rccp Revista Colombiana de Ciencias Pecuarias

Fecal culture and two fecal-PCR methods for the diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* in a seropositive herd¤

Cultivo fecal y dos métodos de PCR en materia fecal para el diagnóstico de Mycobacterium avium subsp. paratuberculosis en un hato seropositivo

Cultivo fecal e dois métodos de PCR em matéria fecal para o diagnóstico de Mycobacterium avium subsp. paratuberculosis em um rebanho soropositivo

Nathalia M Correa-Valencia[1], MV, MSc, DSc(c); Nicolás F Ramírez[1], MV, MSc, Dr An Sci; Michael Bülte[2], Prof. Dr. Med. Vet.; Jorge A Fernández-Silva[1*], MV, MSP, Dr Med Vet.

[1]*Línea de Investigación en Epidemiología y Salud Pública Veterinaria, Grupo de Investigación CENTAURO, Escuela de Medicina Veterinaria, Facultad de Ciencias Agrarias, Universidad de Antioquia, Medellín, Colombia.*

[2]*Institut für Tierärztliche Nahrungsmittelkunde, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Frankfurter Straße 92, Giessen, Germany.*

(Received: June 10, 2016; accepted: October 18, 2016)

doi: 10.17533/udea.rccp.v30n2a02

Abstract

Background: paratuberculosis is a slow-developing infectious disease, characterized by chronic granulomatous enterocolitis. This disease has a variable incubation period from 6 months to over 15 years, and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Its detection by direct and indirect diagnostic techniques has been of special interest. **Objective:** to report the diagnosis and detection of MAP using several diagnostic tests in a herd of the Northern region of Antioquia, Colombia. **Methods:** serum samples from the study herd were analyzed, using a commercial ELISA (enzyme-linked immunosorbent assay) kit. Fecal samples were cultured by duplicate using Herrold´s egg yolk medium (HEYM), and analyzed by an end-point IS*900*-specific nested PCR protocol, and a commercial F*57*-real-time PCR kit. **Results:** eight out of 27 serum samples in the study herd resulted ELISA-positive. None of fecal samples resulted positive to HEYM culture by duplicate and none were found to be positive by F*57*-real-time PCR. Seven of the 27 fecal samples

¤ To cite this article: Correa NM, Ramírez NF, Bülte M, Fernández JA. Fecal culture and two fecal-PCR methods for the diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* in a seropositive herd. Rev Colomb Cienc Pecu 2017; 30:101-115.

* Corresponding author: Jorge Arturo Fernández Silva. Escuela de Medicina Veterinaria, Facultad de Ciencias Agrarias, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia. E-mail: jorge.fernandez@udea.edu.co

were found to be positive by end-point IS*900*-specific nested PCR. Agreement was found between ELISA and end-point IS*900*-specific nested PCR in one of the animals. **Conclusion:** the present study gives information about the agreement between direct and indirect MAP-detection techniques, using different matrixes from animals under the same husbandry conditions.

Keywords: *culture medium, ELISA, Johne´s disease, MAP, molecular diagnosis.*

Resumen

Antecedentes: la paratuberculosis es una enfermedad infecciosa de desarrollo lento, caracterizada por una enterocolitis granulomatosa crónica. Esta enfermedad tiene un periodo de incubación que varía entre los 6 meses hasta por más de 15 años, y es causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Su detección por técnicas diagnósticas directas e indirectas ha sido de interés especial. **Objetivo:** reportar el diagnóstico y detección de MAP utilizando varias técnicas diagnósticas en un hato de la región norte de Antioquia, Colombia. **Métodos:** se analizaron las muestras de suero del hato de estudio utilizando un kit comercial de ELISA (enzyme-linked immunosorbent assay). Las muestras de materia fecal fueron cultivadas por duplicado en Herrold´s egg yolk medium (HEYM), y analizadas mediante un protocolo de PCR anidado específico de IS*900* y un kit comercial de PCR en tiempo real para F*57*. **Resultados:** ocho de las 27 muestras de suero resultaron positivas por ELISA. Ninguna de las muestras de materia fecal resultó positiva al cultivo en HEYM por duplicado ni por PCR en tiempo real para F*57*. Siete de las 27 muestras de materia fecal resultaron positivas a PCR anidado específico de IS*900*. Se encontró concordancia entre el resultado de ELISA y de PCR anidado específico de IS*900* en uno de los animales. **Conclusión:** el presente estudio brinda información acerca de la concordancia entre técnicas directas e indirectas de detección de MAP, utilizando diferentes matrices a partir de animales bajo las mismas condiciones de manejo.

Palabras clave: *diagnóstico molecular, ELISA, enfermedad de Johne, MAP, medio de cultivo.*

Resumo

Antecedentes: a paratuberculosis é uma doença infecciosa de evolução lenta, caracterizada por uma enterocolite granulomatosa crônica. Esta doença tem um período de incubação que varia de 6 meses a 15 anos e é causada pelo *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Sua detecção por técnicas de diagnóstico diretos e indiretos tem sido de especial interesse. **Objetivo:** reportar o diagnóstico e a detecção de MAP utilizando várias técnicas de diagnóstico em um rebanho na região norte de Antióquia, Colômbia. **Métodos:** foram analisadas amostras de soro do rebanho utilizando um kit comercial de ELISA (enzyme-linked immunosorbent assay). As amostras de fezes foram cultivadas em duplicado em Herrold´s egg yolk medium (HEYM) e analisadas utilizando um protocolo de PCR aninhada específico de IS*900* e um kit de PCR em tempo real comercial para F*57*. **Resultados:** oito das 27 amostras de soro foram positivas para ELISA. Nenhuma das amostras testadas na cultura de fezes HEYM duplicado foram positivas ou na PCR em tempo real para F*57*. Sete das 27 amostras de fezes foram positivas na PCR aninhada específica para IS*900*. Foi encontrada concordância entre o resultado de ELISA e PCR aninhada específica para IS*900* em um animal. **Conclusão:** este estudo fornece informações sobre a correlação entre técnicas de detecção direta e indireta do MAP, utilizando diferentes matrizes de animais sob as mesmas condições de condução.

Palavras chave: *diagnóstico molecular, doença de Johne, ELISA, MAP, meio de cultura.*

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow-growing, mycobactin-dependent, acid-fast bacterium that causes Johne's disease or paratuberculosis (PTB) in cattle and other susceptible species (Harris and Barletta, 2001). The disease produces a significant economic impact on the cattle industry, especially on milk and meat production (Sweeney, 1996; Chacon *et al.,* 2004; García and Shalloo, 2015; McAloon *et al.,* 2016). The agent has also been associated to the chronic human enteritis known as Crohn's disease (Atreya *et al.,* 2014; Hanifian, 2014; Liverani *et al*., 2014; Waddell *et al.* 2015; 2016).

For the *ante-mortem* diagnosis of PTB in cattle, several types of test are available and proposed. These include tests to detect antibodies against MAP, detection of MAP genes, bacterial culture of fecal samples and test to detect MAP on tissue samples (Collins *et al.,* 2006; Nielsen and Toft, 2008; Stevenson, 2010a; 2010b). Sensitivity and specificity of tests for the *ante-mortem* diagnosis of PTB vary significantly depending on MAP infection stage and intrinsic characteristics of each test (Nielsen and Toft, 2008).

The antibody detection test known as enzyme-linked immunoassay (ELISA) is the most popular test to detect an immune response to infection by MAP. The ELISA is also the most widely used technique to establish PTB status of herds, but it has shown limitations in some extend relating low sensitivity, primarily because of the slow progression of MAP infection. This does not ensure an adequate detection capacity of animals in an early stage of infection when fecal shedding is low (Kalis *et al.,* 2002; McKenna *et al.,* 2006; Nielsen, 2010). On the contrary, ELISA is highly specific, with a low presentation of false positive results (Harris and Barletta, 2001).

