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Identification of *Mycobacterium avium* subspecies *paratuberculosis* by PCR techniques and establishment of control programs for bovine paratuberculosis in dairy herds[✉]

Identificación de Mycobacterium avium subspecies paratuberculosis mediante pruebas de PCR y definición de programa de control de la paratuberculosis bovina en hatos lecheros

Identificação de Mycobacterium avium subespécie paratuberculosis por PCR e definição do programa de controle da paratuberculose em rebanhos bovinos leiteiros

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Summary

The aim of this study was to establish the protocol of conventional and real time PCR for amplification of Mycobacterium avium subsp. paratuberculosis (MAP) genome from bovine fecal samples, as a way to define strategies for establishing a prevention and control program in a dairy herd at the Universidad de Antioquia (Medellín, Colombia). Fecal samples were individually taken of clinical healthy cows or cows with diarrhea bred in a herd enzootic for Johne's disease, were processed them for culture in liquid Middlebrook 7H9 media supplemented with mycobactin under two different protocols: with or without inhibitors. Fecal samples from clinically healthy cows were used as negative control. Conventional and real time PCR were performed with MAP DNA obtained of fecal or cultured samples. The MAP- specific IS900 segment was amplified by using the respective forward and reverse primers. DNA isolated from a reference MAP strain was used as positive control of PCR. Data were analyzed by descriptive statistics and simple regression analysis between PCR and culture results were performed. All samples cultured in media with or without mycobactin gave a positive result compatible with MAP growth. However, only 13,3% of samples were positive by real time PCR. There was no relationship neither between PCR and

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culture results, nor between clinical condition of the cow and MAP positivity. These results support the need combine culture of feces with PCR diagnosis for identification of MAP-excreting cows in a dairy herd. Finally, a strategy of prevention and control of bovine paratuberculosis is proposed for this enzootic herd and for dairy herds in Colombia.

Key words: *bacterial culture, bovine paratuberculosis, caquexia in cows, dairy cows, Johne's disease, real-time PCR.*

Resumen

El objetivo de este trabajo fue establecer un sistema de detección y amplificación del Mycobacterium avium paratuberculosis (MAP) a partir de muestras de materia fecal bovina, mediante el uso de la técnica de PCR en tiempo real, como estrategia de apoyo para el establecimiento de un programa de prevención detección y control de la paratuberculosis bovina en el hato lechero de la Universidad de Antioquia. Muestras de materia fecal fueron tomadas de bovinos provenientes de un hato enzootico para la enfermedad de Johne, fueron cultivadas en medio de cultivo líquido Middlebrook 7H9 suplementado con micobactina, bajo dos protocolos de aislamiento: con o sin inhibidores. Como control negativo fue utilizada muestras de materia fecal de bovinos clínicamente sanos. Adicionalmente, con el DNA de las muestras aisladas en cultivo y de las muestras de materia fecal, fueron hechas pruebas de PCR convencional y en tiempo real, para la amplificación del elemento de inserción IS900 del MAP, mediante el uso de cebadores específicos para este elemento. Como control positivo se utilizó DNA de MAP de una cepa de referencia. Los resultados fueron evaluados mediante estadística descriptiva y la comparación entre el resultado del cultivo y de la prueba de PCR fue evaluado mediante regresión simple. Los resultados del cultivo bacteriológico, mostraron un total de 15 muestras con crecimiento compatible con MAP, el cual fue verificado por prueba de PCR en el 13.3% de los casos. No hubo correlación entre el resultado de cultivo y la prueba de PCR ni entre el estado clínico y la positividad al MAP. Los resultados confirman la necesidad de combinar el diagnóstico molecular con el cultivo bacteriológico para identificar las vacas positivas en un hato. Finalmente, una estrategia de prevención y control de la enfermedad aplicable a este hato enzootico y a los hatos lecheros en Colombia es discutida.

