommends confirming individual cows are faecal culture positive before making management changes. Guidelines do not direct veterinarians to standard sample handling procedures prior to laboratory processing and testing.

A review of Johne's-related peer-reviewed publications shows a wide variation in ELISA sample handling techniques. Some reports do not state how the samples were transported or when they were processed (Hendrick et al., 2005; Van Schaik et al., 2003; Eamens et al., 2000; Whitlock et al., 2000). Other authors report immediate laboratory submission (Nordlund et al., 1996; Goodger et al., 1996; Pavlik et al., 2000). The third group of publications report delays in sample processing; these reports state samples were stored at either −20 °C or −70 °C for unknown durations, (Sockett et al., 1992; Johnson-Ifearulundu and Kaneene, 1998; Tavornpanich et al., 2004). One author reports storage of up to one year (Strickland et al., 2005). Thus, the evidence is inconclusive regarding whether storage temperature and/or duration have an affect on researchers' ability to detect MAP.

While it is unclear how sample handling may affect MAP detection techniques, delayed sample processing is known to lead to substantial differences in ELISA values for blood components other than serum antibodies. Decreased ELISA values to interferon gamma, in response to infection with *M. tuberculosis*, as storage time increased have been reported (Doherty et al., 2005). Other authors found an increase in serum levels of tissue inhibitor of metalloproteinases-1 with increased storage time (Holten-Andersen et al., 2003). However, not all ELISAs are sensitive to handling methods. An ELISA to measure antibodies to human thymus and activation-regulated chemokine concentrations did not show fluctuations in values in response to storage times (Morita et al., 2002). To our knowledge, effects of sample handling on serum antibody levels to MAP, as measured by ELISA, have not been published in the peer-reviewed literature.

The aim of this study was to test the hypothesis that sample handling substantially (both statistically significant and biologically relevant) affects MAP ELISA scores. To test this hypothesis, we excluded potential confounding by restricting this study to one test kit, one laboratory and one technician.

2. Materials and methods

Briefly, three sample handling scenarios were evaluated. In the first, blood samples from 9 cows ELISA test strong-positive were evaluated for effects of sample preparation and transportation temperature. These samples were ELISA tested the day after collection. In the second scenario, blood samples from the same 9 cows were evaluated for sample preparation and storage temperature; these samples were stored for one week before laboratory testing. In the third scenario, whole blood samples from 21 cows with a broad range of ELISA scores (negative to strong-positive) were evaluated for the effects of storage temperature and storage duration. Subject animals were identified from previous herd testing as part of a Johne's disease surveillance program; strong-positive animals were known before the start of this study.

2.1. Study design/sample collection and preparation

The study was conducted in three parts. In the first part, blood samples from 9 cows previously tested strong-positive (score > 3.5) by ELISA (Biocor ELISA, Omaha, NE) to MAP were subjected to different preparation (serum separated, hemolyzed and serum separated, serum not separated from clotted whole blood—hereafter referred to as whole blood) methods and transportation temperatures (0 °C, 26 °C) with overnight storage at 4 °C. These samples were ELISA tested the day after collection.

In the second part of the study, blood samples from the same 9 strong-positive cows were stored at different temperatures (4 °C, −20 °C) for one week after sample collection; these samples were also subjected to the same handling methods (serum separated, hemolyzed and serum separated, serum not separated from clotted whole blood) as the initial phases of the study and were transported to the laboratory at 0 °C.

Once it was determined there was a statistical difference between the treatment methods, the study group was broadened to include cows of all ELISA scores (from 0.4 [negative] to 14.7 [strong-positive]) and determine the biological significance of storage length and temperature. In the third part of the study, whole blood samples were collected from 21 cows and subjected to 3 treatment protocols: ELISA testing the day after collection with overnight storage at 4 °C, ELISA testing after one week of storage at either 4 °C, or ELISA testing after one week of storage at −20 °C.

All blood samples were collected into separate 10 ml red-topped, vacutainer tubes, via one jugular venipuncture per cow. Hemolyzed samples were obtained by vigorous shaking at the time of collection for 2 min before blood coagulation occurred. For all tested cows, faecal samples were also collected per rectum and submitted for MAP culture. Collection of blood and faecal samples was approved by the Purdue Animal Care and Use Committee.