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Lymphatic fluid for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in cows by PCR, compared to fecal sampling and detection of antibodies in blood and milk

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ABSTRACT

Johne's disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*), can cause considerable economic losses in affected herds. Early diagnosis of JD is hampered by the chronic nature of the disease with a slow subclincal progression. The aim of the present study was to challenge the hypothesis that lymphatic fluid is of diagnostic value in the early stages of the disease. Lymphatic fluid from 122 animals was collected and tested for *MAP* by nested PCR for IS*900* and compared to the results of testing for *MAP* in feces (culture), blood and milk (ELISA) in 110 of these samples. *MAP* was detected by PCR in 27.1% of the lymph samples. Agreement between the tests was poor: 6.9% of the lymph positive cows were also positive in all other tests applied, and 69.0% had negative results in fecal culture, blood and milk ELISA. Resampling of 25 cows after 8 to 12 and 16 to 20 months revealed 20.0% lymph positive animals at the first, 5.5% at the second and 27.8% at the third sampling, respectively. Only one cow showed positive lymph-PCR results at more than one sampling date. Lymph-positive cows had a 7.2 times greater likelihood of being culled within 8 to 12 months after sampling, compared to negative cows, mainly due to other health issues than JD. It can be concluded, that lymphatic fluid might be promising for the detection of early *MAP*-infection in cows, but further studies to elucidate the potential of this diagnostic approach are needed.

1. Introduction

Johne's disease (JD), also called paratuberculosis, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) and occurs in ruminants all over the world (Barkema et al., 2001; Hirsh and Biberstein, 2004). The estimated prevalence of JD is about 68% of the dairy operations, both in the U.S. and parts of Europe (APHIS Info Sheet April 2008, http://www.aphis.usda.gov; Nielsen and Toft, 2009). The disease can cause considerable economic losses in affected farms, including a loss of ~$200 (±$160 U.S.) million due to reduced milk yield (Losinger, 2005) and is listed as notifiable by the World Organization of Animal Health (OIE, www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2013/).

Animals infected with *MAP* shed high amounts of the bacterium with their feces (Manning and Collins, 2010) with additional shedding in milk and colostrum in

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advanced cases of JD (Slana et al., 2008). Transmission of JD mostly takes place by ingestion of the organism in infected feces, milk or colostrum, followed by uptake of *MAP* by M cells in the Peyer patches of the small intestine (Momontani et al., 1988). The bacterium is then passed to macrophages and migrates within the macrophages to mesenteric lymph nodes and to the blood stream, passing the *ductus thoracicus*, leading to distribution of *MAP*-containing macrophages throughout the animal (Chiodini, 1996; Lugton, 1999). Due to a mean incubation period of 5 years in dairy cows (Koenig et al., 1993), clinical signs of JD such as diarrhea and weight loss, occur late after infection with *MAP*. A late and intermittent shedding of *MAP* at low levels in cattle with subclinical JD (Waters et al., 2003), as well as a delayed and variable immune response (Nielsen, 2008) hampers the diagnosis of JD in early stages of the infection.

Bacterial culture of *MAP* in feces is considered the gold standard in the diagnosis of JD (World Organization for Animal Health (OIE), 2012) with a high specificity. However, sensitivity of fecal culture has been reported between 39.0% and 92.0%, depending on the stage of infection of the tested animals (Waters et al., 2003). Polymerase chain reaction (PCR) is routinely used for the diagnosis of JD in fecal and tissue samples but can also be applied for *MAP* detection in milk and blood. The specificity of the PCR is about 97.0%, with a reported sensitivity of 60.0% (Alinovi et al., 2009), depending on primers, techniques, protocols, and study designs used (Bölske and Herthnek, 2010). The Enzyme-linked immunosorbent assays (ELISA) can be used as an indirect test for JD by detection of *MAP*-specific antibodies and is mostly used as screening test for *MAP*. The sensitivity and specificity of the ELISA range between 7.0% and 94.0%, and 41.0% and 100% (Buergelt and Williams, 2003), respectively, depending on the test kit and study population used.