Palabras clave: *bovinos de leche, caquexia en vacas, cultivo bacteriológico, enfermedad de Johne, paratuberculosis bovina, PCR en tiempo real.*

Resumo

O objetivo deste estudo foi estabelecer um sistema de detecção e amplificação do Mycobacterium avium paratuberculosis (MAP) a partir de amostras fecais bovinas, utilizando a técnica de PCR em tempo real, como uma estratégia para apoiar o estabelecimento de um programa rastreio para a prevenção e controle de la paratuberculosis bovina no rebanho leiteiro da Universidade de Antioquia. Foram colhidas amostras de fezes de gado bovino a partir de uma fazenda enzootica para a doença de Johne, e foram cultivadas em meio de cultura líquida Middlebrook 7H9 suplementado com micobactina, sob dois protocolos de isolamento: com ou sem inibidores. Foram utilizadas como controle negativo amostras fecais de bovinos saudáveis. Além disso, as amostras de DNA isoladas e cultivadas a partir de amostras fecais foram realizadas testes de PCR convencional e em tempo real, para amplificação elemento de inserção IS900 no MAP. Foi utilizado como controle positivo do teste DNA de uma estirpe de referencia. Os resultados foram avaliados por estatística descritiva e as comparações entre os resultados de cultura e teste PCR foram avaliadas por regressão simple. Os resultados de cultura bacteriológica mostraram 100% de crescimento em amostras com MAP, que foi verificada por PCR em 13,3% dos casos. Não houve correlação entre o resultado do cultivo e teste PCR ou entre a clínica e a positividade para MAP. Os resultados confirmam a necessidade de combinar o diagnóstico molecular com cultura bacteriológica para identificação positiva de vacas em um rebanho. Finalmente, uma estratégia de prevenção e controle da doença aplicável neste rebanho enzootico e os rebanhos leiteiros e na Colômbia é discutida.

Palavras chave: *a doença de Johne, caquexia em vacas, cultura bacteriológica, gado leiteiro, paratuberculose bovina, real-time PCR.*

Introduction

Bovine paratuberculosis also known as Johne's disease (JD), is a worldwide distributed disease of bovine, particularly of domestic ruminants, that is characterized by a chronic granulomatous inflammation of small intestine. The causing agent of JD is an intracellular bacteria belonging to the avium complex of mycobacterium family, known as *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Sigurðardóttir y Valheim, 2004). Bovine paratuberculosis is an immune-mediated disease of a susceptible host in which clinical signs are mostly related to the host immune response elicited against the MAP. As a consequence, clinical signs of the disease may fluctuate between episodes of exacerbation and remission of clinical signs (Abalos, 2001; Sweeney, 1996). MAP is an acid-alcohol resistant agent, sharing several characteristics with other members of the avium complex of Mycobacterium genus. MAP has a key genetic difference consisting on the presence of 14 to 18 copies of the insertion sequence 900 (IS900), a characteristic that is usually used as a diagnostic criteria when using PCR techniques for amplification of MAP genome (Bannantine y Stabel 2002; Clarence, 1993). In Addition, MAP exhibits a particular characteristic for growing in culture, consisting of its dependence of mycobactin, a component of the bacterial cell wall that function as an iron chelating agent that is produced by most Mycobacteria but not produced by MAP (Valentin y Goethe, 1999). A key factor determining the permanence of MAP in a herd is that it maintains its infecting potential during long periods of time, although it is susceptible to soil having basic pH and high calcium concentrations (Chi *et al.*, 2002). The most important route of infection of a cow is the fecal-oral route, through ingestion of MAP contaminated food or milk (Valentin y Goethe, 1999). Excretion of MAP in feces can occur at any time along the course of the disease, although it is rarely detected before the second year of life (Valentin y Goethe, 1999).

Johne's disease has four clinical states that are defined according to the severity of clinical signs, the potential excretion of MAP into the

environment, and the probability to diagnose the disease by the current laboratory methods. Stage I, is a silent infection with no clinical signs, and is the characteristic stage of young animals up to two years of age. In this stage MAP infection is only detectable by tissue culture or histopathological examination of tissue samples, MAP could be excreted by feces, but their concentration in fecal samples is under the detection limit of routine laboratory tests (Valentin y Goethe, 1999). Stage II, is characterized by a subclinical infection, with no diarrhea or other clinical signs. Impaired cellular immune responses and MAP specific antibodies could be detected in serum. Mastitis and infertility can also be detected. Affected cows could eliminate MAP but only 15% to 25% are detected by bacterial culture and most of the cows could not be detected by current diagnosis techniques and evolve to clinical stage III, whereas others are culled by other causes not related to Johne's disease (Valentin y Goethe, 1999). Stage III, or clinical disease is established after a prolonged incubation period lasting between 2 and 10 years, it includes a gradual weight lost, with intermittent episodes of diarrhea especially at the beginning of the signs. Rarely the infected animals stay in this stage for more than 4 months without progression to Stage IV, some few animals go back to Stage II, in which they stay for an undetermined period of time (Valentin y Goethe, 1999). Most animals in stage III can be diagnosed by ELISA test and Agar Gel Immunodifusion (AGID) (Valentin y Goethe, 1999). Stage IV, is the advanced clinical form of the disease. As much as disease progresses lethargy, debility and emaciation increase in the affected animal, with diarrhea and caquexia being the main terminal signs of the disease. Because of impaired milk yield and/or severe weight loss, most affected animals are culled off before they reach stage IV. Some animals in stage II can rapidly progress to stage IV in a few weeks (Valentin y Goethe, 1999). Interestingly, during clinical stages high quantities of MAP can be eliminated in feces and can exceed 1,010 organisms/gram of feces (Bögli-Stuber *et al.*, 2005). It has been established that for each animal exhibiting an advanced stage of the disease there could be 25 subclinical infected animals in a farm (Valentin y Goethe, 1999). Therefore, the early detection of