Cultivation of MAP from tissues and fecal samples (individual, in pool, and environmental) is the most reliable method of detecting infected animals (Nielsen and Toft, 2008; 2009; Fecteau and Whitlock, 2010). Usually, the specificity of fecal culture is considered to be almost 100% if the isolates obtained are confirmed to be MAP by molecular methods such as polymerase chain reaction (PCR; Nielsen and Toft, 2008; Schönenbrücher *et al.,* 2008; Whittington *et al.,* 2011). Fecal culture has been used as an acceptable standard technique for detecting the infection status of animals —related to elimination rate—, for estimating the sensitivity of other diagnostic tests (e.g. ELISA, PCR), and as an excellent confirmatory test for animals that tested positive with immunological tests (Motiwala *et al.,* 2005; Aly *et al*., 2012). Herrold´s egg yolk medium (HEYM) is the most frequently used technique for the primary cultivation of MAP from clinical samples (feces and tissue), and its sensitivity has been reported from 39 to 82%, compared to liquid media (Collins *et al.,* 1990; Eamens *et al.,* 2000; Stich *et al.,* 2004, Motiwala *et al.,* 2005; Cernicchiaro *et al.,* 2008; Whittington, 2009).

Special aspects of MAP and the disease dynamics can affect the fecal culture accuracy, for example, MAP´s elimination through feces is intermittent and occurs in an advanced stage (stages III and IV) of the disease, mainly when the animals have clinical symptoms (Clarke, 1997; Whittington, 2010; Salem *et al.,* 2013). Although the fecal culture has many limitations, such as a long incubation period (18 to 24 weeks), high costs, risk of contamination with other mycobacteria or fungi, and time required to report the results, it is still considered to be the gold standard for the detection of MAP (van Schaik *et al.,* 2007; Nielsen and Toft, 2008; Whittington, 2010).

The detection of MAP genes by PCR has shown advantages (rapidity, identification of agent, and lack of contamination) and disadvantages (moderate sensitivity, high cost, special equipment, and skilled personnel required; Collins, 1996). However, due to recent developments, PCR has been suggested for herd screening (Collins *et al.,* 2006; Anonymous, 2010), and it has been recently discussed as a possible new gold standard for PTB (Stevenson, 2010a; 2010b). The PCR technique is rapid and specific, and in contrast to a culture-based diagnostic, no additional tests are required to confirm the identity of the organism detected (Collins, 1996).

The most popular target gene for the detection of MAP is the multi-copy element IS*900* (Bolske and Herthnek, 2010; National Advisory Committee on Microbiological Criteria for Foods, 2010; Stevenson, 2010b; Gill *et al.,* 2011). However, mycobacteria other than MAP have been found to carry IS*900*-like elements with nucleotide sequences that are up to 94% identical to the nucleotide sequence of MAP IS*900* (Cousins *et al.,* 1999; Ellingson *et al.,* 2000; Englund *et al.,* 2002; Kim *et al.,* 2002; Taddei *et al.,* 2008). Some PCR systems that target IS*900* also can give false-positive results with DNA from mycobacteria other than MAP and with DNA from other types of organisms (Möbius *et al.,* 2008a; 2008b). Due to this, new protocols avoiding cross-reactions have been reported (Bull *et al.,* 2003; Herthnek and Bölske, 2006; Kawaji *et al.,* 2007). In response to the uncertainty about the specificity of PCR systems that target IS*900* for the identification of MAP, the use of several other target sequences for MAP identification systems have been proposed: IS*Map02*, IS*Mav2*,

hspX, locus 255, and F*57* (Stabel and Bannantine, 2005; Slana *et al.,* 2009; Kralik *et al.,* 2010; Sidoti *et al.,* 2011; Keller *et al.,* 2014).

The PCR performs well as a confirmatory test on cultures, being its sensitivity close to 100% (Manning and Collins, 2001), but its application to clinical samples has been problematic, mainly due to the problems associated with DNA extraction from complex matrices such as milk, feces, and blood, and the presence of PCR inhibitors (Stevenson and Sharp, 1997; Grant *et al.,* 1998; Aly *et al.,* 2010; Stevenson, 2010b), decreasing its sensitivity. The limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, the matrix tested, and the PCR format (conventional gel-based PCR, reverse transcriptase PCR, nested PCR, real-time PCR, or multiplex PCR; Möbius *et al.,* 2008a; Bolske and Herthnek, 2010; National Advisory Committee on Microbiological Criteria for Foods, 2010; Stevenson, 2010b). Ideally, sampling all adult cattle in every herd, environmental sampling, serial testing, and the use of two to three diagnostic tests would be the recommendation for herd screening, to increase the accuracy of MAP diagnosis (Collins *et al.,* 2006; Clark *et al.,* 2008; Stevenson, 2010b).

The aim of this study was to diagnose MAP using fecal culture, F*57*-real-time PCR and end-point IS*900*-specific nested PCR in one herd previously screened positive for MAP antibodies by an indirect serum-ELISA.

Materials and methods

Ethical considerations

This research was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act number 88, March, 2014).

Study herd

The study herd was located in the municipality of San Pedro de los Milagros, Antioquia (Colombia), one of the main dairy municipalities of the country, located in the Andean region of Colombia, with an area of 229 Km^2, an altitude of 2,468 m.o.s.l, a mean annual temperature of 16 °C, and a cattle population of approximately 71,395 animals. The study herd was visited only once as part of a previous study in 2015, that aimed the determination of the seroprevalence of MAP and the exploration of the main risk factors associated with ELISA positive results in dairy cows of the municipality of interest (Correa-Valencia *et al.,* 2016). The study herd, reported a cattle population of 39 bovines, including 27 cows over 2 years of age at the moment of the sampling, the predominant breed was classified as *other* in the previous study (different from Holstein and Jersey), without history of farming other ruminants different from bovines (i.e. goats, sheep, buffaloes), spreading manure as a fertilizer in the pastures was a common practice in the herd, as well as, leaving the calves with their dams after parturition in direct contact, certified as free of tuberculosis and brucellosis, and never reported any compatible clinical case and/or followed any structured control program for prevention or control of PTB before the sampling in 2015.

Blood and fecal samples were taken from all animals over 2 years of age (n = 27). The sample collection was conducted according to standard methods to avoid unnecessary pain or stress to animals. Blood samples were taken from the coccygeal or jugular vein, collected in red-top plastic Vacutainer® (Becton Dickinson, Sparks, NV, USA) tubes and transported refrigerated to the laboratory, where they were centrifuged at 1,008 RCF for 5 min. Fecal samples were taken with a clean glove directly from the rectum of every adult animal, and then, transported refrigerated to the laboratory. The obtained serum and the fecal samples were stored at -20 °C until analysis.

ELISA

Serum ELISA was performed using the pre-absorbed ELISA kit Parachek®2 (Prionics AG, Schlieren, Switzerland) following the manufacturer's instructions. The samples were read using Epoch Microplate Spectrophotometer® (BioTek, Winooski, VT, USA). The ELISA test included a pre-absorption step with *Mycobacterium phlei* to reduce cross-reactions. An animal was considered ELISA-positive

if serum sample was above or equal to the cut-off of 15 percent positivity (%P), as it is defined by the manufacturer of the diagnostic test used.