The bovine udder has a distinct system of afferent lymph vessels (Fürll et al., 2006) with a flow of 13 to 45 ml of lymphatic fluid per hour in healthy cows (Gorewit et al., 1993). The large subcutaneous lymph vessels of the bovine udder are visible through the skin (Dyce et al., 1996) and can be used for collection of lymphatic fluid by puncture (Fürll et al., 2006; Hagen and Fürll, 2011; Khol et al., 2012). Khol et al. (2012) tested lymph samples of 51 cows originating from herds with a history of JD and found 43.1% of the samples positive for *MAP* by PCR.

The aim of the study was to test the hypothesis that lymphatic fluid from the udder of cows is of advantage over fecal culture and ELISA for the diagnosis of JD, because of the occurrence of *MAP*-containing macrophages within lymphatic fluid in the early stages of the disease. Therefore, results of lymph-PCR were compared to those of fecal culture, as well as to blood and milk ELISA results in single and consecutive samples taken over a period of 20 months.

2. Materials and methods

2.1. Study population

Altogether, 86 cows originating from 3 dairy farms located in north central Florida and known to be infected with *MAP* were included in the study. In another 11 cows no lymph sample could be gained and therefore the animals are not included in the study population. The majority (79) of the cows included was selected from the University of Florida Dairy Unit (DU) facility (Gainesville, FL); the remaining seven cows belonged to two large commercial dairies. The DU-farm consisted of approximately 450 lactating cows, while the two commercial farms housed approximately 3500 and 1500 lactating cows, respectively. Animals were kept in free stall barns and were milked in a double herringbone milking parlor in all three farms. The predominant breed was Holstein, with a study population of 96.5% (83/86) Holstein cows, the remaining animals consisted of 2.3% (2/86) Red Holstein, and 1.2% (1/86) Holstein crossbred cows. The sampled animals had a mean age of 5.1 years (1.8 to 9.2); all cows were lactating at the sampling date.

The majority of the cows were not randomly selected, but chosen with the aim to test animals with an increased likelihood to be infected with JD. Clinical signs of JD, such as diarrhea and weight loss were present in 7.0% (6/86) of the cows, another 8.1% (7/86) where included in the study because they were among the oldest cows of the herd and had positive or suspicious *MAP* serum ELISA results. Most of the cows, 80.2% (69/86), were sampled because of a low body condition score. The remaining 4.7% (4/86) cows included in the study were sampled at random occasions.

To follow the detection of *MAP* in lymph over time, cows housed at the DU-farm were resampled twice if they were still in the herd and lactating at sampling date, leading to 25 resampled animals. The first resampling was performed 8–12 months after the first sampling and the second 8 months after that time, resulting in three consecutive lymph samplings within 16–20 months. Consecutive samples were collected three times in 44.0% (11/25) and two times in 56.0% (14/25) of these cows. Herd records were used to evaluate the culling rate of sampled cows within 8 to 12 months after sampling.

Overall, lymph puncture was performed 122 times in the cows enrolled; specimens from cows sampled more than one time were classified as a new sample in the data set, when collected more than 6 months apart.

For comparison of the lymph-PCR results to those of fecal culture and blood and milk ELISA, only the 110 samplings in which all tests were performed were considered for evaluation. A description of the study population and sampling scheme are given in Fig. 1.

2.2. Sample collection

All sampling procedures were approved by the Institutional Animal Care and use Unit of the University of Florida (IACUC protocol no. 200903646). Lymph samples were collected after milking with cows restrained in an examination parlor. An 18-gauge (1.27-mm) needle was used after cleaning of the puncture site with an iodophor scrub and 70% alcohol. Cows were not sedated for sampling, puncture of the lymph vessel was performed as described by Khol et al. (2012). The lymphatic fluid was collected in a sterile tube containing 0.75 ml of ethylenediamine tetra-acetic acid (EDTA, Vacutainer® Becton,