MAP-excreting infected animals is an obligation for establishing strategies of prevention and control on an infected farm (Zapata *et al.*, 2008).

One of the most limiting factors for culture and isolation of MAP is its low rate of growth in culture, which generally last between 8 to 12 weeks (De Juan, 2006; Valentin y Goethe, 1999), a fact that difficult the implementation of bacterial culture as a current test for MAP diagnosis. For instance, the best approach for detecting infected animals is to combine bacterial culture with molecular techniques such as conventional or real time PCR (Taylor JH, 2009). Because in La Montaña dairy Herd (from Universidad de Antioquia, Medellín, Colombia) several cows with clinical signs compatible with Johne's disease have been identified by clinical and histopathology examination (Ramírez *et al.* 2002), the aim of the present study was to detect MAP-secreting cows by amplification of the MAP-specific IS900 segment by conventional and real time PCR, as a way to establish a control program for that herd and to provide insights for the study of Johne's disease in dairy herds in Colombia.

Materials and methods

Institutional Board Approval

This study was approved by "Comité de Ética para la Experimentación Animal" at the Universidad de Antioquia (Act # 8714028, 2006)

Type of study and sample

A descriptive study was performed in which feces from clinical healthy cows or cows exhibiting clinical signs compatible with Johne's disease (chronic emaciation and diarrhea) were sampled and processed for bacterial culture and conventional and real time PCR. In this study cows exhibiting clinical signs compatible with Johne's disease and clinical healthy cows were selected at "La Montaña" dairy herd (Universidad de Antioquia, Medellín, Colombia), located at San Pedro de los Milagros Municipality (Antioquia region, Colombia). The farm has an average annual rain fall of 2.500 mm, average temperature of 16 °C, and it is located at 2.360 m above the sea level.

Fecal sampling

Fecal samples were taken individually from the rectum of each cow using a plastic glove, previous lavage of perianal region with clean water. Each fecal sample was placed in a sterile plastic recipient and transported to the laboratory under ice temperature in a transport vessel. Once in the laboratory all samples were storage at -20 °C until processing.

Bacterial culture

Sample decontamination. Approximately 2 g of feces were taken with a plastic device and mixed in a glass flask containing 40 ml of sterile distilled water, and shaken 30 min/room temperature. Then, 5 ml of solution were taken from the upper region of suspension and mixed with 0.75% Hexadecylpyridinio-chloride in a glass flask. The flask containing that suspension was inverted several times and was left under incubation for 24 h/ room temperature.

Culture media. Liquid media Middlebrook 7H9 Broth base (Becton Dickinson, Franklin Lakes, NJ, USA), was prepared according to manufacturer's instruction. Briefly: 2.35 g of 7H9 Broth base was re-suspended in 450 ml distilled water and added with 2 ml glycerol. The suspension was heated until complete dilution. The media was autoclaved at 15l pounds/121 °C/10 min, and then cooled to 45 °C. Under aseptic conditions 1 vial of Middlebrook ADC Growth Supplement was added (Becton Dickinson, Franklin Lakes, NJ, USA) and mixed vigorously.