Fecal culture

Feces from all animals were thawed leaving the samples under 4 °C for 24 h prior to the decontamination procedure. Fecal culture was carried out according to the protocol reported previously by Fernández-Silva *et al.* (2011a). Briefly, 3 g of feces were added to a 50 mL sterile tube containing 30 mL of a 0.75% HPC (hexadecyl pyridinium chloride) weight/volume (w/v) solution. This suspension was manually mixed by shaking, and let in a vertical position for 5 min at room temperature to allow precipitation and sedimentation of big particles. Approximately 20 mL of the upper portion of the supernatant was transfer to another 50 mL sterile tube, in which the whole suspension was agitated at 200 RPM for 30 min. Tubes were place in vertical position in the dark for 24 h at room temperature. Decontaminated pooled fecal samples were centrifuged at 900 x g during 30 min, supernatant was discarded. Duplicated HEYM slants, supplemented with mycobactin J and amphotericin B, nalidixic acid, and vancomycin mix (Becton Dickinson, New Jersey, USA) were inoculated with 300 µL of the decontaminated pellet. All culture media were incubated at 37 °C for 24 weeks and were checked weekly for mycobacterial growth or contamination with undesirable germs. MAP growth was visually monitored for typical slow growth rate and colony morphology according to previous descriptions (colonies developing after ≥ 3 weeks of incubation, initially round, smooth and white, then tending to heap up slightly and becoming dull light yellow with wrinkling of the surface; Whittington, 2010).

DNA isolation from individual fecal samples

Each fecal sample was homogenized for 5 min prior to DNA extraction procedure. DNA from individual fecal samples was extracted according to the following procedure reported previously by Leite *et al.* (2013) using a commercial DNA preparation kit (ZR Fecal DNA Kit™, Zymo Research, Irvine, CA, USA). Processing was done according to kit´s protocol for isolation of nucleic acids from bacteria and yeast. A mechanical cell disruptor step was carried out in an automated biological sample lyzer (Disruptor Genie® 120V, Thomas Scientific, Swedesboro, NJ, USA) to achieve a more efficient cell lysis.

End-point IS900-specific nested PCR

DNA from individual fecal samples was tested for MAP by end-point IS*900*-specific nested PCR, using primers targeting IS*900* designated TJ1-4 [TJ1 (5´-GCT GAT CGC CTT GCT CAT-3´) and TJ2 (5´-CGG GAG TTT GGT AGC CAG TA-3´) in the first-round-PCR, and primer pair TJ3 (5´-CAG CGG CTG CTT TAT ATT CC-3´) and TJ4 (5´-GGC ACG GCT CTT GTT GTA GT-3´) in the second round-PCR] according to Bull *et al.* (2003), modified by Füllgrabe (2009) and Bulander (2009). The first and second-round PCR mixture comprised the same mix volumes in a final volume of 50 µL with 5 µL of TaqDNA polymerase buffer- $MgCl_2$, 1 µL of dNTP mix, 1 µL of each primer, and 0.4 µL of TaqDNA polymerase (AmpliTaq Gold® DNA Polymerase LD, recombinant; 5 U/µL; Applied Biosystems™, Foster City, CA, USA), and 5 µL of DNA (at a 1:10 dilution) from sample or from the first-round-PCR. Additionally to the samples, a positive (*Mycobacterium avium* subsp. *pararuberculosis*, strain K10; ATCC® BAA-968™) and a negative control, as well as, a blank control were included. Cycling conditions for both rounds were: 1 cycle of 95 °C for 10 min and then 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, followed by 1 cycle of 72 °C for 7 min. Amplicons of the expected size (355 and 294 bp, for the first and second round, respectively) were visualized with ethidium bromide on 1.5% agarose gels.

F57-real-time PCR

DNA from individual fecal samples was tested for MAP confirmation by F*57* using a commercial Real-Time PCR kit, which includes an internal amplification control (IAC) to avoid the misinterpretation of false negative results (MAPsureEasy® Kit-MSE, TransMIT, Giessen, Germany). The components of the MAPsureEasy® Kit-MSE are the 25x MAP Oligonucleotide Mix including primers [F*57po*-244 F 5‘– TAC GAG CAC GCA GGC ATT C – 3‘ and F*57po*-306 R 5‘– CGG TCC AGT TCG CTG TCA T – 3‘] and probes [F*57*po-TaqMan® Probe VIC-CCT GAC CAC CCT TC-MGB and IAC MSE TaqMan®

Probe FAM-AGC AAT AAA CCA GCC AGC-MGB]; the 2x qPCR Master Mix (from qPCR Mastermix plus w/o UNG* of Eurogentec, Ireland, 2x PCR MM for Probe assay); the IAC (DNA IAC); and the positive control DNA of MAP strain K10 (ATCC® BAA-968™). The PCR mixture was prepared according to the protocol, one sample in a final volume of 25 µL: 5.5 µL of molecular grade water, 12.5 µL of 2x qPCR Master Mix, 1 µL of 25x MAP Oligonucleotide Mix; 1 µL of the IAC-DNA, 5 µL of DNA probe, and 5 µL of DNA.

Results

ELISA

Eight of the 27 (29.6%) animals were positive by serum-ELISA in the study herd (Table 1).

Fecal culture

None of the 27 fecal samples from animals of the study herd were positive by fecal culture based on growth rate and colony morphology (Table 1). Two duplicated cultures (four slants) presented contamination (7.4%).

End-point IS900-specific nested PCR and F57-real-time PCR

All samples resulted negative by F*57*-real-time PCR, and seven (25.9%) resulted positive by end-point IS*900*-specific nested PCR (Table 1). Amplifications for end-point IS*900*-specific nested PCR in agarose gel results are shown in Figures 1 and 2. A compilation of individual information and tests results for animals tested (n = 27) of the study herd are shown in Table 1.

Discussion

The present study aimed to diagnose MAP using fecal culture, F*57*-real-time PCR, and end-point IS*900*-specific nested PCR in one herd previously screened positive for MAP antibodies by an indirect serum-ELISA.

The confirmation of ELISA test results using fecal culture and PCR was considered necessary to obtain a precise detection of PTB infected animals in an ELISA positive herd. Nevertheless, we expected to find a higher proportion of MAP-positive animals (by ELISA, as well as, by fecal culture and PCR) in the study herd, considering inappropriate herd management practices present and known to be risk factors for the disease (e.g. presence of animals born at other dairies, exposure of calves 0-6 weeks to adults feces, feces spread on forage fed to any age group (Collins *et al.,* 1994; Goodger *et al.,* 1996; Jakobsen *et al.,* 2000; Wells and Wagner, 2000; Diéguez *et al.,* 2008; Tiwari *et al.,* 2009; Sorge *et al.,* 2012; Künzler *et al.,* 2014; Fernández-Silva and Ramírez-Vásquez, 2015; Vilar *et al*., 2015). When a test combination is considered, it must be taken into account that some infected cows produce antibodies for several years prior to the fecal-shedding of detectable quantities of MAP. However, in other animals, antibodies may not be detectable during the early stages of infection when MAP fecal-shedding is minimal (Kalis *et al.,* 2002; McKenna *et al.,* 2006; Nielsen, 2010).

The ELISA results should be analyzed cautiously, mainly considering its sensitivity because of the silent and long-lasting behavior of the disease, more than as a failure of the test itself (Sweeney *et al.,* 1996; Collins *et al.,* 2005; Mon *et al.,* 2012; Sorge *et al*., 2012). According to Lavers *et al.* (2015), the sensitivity of serum ELISA is approximately 25.4-45.3% and its specificity of 97.6-98.9% in asymptomatic animals, which can lead to a misclassification of the cows and reporting infected cows as negative. On the other hand, the results could be related to sample handling. In the present study, the serum samples were frozen for 30 to 45 days at -20 °C, which could have led to lower scores for the MAP ELISA (Alinovi *et al.,* 2009).

Fecal culture did not report any positive result, which could be explained, among other aspects, by the storage conditions (4 °C for 12 h max, and then at -20 °C for 7 months). According to Khare *et al*. (2008), to store fecal samples at 4 °C for 48 h, and then at -20 °C for at least one week is limiting for the culture sensitivity, contrary to short-term storage at 4 °C and longer term storage at -70 °C, which appear to have no damaging effects on MAP viability in the fecal sample.

