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-Valencia¹, MV, MSc, DSc(c); Nicolás F Ramírez¹, MV, MSc, Dr An Sci; Michael Bülte², Prof. Dr. Med. Vet.; Jorge A Fernández-Silva¹*, MV, MSP, Dr Med Vet.

¹Línea 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.

²Institut 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 endpoint IS900-specific nested PCR protocol, and a commercial F57-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 F57-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

Rev Colomb Cienc Pecu 2017; 30:101-115
were found to be positive by end-point IS900-specific nested PCR. Agreement was found between ELISA and end-point IS900-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 matrices from animals under the same husbandry conditions.

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

**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 Shallow, 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; Schönénbrücher *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 IS900 (Bölske 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 IS900-like elements with nucleotide sequences that are up to 94% identical to the nucleotide sequence of MAP IS900 (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 IS900 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 IS900 for the identification of MAP, the use of several other target sequences for MAP identification systems have been proposed: ISMap02, ISMav2,
hspX, locus 255, and F57 (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, F57-real-time PCR and end-point IS900-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 Km², an altitude of 2,468 m.o.s.l, a mean annual temperature of 16 ºC, 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 ºC 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 IS900-specific nested PCR, using primers targeting IS900 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-MgCl₂, 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. paratuberculosis, strain K10; ATCC® BAA-968(TM)) 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 F57 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 [F57po-244 F 5’– TAC GAG CAC GCA GGCG ATT C – 3’ and F57po-306 R 5’– CGG TCC AGT TCG CTG TCA T– 3’] and probes [F57po-TaqMan® Probe VIC-CCT GAC CAC CCT TC-MGB and IAC MSE TaqMan®...
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 (Kalisi 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).

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Breed*</th>
<th>Parity</th>
<th>Days in milk</th>
<th>Milk production per day (L)</th>
<th>Productive stage</th>
<th>Born in herd</th>
<th>Serum ELISA</th>
<th>Fecal culture</th>
<th>IS900-nested PCR</th>
<th>F57-real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Other</td>
<td>2</td>
<td>192</td>
<td>23</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>Other</td>
<td>6</td>
<td>163</td>
<td>33</td>
<td>Milking</td>
<td>Yes</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>Other</td>
<td>2</td>
<td>372</td>
<td>n.d.</td>
<td>Dry</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>Other</td>
<td>5</td>
<td>72</td>
<td>34</td>
<td>Milking</td>
<td>No</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>Holstein</td>
<td>1</td>
<td>4</td>
<td>n.d.</td>
<td>Dry</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>Other</td>
<td>4</td>
<td>214</td>
<td>24</td>
<td>Milking</td>
<td>No</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>Other</td>
<td>6</td>
<td>182</td>
<td>21</td>
<td>Milking</td>
<td>No</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>Other</td>
<td>2</td>
<td>133</td>
<td>25</td>
<td>Milking</td>
<td>No</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>Other</td>
<td>2</td>
<td>235</td>
<td>14</td>
<td>Milking</td>
<td>No</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>Other</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Heifer</td>
<td>Yes</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>11</td>
<td>Other</td>
<td>1</td>
<td>37</td>
<td>27</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>12</td>
<td>Other</td>
<td>2</td>
<td>299</td>
<td>16</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>13</td>
<td>Holstein</td>
<td>2</td>
<td>88</td>
<td>31</td>
<td>Milking</td>
<td>Yes</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>Holstein</td>
<td>1</td>
<td>215</td>
<td>25</td>
<td>Milking</td>
<td>Yes</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>Other</td>
<td>1</td>
<td>52</td>
<td>21</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>16</td>
<td>Other</td>
<td>2</td>
<td>227</td>
<td>16</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>17</td>
<td>Holstein</td>
<td>6</td>
<td>324</td>
<td>n.d.</td>
<td>Dry</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>18</td>
<td>Holstein</td>
<td>2</td>
<td>197</td>
<td>19</td>
<td>Milking</td>
<td>Yes</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>19</td>
<td>Holstein</td>
<td>7</td>
<td>72</td>
<td>51</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>20</td>
<td>Other</td>
<td>5</td>
<td>18</td>
<td>25</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>21</td>
<td>Other</td>
<td>3</td>
<td>192</td>
<td>25</td>
<td>Milking</td>
<td>No</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>22</td>
<td>Holstein</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Heifer</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>23</td>
<td>Other</td>
<td>5</td>
<td>161</td>
<td>22</td>
<td>Milking</td>
<td>Yes</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>24</td>
<td>Holstein</td>
<td>5</td>
<td>89</td>
<td>37</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>25</td>
<td>Other</td>
<td>3</td>
<td>409</td>
<td>18</td>
<td>Milking</td>
<td>Yes</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>26</td>
<td>Other</td>
<td>3</td>
<td>184</td>
<td>24</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>27</td>
<td>Jersey</td>
<td>1</td>
<td>40</td>
<td>23</td>
<td>Milking</td>
<td>Yes</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

* 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.
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 (Whittington 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; Zeconci 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

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).
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 IS900-specific nested PCR used to test our samples are reported to be increased (Englund et al., 2001; Ikonomopoulou 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 (IS900 and F57), leading to non-comparable results related to their specificity and sensitivity. The IS900 is a repetitive DNA sequence present in 15-18 copies of MAP genome (Collins et al., 1989; Green et al., 1989). However, IS900-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, F57, 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 IS900 assay can detect 0.01 pg of DNA (corresponding to 10 genomes) when extracted from a pure culture, while the F57 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 Schönöbrücher et al. (2008) recommend including the F57-PCR assay to confirm the presence of MAP after a positive IS900-PCR. According to this, our results (F57-PCR negative results and some positive results by IS900-PCR), can be considered MAP-unspecific by IS900-PCR, and confirmed as negative by the F57 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; Schönöbrücher 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 IS900-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


Correa NM et al. Diagnosis of Mycobacterium avium subsp. paratuberculosis.
Correa NM et al. Diagnosis of Mycobacterium avium subsp. paratuberculosis


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.


Rev Colomb Cienc Pecu 2017; 30:101-115
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.


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