Sample inoculation in liquid media. From the bottom of the fecal dilution-containing tube that was previously incubated for 24 hours, 0.1 ml of sediment were taken with a sterile Pasteur pipette and was added to the 7H9 liquid media-containing tube, and was then supplemented with 1ml/500ml mycobactine (Allied Monitor Inc, Fayette, MO, USA), 10 mg/l Amphotericine-B (Dr. Ehrenstofer GmbH, Augsburg, Germany), 100 mg/l carbeniciline (USP, Rockville, MD, USA), 200.000 U/l Piperaciline (USP, Rockville, MD, USA), and 10 mg/l Trimethoprim (USP, Rockville, MD, USA). Samples were cultured at 37 °C until observation of turbidity.

Acid-alcohol resistant staining. In order to verify the presence of Acid-alcohol resistant (AAR) bacilli, a sample of the sediment of culture was taken with a sterile device and a smear was stained according to the method for Ziehl-Neelsen.

DNA extraction from fecal samples and from liquid culture media. Total DNA was extracted according to the protocol established for DNA from fecal samples (QIAamp® DNA Stool Mini Kit, Qiagen, Valencia, California, USA). DNA concentration and purity was determined by optical densities in a Nanodrop® ND 1000 (Thermo Scientific, Wilmington, Denver, USA), samples were stored at -80 °C until processing.

Amplification of MAP DNA by PCR

Conventional PCR. Conventional PCR was processed in order to verify the specificity of primers chosen for IS900 amplification, before its use in real time PCR. Ten nanograms (10 ng) of DNA from each sample were incubated with IS900 primers F58: ACGTCGGGTATGGCTTTCAT (Forward) and SF109: AATCTCCTTCGGCCATCCA (Reverse) (O'Mahony y Hill, 2002). Primers sequence was verified in Silica for corroboration of denaturation, annealing and extension values reported in the literature for these primers. Accordingly, the following values were established in our laboratory: one cycle at 95 °C/5min; 40 cycles at 95 °C/30s, 63 °C/30s and 72 °C 30s, and a final cycle at 72 °C /5 min. The mix containing 50 mM de KCl, and 15 mM de MgCl₂, was added with the sample DNA. A DNA from a reference MAP strain (ATCC BAA-968) (A gift of Institute of Veterinary Food Sciences, Faculty of Veterinary Medicine. University of Gießen, Germany) was used as a positive control. The mix and primers without sample DNA was used as a negative control.

Electroforesis. PCR products were analyzed in 2% agarose (Agarosa I Amresco® Ohio, USA) added with (5 µl) Ethidium bromide. Gels were run in a Biorat Universal Hood II (laboratory Segrate, Milan Italia) equipment at (100mv) during (60 min). The resulting bars were photographed and recorded for densitometry analysis.

Real time PCR. Five microlitres (5 µl) of purified DNA were added with specific forward and reward primers for amplification of the IS900 MAP sequence, under the following conditions: one cycle at 95 °C/15 min, 45 cycles at 95 °C/30 sec, 63 °C/30 sec, and 72 °C/30 sec, and a final step at 72 °C/3 min. A 25µl mixed reaction containing 62.5 nM of each primer SF58 (Forward) ACGTCGGGTATGGCTTTCAT and SF109 (Reverse) AATCTCCTTCGGCCATCCA (O'Mahony y Hill, 2002), were added with 5 µl DNA and 15.75 µl SYBR GREEN PCR Master Mix (3mM MgCl₂, 200µM of each dATP, dGTP, dCTP, and 400 µM dUTP, 1.25 IU DNA polymerase and 0.5 IU Uracil N-glucosilasa (UNG). Each sample was processed with a negative control of extraction and both a negative and a positive control were used. For the negative control of extraction an equal volume of deionized distilled water instead of a bacterial suspension was used. Equal volumes of cultured MAP DNA or deionized distilled sterile water were added to positive or negative control, respectively. Detection of the amplified IS900 product was performed with a Corbett 1000 (Corbett Robotics, San Francisco, CA, USA) equipment. The resulting PCR product consisted of a 52 bp DNA fragment (O'Mahony y Hill, 2002). The threshold cycle (Tc) for amplification of samples was compared with the Tc of a standard DNA of MAP obtained by culture.

Statistic analysis

Data from the period of time elapsed between sampling and positive culture results, population variables of sampled animals were analyzed by descriptive statistics, and simple regression analysis between PCR and culture results were performed.