Table 1. Animal-level information and MAP-diagnostic tests results in a study herd in the municipality of San Pedro de los Milagros, Antioquia (Colombia).

Animal ID	Breed*	Parity	Days in milk	Milk production per day (L)	Productive stage	Born in herd	Serum ELISA	Fecal culture	IS*900*-nested PCR	F*57*-real-time PCR
1	Other	2	192	23	Milking	Yes	−	−	+	−
2	Other	6	163	33	Milking	Yes	+	−	−	−
3	Other	2	372	n.d.	Dry	Yes	−	−	−	−
4	Other	5	72	34	Milking	No	−	−	−	−
5	Holstein	1	4	n.d.	Dry	Yes	−	−	−	−
6	Other	4	214	24	Milking	No	−	−	−	−
7	Other	6	182	21	Milking	No	+	−	−	−
8	Other	2	133	25	Milking	No	−	−	−	−
9	Other	2	235	14	Milking	No	−	−	−	−
10	Other	n.d.	n.d.	n.d.	Heifer	Yes	+	−	−	−
11	Other	1	37	27	Milking	Yes	−	−	−	−
12	Other	2	299	16	Milking	Yes	−	−	+	−
13	Holstein	2	88	31	Milking	Yes	+	−	−	−
14	Holstein	1	215	25	Milking	Yes	+	−	−	−
15	Other	1	52	21	Milking	Yes	−	−	+	−
16	Other	2	227	16	Milking	Yes	−	−	−	−
17	Holstein	6	324	n.d.	Dry	Yes	−	−	−	−
18	Holstein	2	197	19	Milking	Yes	+	−	−	−
19	Holstein	7	72	51	Milking	Yes	−	−	−	−
20	Other	5	18	25	Milking	Yes	−	−	−	−
21	Other	3	192	25	Milking	No	−	−	−	−
22	Holstein	n.d.	n.d.	n.d.	Heifer	Yes	−	−	+	−
23	Other	5	161	22	Milking	Yes	+	−	−	−
24	Holstein	5	89	37	Milking	Yes	−	−	+	−
25	Other	3	409	18	Milking	Yes	+	−	+	−
26	Other	3	184	24	Milking	Yes	−	−	−	−
27	Jersey	1	40	23	Milking	Yes	−	−	+	−

* Other breeds included Guernsey, Ayrshire, Swedish Red, Swiss Brown, Jersey, and several crossbreeds of Holstein with Jersey, Ayrshire, Angus, Blanco Orejinegro, Brahman, and Gir.

n.d.: no data available at the moment of sampling; +: positive result; −: negative result.

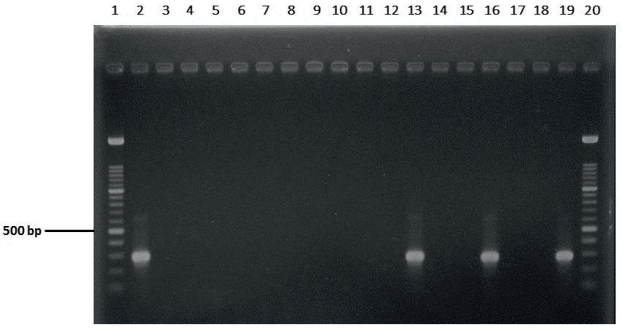

Figure 1. End-point IS*900*-specific nested PCR in agarose gel (final product of 294 bp), samples of cows 1-17. Molecular size marker (100 bp DNA ladder; Roche, Mannheim, Germany; lane 1 and 20), animal 1 (lane 2), animal 12 (lane 13), animal 15 (lane 16), positive control (*Mycobacterium avium subsp. pararuberculosis*, strain K10, ATCC® BAA-968™; lane 19), negative results (lanes 3-12, 14-15, and 17-18).

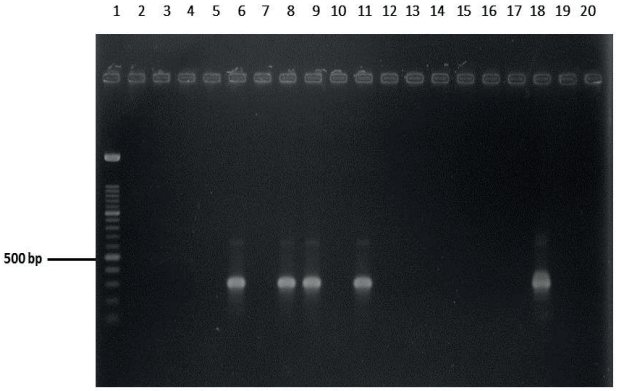

Figure 2. End-point IS*900*-specific nested PCR in agarose gel (final product of 294 bp), samples of cows 18-27. Molecular size marker (100 bp DNA ladder; Roche, Mannheim, Germany; lane 1 and 20), animal 22 (lane 6), animal 24 (lane 8), animal 25 (lane 9), animal 27 (lane 11), positive control (*Mycobacterium avium subsp. pararuberculosis*, strain K10, ATCC® BAA-968™; lane 18), blank control (master mixture blank; lane 19), negative results (lanes 2-5, 7, 10), empty lanes (12-17).

On the other hand, there would be false-negative fecal culture for samples that contain few organisms due to less of MAP during the culturing as a direct consequence of the process (Whittington, 2010). Dehydration and the possible reduction of viable microorganism by chemical decontamination are important data to interpret negative results, especially in low intensity fecal shedders (Reddacliff *et al.,* 2003).

Another point that should be considered to explain some of our results is the low-shedder status, considering that literature reports that about 75% of positive animals are either low or very low shedders (van Schaik *et al.,* 2003; USDA, APHIS, VS, CEAH, 2008). In view of the minimal amount of detectable MAP (100 CFU/g of feces; Merkal, 1970), only 15-25% of subclinical low and/or moderate fecal shedders can be detected by bacterial culture (Whitlock and Buergelt, 1996). The sensitivity of the fecal culture in clinical stages can be 91% (Álvarez *et al.,* 2009), a value that can be reduced to 45-72% (Crossley *et al.,* 2005; Alinovi *et al.,* 2009) in subclinical stages, whereas the specificity is very good (100%) in all stages (Ayele *et al*., 2001). This information can explain some of our results, considering the seroprevalence results for the whole municipality (3.6 and 2% at herd-level and animal-level, respectively; Correa-Valencia *et al.,* 2016), where no clinical animals were sampled.

The use of direct PCR to fecal DNA has several advantages as for example shorter times to diagnosis compared to culture (3 days vs. 14-22 weeks). In addition, the procedure for the extraction of fecal DNA in preparation for PCR has become easier and less expensive in the recent years (Stabel *et al.,* 2004). Considering an effective method to ensure a complete-DNA extraction, a mechanical disruption step (bead-beating) was included —which breaks up bacterial cell wall mechanically by vibrating bacteria at high speed, in addition to the commercial kit protocol (Odumero *et al.,* 2002; Zecconi *et al.,* 2002; Herthnek, 2009) improving the sensitivity of the protocol applied, also reported by Leite *et al.* (2013) with the comparable performance results.

Special attention should be given to the inhibitory effects of certain components of the samples on Taq polymerase, which could cause false negative results, being a probable explanation for some of our negative outcomes (Tiwari *et al.,* 2006). Feces, especially those from ruminants, are expected to include high levels of PCR inhibitors (Al-Soud and Radstrom, 1998; Inglis and Kalischuck, 2003; Thorton and Passen, 2004), and one of the main difficulties is to remove them to improved PCR sensitivity (Harris and Barletta, 2001). Although no clinical cows were found in our study herd, in some cases is highly probable that feces from

cows with clinical PTB may contain heme (a complex of iron with protoporphyrin IX) and epithelial cells, being these components reported to be inhibitory to PCR (Inglis and Kalischuck, 2003).