Results

Basic data from animals sampled in this study is presented in table 1. A single fecal sample was processed by PCR for most of the animals but one cow from which three samples were processed (Encina). The average age of sampled cows was 6.7 year (+/- 2.77 S.D.). All cultured samples (el 100%) showed growth of colonies characterized by

turbidity of media at week 20th of culture. Aliquots of each sample grown in culture were taken for acid-alcohol resistant (AAR) staining (Zielh Neelsen) and for real time PCR. A total of 9 out of 15 samples (56 %) stained positive for AAR, whereas 3 out of

15 (20%) gave a positive result by real time PCR. Representative samples processed by conventional PCR are presented in figure 1, in which positive bands migrate near to primer. These samples were further analyzed by real time PCR (Figure 1).

Table 1. Basic data of sampled animals and results of bacterial culture, AAR staining and PCR technique

Sample ID	Name	Racial group	Age (years)	Bacterial culture	AAR result	PCR result
MAP-34	Encina	Holstein	4	+	-	+
MAP- 48	Bamba	F1 BxH	6.3	+	+	+
MAP-49	Banana	Holstein	6.93	+	+	-
MAP-50	Bastilla	Holstein	6.84	+	+	-
MAP-52	Belmira	Holstein	6.73	+	+	-
MAP-55	Candela	Holstein	12.4	+	+	-
MAP-10	Carlota	Holstein	5.15	+	+	-
MAP-56	Celia	Holstein	5.8	+	+	-
MAP-11	Demanda	Holstein	5	+	+	-
MAP-01	Diana	Holstein	5	+	-	-
MAP-58	Dolly	Holstein	12.7	+	-	-
MAP-32	Donna	Holstein	5	+	-	-
MAP-16	Electra	Holstein	4	+	+	+
MAP-60	Estafa	Holstein	4	+	-	-
MAP-66	Sensacion	F1 BxH	9.1	+	-	-

+, positive result; -, negative result, F1 BxH, cross-bred BON x holstein

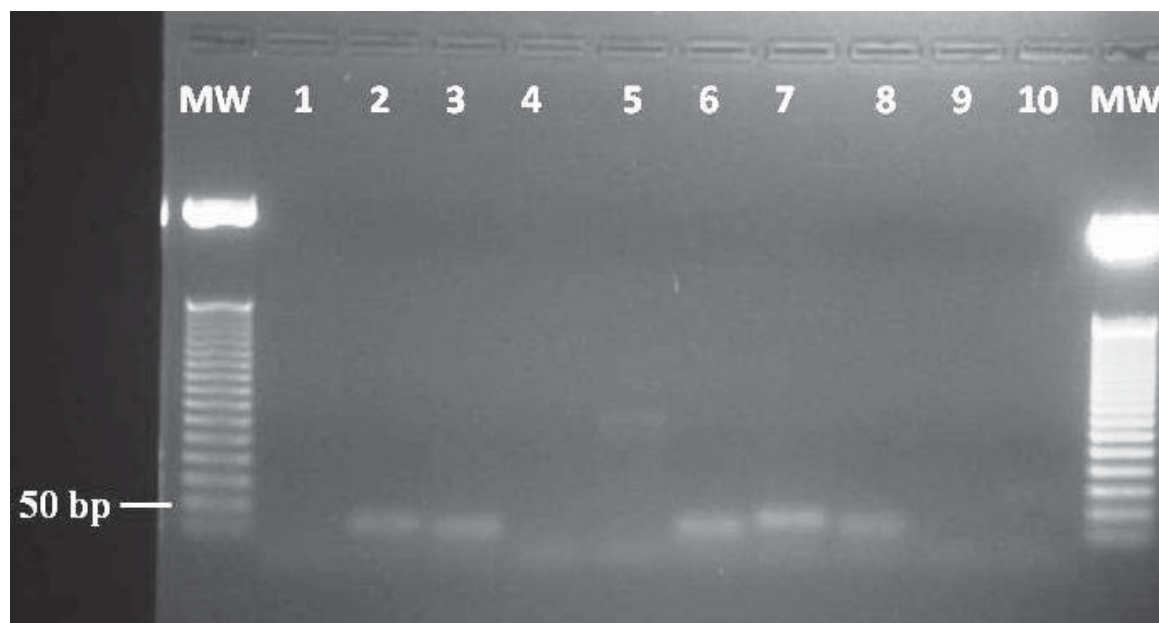


Figure 1. Representative electrophoresis of samples evaluated by conventional PCR for amplification of IS900 MAP-specific sequence from fecal samples of Holstein and F1 BxH animals. Lanes: 1-3, positive control undiluted, or diluted 1/10 or 1/100; Lanes 4-6, Cow Encina, sample undiluted or diluted 1/10 or 1/100; Lanes 7-9, cow Electra sample undiluted or diluted 1/10 or 1/100; Lane 10, negative control. MW, molecular weight marker.

The results of real time PCR showed that positive samples reached the amplification threshold beginning after cycle # 32 (Figure 2), a finding that could be due to the low quantity of MAP specific DNA present in fecal samples.

In order to verify that primer dimers were different from positive sample bands, the melting data showed that only positive samples were indeed amplified rather than primer dimers (Figure 3).

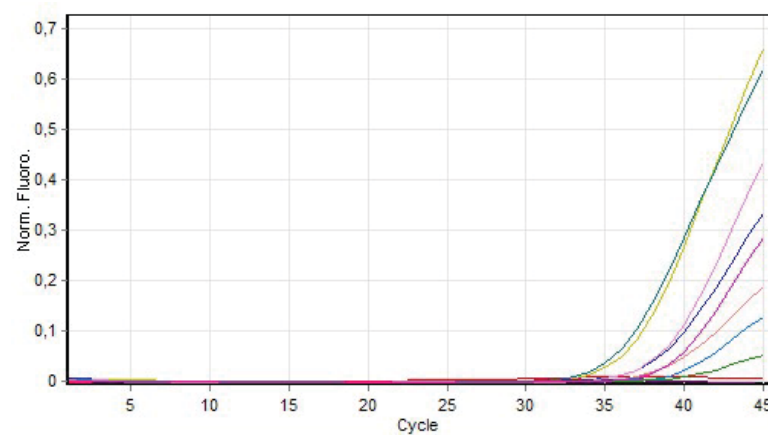


Figure 2. Quantitation data for Cycling A. Green by quantitative PCR of fecal samples obtained from Holstein and F1 BxH animals. Color lines represent the same samples indicated in figure 1, from the left to the right.

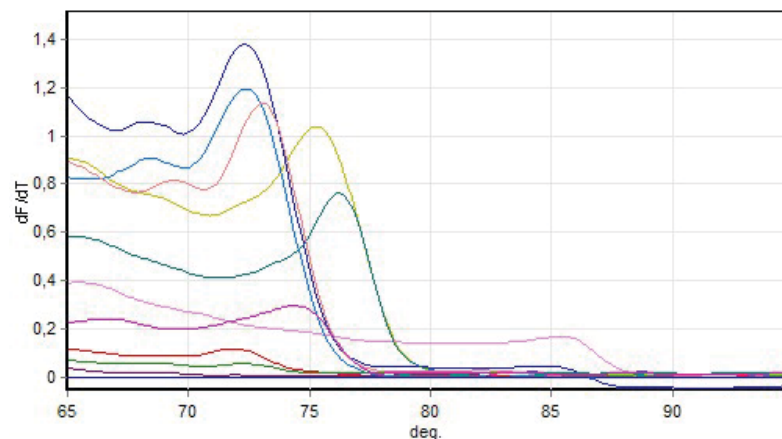


Figure 3. Melting data for quantitative PCR of fecal samples obtained from Holstein and F1 BxH animals. Color lines represent the same samples indicated in Figure 1, from the top to the bottom.

Discussion

There are two classical techniques for the diagnosis of bovine paratuberculosis including the analysis of serum antibodies by ELISA and bacterial culture from fecal samples. Although both of these techniques are highly sensitive the specificity is low particularly when dealing with asymptomatic (subclinical stages) infected animals which usually excrete low quantities of MAP in feces. The sensitivity of ELISA test can vary

between 15% and 95%, depending on the number of bacteria excreted by the infected animal. Bacterial culture is considered the gold standard for MAP detection as it is 100% sensitive (Clark *et al*, 2008; De Juan *et al*, 2006), although it is very expensive, and extremely slow for giving a final result a fact that limits its diagnosis potential (Sigurðardóttir y Valheim, 2004; Sweeney, 1996). In agreement with the reports of the literature the results in bacterial culture in the present study, showed that 20 weeks

elapsed between the initial time of culture until the time in which an alteration of the media suggestive of MAP growth was observed. Interestingly, samples cultured without adding mycobactin to the media also showed bacterial growth after 20 weeks of culture suggesting other mycobacteria different to MAP could have been grown in culture, probably environmental mycobacteria. Bacterial contamination could be discarded because media did not show the turbidity and change of pH typical of non specific contamination. In fact, culture media was added with three different antibiotics and an antifungal compound suggesting that the observed growth could be related to environmental mycobacteria rather than other to non specific bacterial contamination.