The sensitivity and specificity of the end-point IS*900*-specific nested PCR used to test our samples are reported to be increased (Englund *et al.,* 2001; Ikonomopoulos *et al.,* 2004; Bölske and Herthnek, 2010). Any PCR inhibitors in the first run would be diluted when transferred as template to the second PCR (Bölske and Herthnek, 2010).

Our assays used two molecular elements found in different loci and ratios in MAP genome (IS*900* and F*57*), leading to non-comparable results related to their specificity and sensitivity. The IS*900* is a repetitive DNA sequence present in 15-18 copies of MAP genome (Collins *et al.,* 1989; Green *et al.,* 1989). However, IS*900*-like elements have been described at low copy numbers in rarely encountered environmental mycobacteria (Cousins *et al*., 1999; Englund *et al.,* 2002; Tasara *et al.,* 2005), compromising its specificity. On the other hand, F*57,* a single copy-segment, has demonstrated high specificity for the detection of MAP (Coetsier *et al.,* 2000; Ellingson *et al.,* 2000; Harris and Barletta, 2001; Strommenger *et al.,* 2001; Vansnick *et al.,* 2004; Rajeev *et al*., 2005). The nested IS*900* assay can detect 0.01 pg of DNA (corresponding to 10 genomes) when extracted from a pure culture, while the F*57* assay can detect 0.1 pg of DNA (corresponding to 100 genomes; Radomski *et al.,* 2013). Vansnicka *et al.* (2004), Tasara and Stephan (2005), and Schönenbrücher *et al.* (2008) recommend including the F*57*-PCR assay to confirm the presence of MAP after a positive IS*900*-PCR. According to this, our results (F*57*-PCR negative results and some positive results by IS*900*-PCR), can be considered MAP-unspecific by IS*900*-PCR, and confirmed as negative by the F*57* insertion detection.

Nevertheless, our results in the PCR protocols applied could be better explained by the already reported behavior of the disease than to PCR misclassification. According to Withlock *et al.* (2000), the disadvantages of some detection test are due mainly because of the intermittent shedding of microorganisms. This means that the sensitivity of direct tests to detect symptomatic animals is high, but low for detection of infected/subclinical animals (Nielsen and Toft, 2008; Schönenbrücher *et al.,* 2008; Whittington *et al.,* 2011).

On the other hand, the thawing of fecal samples stored at -20 °C was done in different times for fecal culturing process and for DNA extraction what could have affected the detection by PCR, leading to false negative results because of DNA damage during thawing-freezing re-processes, which can explain PCR results in this study (Bölske and Herthnek, 2010; Whittington, 2010).

The low agreement between tests results has been also reported before (Muskens *et al.,* 2003; Glanemann *et al*., 2004; Dreier *et al.,* 2006) and could be explained due to the fact that ELISA negative or ELISA false-positive results have a low probability of delivering a positive culture result if just a single sampling is planned as normally done in a cross-sectional study, which was the case of the present study (Sweeney *et al*., 2006). Similar results on low agreement between ELISA and culture (Fernández-Silva *et al.,* 2011b) and ELISA and PCR to MAP (Fernández-Silva *et al.,* 2011a) were found in previous studies in asymptomatic animals from herds of the same dairy region.

Our results for all the tests used does not necessarily mean that the animals were not really infected, because the shedding phase has probably not yet started (infected animal in a noninfectious phase) or was absent at the moment of fecal sampling (intermittency). Another possibility is that in these animals MAP-antibodies were detected prior to the start of bacterial shedding, which could begin later and could be then detected by PCR or fecal culture (Nielsen, 2008). Considering MAP-shedding characteristics as the major limitation in the detection of infected animals, it should be taken into account that the elimination of the bacteria through feces happens at all stages but at different levels and sporadically, which demands repeated testing to detect animals shedding very low number of MAP, which could anyway go undetected (Stevenson, 2010b). Nevertheless, we found a positive result by serum-ELISA and fecal PCR in one of the cows in the study herd, revealing parallel detectable antibody levels and detectable MAP fecal-shedding, being this a biologically plausible result.

Alinovi *et al*. (2009) reported that test sensitivity for culture methods and real-time PCR, as well as, test accuracy, are comparable. This clearly demonstrates that in field applications, real-time PCR is as useful as solid or liquid culture methods while providing the producer with test results within hours, not weeks. Serum ELISA, although not as accurate as the other tests evaluated, continues to be a useful alternative because of its rapid turn-around. Now, with PCR, results that are more accurate can be available as fast as for ELISA.

Our results in a seropositive herd delivered one asymptomatic ELISA-positive cow with a negative fecal culture, and a positive end-point IS*900*-specific PCR result. In addition, there were 13 asymptomatic ELISA-negative cows, producing negative results by fecal culture, and negative results by two different PCR methods in an infected herd. We detected a low agreement between the diagnostic tests used (ELISA, fecal culture, and PCR). These results evidence the perfect examples of MAP´s detection paradox and the most confounding component in PTB control: the detection of truly infected and uninfected animals. The information in this study indicates the importance of MAP detection and its direct impact in the implementation of strategic management practices to ensure the control of the disease and the dissemination of the agent. Thus, it will be necessary to design risk-based programs in each region in the country, adapted to its specific conditions, even considering production systems. Follow-up studies on herds with PTB over a long time to investigate whether the change of individual and herd-level management practices lead to changes in PTB control on this herd should be performed.

Acknowledgments

This research was funded by Vecol and Estrategia de Sostenibilidad CODI 2013-2014, Universidad de Antioquia (CENTAURO). The authors thank Prof. Dr. Martha Olivera, Julian Londoño, MV (Vecol), and to farmers for allowing animal testing and information collecting. Authors also thank all technical and laboratory team who supported all testing and diagnostic procedures, especially Claudia Walter at IFTN, Giessen.

Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

References

Alinovi CA, Ward MP, Lin TL, Moore GE, Wu CC. Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* subsp. *paratuberculosis*. Vet Microbiol 2009; 14:177-179.

Al-Soud W, Radstrom AP. Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. Appl Environ Microbiol 1998; 64:3748-3753.

Álvarez J, de Juan L Bezos J, Romero B, Sáez JL, Marqués S, Domíngues C, Minguez O, Fernández-Mardomingo B, Mateos A, Domínguez L, Aranaz, A. Effect of paratuberculosis on the diagnosis of bovine tuberculosis in a cattle herd with a mixed infection using interferon-gamma detection assay. Vet Microbiol 2009; 135:389-393.

Aly SS, Mangold BL, Whitlock RH, Sweeney RW, Anderson RJ, Jiang J, Schukken YH, Hovingh E, Wolfgang D, Van Kessel JAS, Karns JS, Lombard JE, Smith JM, Gardner IA. Correlation between Herrold egg yolk medium culture and real-time quantitative polymerase chain reaction results for *Mycobacterium avium* subspecies *paratuberculosis* in pooled fecal and environmental samples. J Vet Diagn Invest 2010; 22(5):677-683.

Aly SS, Anderson RJ, Whitlock RH, Fyock TL, McAdams SC, Byrem TM, Jiang J, Adaska JM, Gardnerl IA. Cost-effectiveness of diagnostic strategies to identify *Mycobacterium avium* subspecies *paratuberculosis* super-shedder cows in a large dairy herd using antibody enzyme-linked immunosorbent assays, quantitative real-time polymerase chain reaction, and bacterial culture. J Vet Diagnostic Invest 2012; 24:821-832.

Anonymous. Uniform program standards for the voluntary bovine Johne´s disease control program. In: United States Department of Agriculture-USDA, Animal and Plant Health Inspection Service-APHIS; 2010. 40 p.

Atreya R, Bülte M, Gerlach GF, Goethe R, Hornef MW, Köhler H, Meensd J, Möbiusf P, Roebg E, Weissh S. Facts, myths and hypotheses on the zoonotic nature of *Mycobacterium avium* subspecies *paratuberculosis*. Int J Med Microbiol 2014; 304(7):858-867.

Ayele WY, Machá ková M, Pavlík I. The transmission and impact of paratuberculosis infection in domestic and wild ruminants. Vet Med Czech 2001; 46:205-224.

Bölske G, Herthnek D. Diagnosis of paratuberculosis by PCR. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 267-278.

Bulander K. Vergleichende Untersuchungen zum Nachweis von *Mycobacterium avium* ssp. *paratuberculosis* in Milchrinderbeständen. Dissertation, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Germany 2009. 214p.

Bull TJ, Sidi-Boumedine K, McMinn EJ, Stevenson K, Pickup R, Hermon-Taylor J. Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex. Mol Cel Probes 2003; 17:157-164.

Bülte M, Schonenbrucher H, Abdulmawjood A. From farm to fork —*Mycobacterium* avium ssp. *paratuberculosis* (MAP) as zoonotic agent? Berl Munch Tierarztl Wochenschr 2005; 118:377-385.

Cernicchiaro N, Wells SJ, Janagama H, Sreevatsan S. Influence of type of culture medium on characterization of *Mycobacterium avium* subsp. *paratuberculosis* subtypes. J Clin Microbiol 2008; 46(1):145-149.

Chacon O, Bermudez LE, Barletta RG. Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis*. Annu Rev Microbiol 2004; 58:329-363.

Clark Jr DL, Koziczkowski JJ, Radcliff RP, Carlson RA, Ellingson JLE. Detection of *Mycobacterium avium* subspecies *paratuberculosis*: comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. J Dair Sci 2008; 91:2620-2627.

Clarke CJ. The pathology and pathogenesis of paratuberculosis in ruminants and other species. J Comp Path 1997; 116:217-261.

Coetsier C, Vannuffel P, Blondeel N, Denef JF, Cocito C, Gala JL. Duplex PCR for differential identification of *Mycobacterium bovis*, *M. avium*, and *M. avium* subsp. *paratuberculosis* in formalin-fixed paraffin-embedded tissues from cattle. J Clin Microbiol 2000; 38:3048-3054.

Collins MT. Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract 1996; 12:357-371.

Collins DM, Gabric DM, de Lisle GW. Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. FEMS Microbiol Lett 1989; 60:175-178.

Collins DM, Gabric DM, de Lisle GW. Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. J Clin Microbiol 1990; 28:1591-1596.

Collins MT, Gardner IA, Garry FB, Roussel AJ, Wells SJ. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. J Am Vet Med Assoc 2006; 229:1912-1919.

Collins MT, Sockett DC, Goodger WJ, Conrad TA, Thomas CB, Carr DJ. Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin. J Am Vet Med Assoc 1994; 204(4): 636-641.

Correa Valencia NM, Ramírez NF, Olivera M, Fernández Silva JA. Milk yield and lactation stage are associated with positive results to ELISA for *Mycobacterium avium* subsp. *paratuberculosis* in dairy cows from Northern Antioquia, Colombia: a preliminary study. Trop Anim Health Prod 2016; 48:1191-1200.

Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Kluver P. Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS*900* -like sequences detectable IS*900* polymerase chain reaction: implications for diagnosis. Mol Cell Probes 1999; 13:431-442.

Crossley BM, Zagmutt-Vergara FJ, Fyock TL, Whitlock RH, Gardner IA. Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* by dairy cows. Vet Microbiol 2005; 107: 257-263.

Dieguez FJ, Arnaiz I, Sanjuan ML, Vilar MJ, Yus E. Management practices associated with *Mycobacterium* avium subspecies *paratuberculosis* infection and the effects of the infection on dairy herds. Vet Rec 2008; 162:614-617.

Dreier S, Khol JL, Stein B, Fuchs K, Gutler S, Baumgartner W. Serological, bacteriological and molecular biological survey of paratuberculosis (Johne's disease) in Austrian cattle. J Vet Med B Infect Dis Vet Public Health 2006; 53:477-481.

Eamens GJ, Whittington RJ, Marsh IB, Turner ML, Saunders V, Kemsley PD, Rayward D. Comparative sensitivity of various fecal culture methods and ELISA in dairy cattle herds with endemic Johne´s disease. Vet Microbiol 2000; 85:243-251.

Ellingson JL, Stabel JR, Bishai WR, Frothingham R, Miller JM. Evaluation of the accuracy and reproducibility of a potential PCR panel assay for rapid detection and differentiation of *Mycobacterium avium* subsp. *paratuberculosis.* Mol Cell Probes 2000; 14:153-161.

Englund S, Bölske G, Ballagi-Pordány A, Johansson KE. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples by single, fluorescent and nested PCR based on the IS*900* gene. Vet Microbiol 2001; 81:257-271.

Englund S, Bolske G, Johansson KE. An IS*900*-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. FEMS Microbiol Lett 2002; 209:267-271.

Fecteau ME, Whitlock RH. Paratuberculosis in cattle. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 144-153.

Fernández Silva JA, Abdulmawjood A, Akineden O, Bülte M. Serological and molecular detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle of dairy herds in Colombia. Trop Anim Health Prod 2011a; 43:1501-1507.

Fernández Silva JA, Abdulmawjood A, Bülte M. Diagnosis and molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* from dairy cows in Colombia. Vet Med Int 2011b; 1-29.

Fernández Silva JA, Ramírez Vásquez NF. Factors associated with the paratuberculosis serum enzyme-linked immunosorbent assay (ELISA) status of dairy cows from two municipalities of the Northern Antioquia, Colombia. Rev Colomb Cienc Pecu 2015; 28(Sup):91.

Füllgrabe RAR. Untersuchungen zum kulturellen und Molekularbiologischen nachweis von *Mycobacterium avium* ssp. *paratuberculosis* (MAP) aus humanen darmbioptaten. Inaugural-Dissertation, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, 2009. 308p. http://geb.uni-giessen.de/geb/volltexte/2009/6826/pdf/FuellgrabeRegina-2008-12-18.pdf

García Ab, Shalloo L. Invited review: the economic impact and control of paratuberculosis in cattle. J Dairy Sci 2015; 98(8):501939.

Gill CO, Saucier L, Meadus WJ. *Mycobacterium avium* subsp. *paratuberculosis* in dairy products, meat, and drinking water. J Food Prot 2011; 74:480-499.

Glanemann B, Hoelzle LE, Bogli-Stuber K, Jemmi T, Wittenbrink MM. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by culture and serology. Schweiz Arch Tierheilkd 2004; 146:409-415.

Godfroid J, Delcorps C, Irenge LM, Walravens K, Marche S, Gala JL. Definitive differentiation between single and mixed mycobacterial infections in red deer (*Cervus elaphus*) by a combination of duplex amplification of p34 and F*57* sequences and Hpy188I enzymatic restriction of duplex amplicons. Clin Microbiol 2005; 43:4640-4648.

Goodger WJ, Collins MT, Nordlund KV, Eisele C, Pelletier J, Thomas CB, Sockett DC. Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle. J Am Vet Med Assoc 1996; 208:1877-1881.

Grant IR, Ball HJ, Rowe MT. Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. Appl Environ Microbiol 1998; 64(9):3153-3158.

Green EP, Tizard MLV, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon Taylor J. Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis.* Nucleic Acids Res 1989; 17:9063-9073.

Hanifian S. Survival of *Mycobacterium avium* subsp. *paratuberculosis* in ultra-filtered white cheese. Lett Appl Microbiol 2014; 16.

Harris NB, Barletta RG. *Mycobacterium avium* subspecies *paratuberculosis* in veterinary medicine. Clin Microbiol Rev 2001; 14:489-512.

Herthnek D. Molecular diagnostic methods for *Mycobacterium avium* subsp. *paratuberculosis:* more than a gut feeling. Inaugural-Dissertation Dr. med. vet. Faculty of Veterinary Medicine and Animal Science, Department of Biomedical Sciences and Veterinary Public Health, Uppsala, 2009, 67p.

Herthnek D, Bölske G. New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis*. BMC Microbiol 2006 4; 6:87.

Ikonomopoulos J, Gazouli M, Pavlik I, Bartos M, Zacharatos P, Xylouri E, Papalambros E, Gorgoulis V. Comparative evaluation of PCR assays for the robust molecular detection of *Mycobacterium avium* subsp. *paratuberculosis*. J Microbiol Methods 2004; 56:315-321.

Inglis GD, Kalischuck LD. Use of PCR for direct detection of *Campylobacter* species in bovine feces. Appl Environ Microbiol 2003; 69; 3435-3447.

Jakobsen MB, Alban L, Nielsen SS. A cross-sectional study of paratuberculosis in 1155 Danish dairy cows. Prev Vet Med 2000; 46:15-27.

Kalis CH, Barkema HW, Hesselink JW, van Maanen C, Collins MT. Evaluation of two absorbed enzyme-linked immunosorbent assays and a complement fixation test as replacements for fecal culture in the detection of cows shedding *Mycobacterium avium* subspecies *paratuberculosis*. J Vet Diagn Invest 2002; 14:219-224.

Kawaji S, Taylor DL, Mori Y, Whittington RJ. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine feces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture. Vet Microbiol 2007; 125(1-2):36-48.

Keller SM, Stephan R, Kuenzler R, Meylan M, Wittenbrink MM. Comparison of fecal culture and F*57* real-time polymerase chain reaction for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss cattle herds with a history of paratuberculosis. Acta Vet Scand 2014; 56:68.

Khare S, Adams LG, Osterstock J, Roussel A, David L. Effects of shipping and storage conditions of fecal samples on viability of *Mycobacterium paratuberculosis*. J Clin Microbiol 2008; 46(4):1561-1562.

Kim SG, Shin SJ, Jacobson RH, Miller LJ, Harpending PR, Stehman SM, Rossiter CA, Lein DA. Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. J Vet Diagn Invest 2002; 14(2):126-131.

Kralik P, Nocker A, Pavlik I. *Mycobacterium avium* subsp. *paratuberculosis* viability determination using F*57* quantitative PCR in combination with propidium monoazide treatment. Int J Food Microbiol 2010; 141 Suppl 1:S80-6.

Künzler R, Torgerson P, Keller S, Wittenbrink M, Stephan R, Knubben-Schweizer G, Berchtold B, Meylan M. Observed management practices in relation to the risk of infection with paratuberculosis and to the spread of *Mycobacterium avium* ssp. *paratuberculosis* in Swiss dairy and beef herds. BMC Vet Res 2014; 10:132.

Lavers CJ, Dohoo IR, McKenna SL, Keefe GP. Sensitivity and specificity of repeated test results from a commercial milk enzyme-linked immunosorbent assay for detection of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. J Am Vet Med Association 2015; 246(2):236-244.

Leite FL, Stokes KD, Robbe-Austerman S, Stabel JR. Comparison of fecal DNA extraction kits for the detection of *Mycobacterium avium* subsp. *paratuberculosis* by polymerase chain reaction. J Vet Diagn Invest 2013; 25(1):27-34.

Liverani E, Scaioli E, Cardamone C, Dal Monte P, Belluzzi A. *Mycobacterium avium* subspecies *paratuberculosis* in the etiology of Crohn's disease, cause or epiphenomenon? World J Gastroenterol 2014; 20(36):13060-130670.

Manning EJ, Collins MT. *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. Rev Sci Tech 2001; 20:133-150.

McAloon Cg, Whyte P, More Sj, Green Mj, O'grady L, Garcia A, Doherty Ml. The effect of paratuberculosis on milk yield —A systematic review and meta-analysis. J Dairy Sci 2016; 99(2):1449-1160.

McKenna SL, Barkema HW, Keefe GP, Sockett DC. Agreement between three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. Vet Microbiol 2006; 114:285-291.

Merkal RS. Diagnostic methods for detection of paratuberculosis (Johne's disease). 74th Annual Meeting of the US Animal Health Association, 1970, Abstract 74, p. 620-3, USA.

Möbius P, Hotzel H, Rassbach A, Kohler H. Comparison of 13 single-round and nested PCR assays targeting IS*900*, IS*Mav2*, F*57* and locus 255 for detection of *Mycobacterium avium* subsp. *paratuberculosis*. Vet Microbiol 2008a; 126:324-333.

Möbius P, Luyven G, Hotzel H, Köhler H. High genetic diversity among *Mycobacterium avium* subsp. *paratuberculosis* strains from German cattle herds shown by combination of IS*900* restriction fragment length polymorphism analysis and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing. J Clin Microbiol 2008b; 46(3):972-981.

Mon ML, Viale M, Baschetti G, Alvarado Pinedo F, Gioffre A, Travería G, Willemsen P, Bakker D, Romano MI. Search for *Mycobacterium avium* subspecies *paratuberculosis* antigens for the diagnosis of paratuberculosis. Vet-Med International 2012, 860362.

Motiwala AS, Amonsin A, Strother M, Manning EJ, Kapur V, Sreevatsan S. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* isolates recovered from wild animal species. J Clin Microbiol 2004; 42:1703-1712.

Motiwala AS, Strother M, Theus NE, Stich RW, Byrum B, Shulaw WP, Kapur V, Sreevatsan S. Rapid detection and typing of strains of *Mycobacterium avium* subsp. *paratuberculosis* from broth cultures. J Clin Microbiol 2005; 43:2111-2117.

Muskens J, Mars MH, Elbers AR, van Maanen K, Bakker D. The results of using faecal culture as confirmation test of paratuberculosis-seropositive dairy cattle. J Vet Med B Infect Dis Vet Public Health 2003; 50:231-234.

National Advisory Committee on Microbiological Criteria for Foods. Assessment of food as a source of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP). J Food Prot 2010; 73:1357-1397.

Nielsen SS. Transitions in diagnostic tests used for detection of *Mycobacterium avium* subsp. *paratuberculosis* infections in cattle. Vet Microbiol 2008; 132(3-4):274-282.

Nielsen SS. Immune-based diagnosis of paratuberculosis. In: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control. CAB International, Oxfordshire, 2010. pp. 284-293.

Nielsen SS, Toft N. *Ante-mortem* diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gama assay, and fecal culture techniques. Vet Microbiol 2008; 129:217-235.

Nielsen SS, Toft N. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev Vet Med 2009; 88:1-14.

Radomski N, Kreitmann L, McIntosh F, Behr MA. The critical role of DNA extraction for detection of mycobacteria in tissues. PLoS One 2013; 8:e78749.

Rajeev S, Zhang Y, Sreevatsan S, Motiwala AS, Byrum B. Evaluation of multiple genomic targets for identification and confirmation of *Mycobacterium avium* subsp. *paratuberculosis* isolates using real-time PCR. Vet Microbiol 2005; 105:215-221.

Reddacliff LA, Vadali A, Whittington RJ. The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. Vet Microbiol 2003; 24:271-282.

Odumeru J, Gao A, Chen S, Raymond M, Mutharia L. Use of the bead beater for preparation of *Mycobacterium paratuberculosis* template DNA in milk. Can J Vet Res 2001; 65(4):201-205.

Salem M, Heydel C, El-Sayed A, Ahmed SA, Zschöck M, Baljer G. *Mycobacterium avium* subspecies *paratuberculosis*: an insidious problem for the ruminant industry. Trop Anim Health Prod 2013; 45:351-366.

Schönenbrücher H, Abdulmawjood A, Failing K, Bülte M. New triplex real-time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. Appl. Environ Microbiol 2008; 74:2751-2758.

Sidoti F, Banche G, Astegiano S, Allizond V, Cuffini AM, Bergallo M.Validation and standardization of IS*900* and F*57* real-time quantitative PCR assays for the specific detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. Can J Microbiol 2011; 57(5):347-354.

Slana I, Liapi M, Moravkova M, Kralova A, Pavlik I. *Mycobacterium avium* subsp. *paratuberculosis* in cow bulk tank milk in Cyprus detected by culture and quantitative IS*900* and F*57* real-time PCR. Prev Vet Med 2009; 89(3-4):223-226.

Sorge US, Lissemore K, Godkin A, Jansen J, Hendrick S, Wells S, Kelton DF. Risk factors for herds to test positive for *Mycobacterium avium* ssp. *paratuberculosis*-antibodies with a commercial milk enzyme-linked immunosorbent assay (ELISA) in Ontario and western Canada. Can Vet J 2012; 53:963-970.

Stabel JR., Bosworth TL, Kirkbride TA, Forde RL, Whitlock RH. A simple, rapid, and effective method for the extraction of *Mycobacterium paratuberculosis* DNA from fecal samples for polymerase chain reaction. J Vet Diagn Investig 2004; 16:22-30.

Stabel JR, Bannantine JP. Development of a nested PCR method targeting a unique multicopy element, IS*Map02*, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. J Clin Microbiol 2005; 43:4744-4750.

Stevenson K. Comparative differences between strains of *Mycobacterium avium* subsp. *paratuberculosis*. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010a. p. 126-132.

Stevenson K. Diagnosis of Johne´s disease: current limitations and prospects. Cattle Practice 2010b; 18:104-109.

Stevenson K. Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* and the influence of strain type on infection and pathogenesis: a review. Vet Res 2015; 46:64.

Stevenson K, Sharp JM. The contribution of molecular biology to *Mycobacterium avium* subspecies *paratuberculosis* research. Vet J 1997; 153(3):269-286.

Stich RW, Byrum B, Love B, Theus N, Barber L, Shulaw WP. Evaluation of an automated system for non-radiometric detection of *Mycobacterium avium paratuberculosis* in bovine feces. J Microbiol Methods 2004; 56(2):267-275.

Strommenger B, Stevenson K, Gerlach GF. Isolation and diagnostic potential of ISMav2, a novel insertion sequence-like element from *Mycobacterium avium* subspecies *paratuberculosis*. FEMS Microbiol Lett 2001; 196:31-37.

Sweeney RW. Transmission of paratuberculosis. Vet Clinic North Am Food Anim Pract 1996; 12(2):305-312.

Sweeney RW, Whitlock RH, McAdams S, Fyock T. Longitudinal study of ELISA seroreactivity to *Mycobacterium avium* subsp. *paratuberculosis* in infected cattle and culturenegative herd mates. J Vet Diagn Invest 2006; 18:2-6.

Sweeney RW, Collins MT, Koets AP, McGuirk SM, Roussel AJ. Paratuberculosis (Johne's disease) in cattle and other susceptible species. J Vet Intern Med 2012; 26(6):1239-1250.

Taddei R, Barbieri I, Pacciarini ML, Fallacara F, Belletti GL, Arrigoni N. Mycobacterium porcinum strains isolated from bovine bulk milk: implications for *Mycobacterium avium* subsp. *paratuberculosis* detection by PCR and culture. Vet Microbiol 2008; 130(3-4):338-347.

Tasara T, Hoelzle LE, Stephan R. Development and evaluation of a *Mycobacterium avium* subspecies *paratuberculosis* (MAP) specific multiplex PCR assay. Int J Food Microbiol 2005; 104:279-287.

Tiwari A, VanLeeuwen JA, McKenna LB, Keefe GP, Barkema HW. Johne's disease in Canada. Part I. Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. J Vet Can 2006; 47:874-882.

Tiwari A, VanLeeuwen JA, Dohoo IR, Keefe GP, Haddad JP, Scott HM, Whiting T. Risk factors associated with *Mycobacterium avium* subspecies *paratuberculosis* seropositivity in Canadian dairy cows and herds. Prev Vet Med 2009; 88:32-41.

Thorton CG, Passen S. Inhibition of PCR amplification by phytic acid, and treatment of bovine fecal specimens with phytase to reduce inhibition. J Microbiol Methods 2004; 100:97-204.

USDA, APHIS, VS, and CEAH. National Animal Health Monitoring System (NAHMS) dairy 2007: Johne's disease on U.S. dairy operations. United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services National Animal Health Monitoring System, Washington, DC. 2008. [Access date: February 1st, 2016]. URL: https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_is_Johnes.pdf

Vansnick E, De Rijk P, Vercammen F, Geysen D, Rigouts L, Portaels F. Newly develop primers for the detection of *Mycobacterium avium* subsp. *paratuberculosis*. Vet Microbiol 2004; 100:197-204.

van Schaik G, Stehman SM, Schukken YH, Rossiter CR, Shin SJ. Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence. J Vet Diagn Investig 2003; 15:233-241.

van Schaik G, Pradenas F M, Mella N A, Kruze V J. Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow- and herd-status for *Mycobacterium avium* subsp. paratuberculosis. Prev Vet Med 2007; 82(1-2):159-165.

Vansnicka E, de Rijkb P, Vercammenc F, Geysena D, Rigoutsb L, Portaelsb F. Newly developed primers for the detection of *Mycobacterium avium* subspecies *paratuberculosis*. Vet Microbiol 2004; 100(3-4):197-204.

Vilar AL, Santos CS, Pimenta CL, Freitas TD, Brasil AW, Clementino IJ, Alves CJ, Bezerra CS, Riet Correa F, Oliveira TS, Azevedo SS. Herd-level prevalence and associated risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in cattle in the State of Paraíba, Northeastern Brazil. Prev Vet Med 2015; 121(1-2):49-55.

Waddell LA, Rajić A, Stärk KD, McEwen SA. The zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: a systematic review and meta-analyses of the evidence. Epidemiol Infect 2015; 143(15):3135-3157.

Waddell LA, Rajić A, Stärk KD, McEwen SA. The potential public health impact of *Mycobacterium avium* ssp. *paratuberculosis*: Global opinion survey of topic specialists. Zoon Pub Health 2016; 63(3):212-222.

Wells SJ, Wagner BA. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J Am Vet Med Assoc 2000; 216:1450-1457.

Whitlock RH, Buergelt C. Preclinical and clinical manifestation of paratuberculosis (including pathology). Vet Clin North Am Food Anim Pract 1996; 12:345-356.

Whitlock RH, Wells SJ, Sweeney RW, Van Tiem J. Vet Microbiol 2000; 77(3-4):387-398.

Whittington RJ. Factors affecting isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* from fecal and tissue samples in a liquid culture system. J Clin Microbiol 2009; 47(3):614-622.

Whittington RJ. Cultivation of *Mycobacterium avium* subsp. *paratuberculosis*. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 244-260.

Whittington RJ, Marsh IB, Saunders V, Grant IR, Juste R, Sevilla IA, Manning EJ, Whitlock RH. Culture phenotypes of genomically and geographically diverse *Mycobacterium avium* subsp. *paratuberculosis* isolates from different hosts. J Clin Microbiol 2011; 49:1822-1830.

Zecconi A, Mosca A, Piccinini R, Robbi C. A comparison of seven different protocols to extract *Mycobacterium avium* subspecies *paratuberculosis* DNA from bovine feces. In: Juste RA, Geijo MV, Garrido JM (Eds) Proceedings of the seventh international colloquium on paratuberculosis. International Association for Paratuberculosis, Madison, WI, pp. 270-273.