As PCR was proposed to be the most promising technique to rapidly amplify and detect MAP genome from infected-animal fecal samples, by amplification of IS900 MAP-specific sequence (Bull *et al.*, 2000; Harris y Barletta, 2001), this technique have been developed for a more accurate and rapid than bacterial culture diagnosis of MAP-infected animals (Collins y Stephens, 1993b; Góngora y Villamil, 1999; Whitlock y Buergelt, 1996). Bovine paratuberculosis is characterized to be a chronic progressive disease during which MAP excretion in feces is proportionally related to clinical stage of the disease (Crossley *et al.*, 2005; Raizman *et al.*, 2007). However, in the present study cows were positive by PCR instead of being asymptomatic for Johne's disease. This fact could suggest that MAP-infected animals under the conditions of this enzootic herd can excrete considerable quantities of MAP in feces instead of being in a subclinical or early stage of the disease, in agreement with reports by several authors (Raizman *et al.*, 2007, Mercado *et al.*, 2000.). Additional evidence is provided by a case of a cow (Encina), who was sampled during the present study when it had no clinical symptoms compatibles with JD, but it died some weeks after sampling it after a short period of clinical diarrhea. At necropsy this cow exhibited gross macroscopic lesions in ileum and jejunum compatible with JD, and confirmed by histopathology in which abundant AAR positive bacilli were found in lamina propia of ileum and jejunum (Dr. Dubel I. Balvin, Universidad de Antioquia, unpublished case report). Based on this finding and taken into consideration that this

herd is considered enzootic for JD, the finding of clinical healthy animals that resulted positive for MAP by bacterial culture and/or PCR techniques, are in agreement with the report of the literature about the existence of 25 potentially-infected animals (in subclinical stages of the disease) for each clinical case diagnosed in a herd (Fang *et al.*, 2002). On the other hand, a negative result of a bacterial culture or a PCR performed on fecal samples do not necessary mean that the animal in question is not infected, because other factors related to the clinical stage of the disease and the quantity of excreted mycobacteria at the time of sampling, can be a bias for an appropriate diagnosis of MAP-infected animals.

The results on diagnosis of MAP-infected animals should be interpreted with caution regarding the establishment of a control program for JD in a given herd, because the high variability in the probability of finding MAP-excreted in feces is not a confidence variable to be considered for control purposes or for considering a herd as a MAP-infected or MAP-free herd, excepting when serial samples from the same animal are analyzed. In addition, caution should be kept when analyzing only a portion of the fecal sample that is contaminated with MAP in a given moment, because low excreting animals could not be appropriately detected when only a sample is taken instead of a gross quantity of fecal material because of obvious (economical and practical) limitations.

Several reports have shown the disparity between bacterial culture and PCR results (Bögli-Stubler *et al.*, 2005; Sevilla, 2007). This is due, at least in part, to the different stages of the disease that are diagnosed for each technique, and its sensitivity. E.g., sensitivity of PCR technique depends on the quantity of DNA present in the sample and the efficiency of the protocol used for DNA extraction its purity, and the presence of PCR inhibitors in the fecal sample. On the other hand, bacterial culture requires a previous treatment of the sample for avoiding contamination by other microorganisms growing faster than MAP (De Juan *et al.* 2006). In this study we also found such a lack of concordance between bacterial culture and PCR results. This would be due to factors related to the method used for previous decontamination of the sample, the

presence of other microorganisms interacting with MAP, metabolic stages of the bacteria, the use of thawed samples, the presence of various strain of MAP, and differences between these, etc.

An integral strategy for identification of infected animals in a herd, prevention and control of the disease

Because treatments of infected animals suffering Johnes's disease are hardly inefficient and expensive (O'Mahony y Hill, 2002), no treatments are recommended for infected animals in a dairy herd. Treated animals may exhibit improvement of their clinical signs but a complete remission of the entity is not achieved, animals can rapidly become ill and MAP can be newly excreted in feces (Collins y Stephens, 1993b; Whipple *et al.* 1991). Therefore, the best choice for farmers is to define specific strategies for control of the disease in dairies, by avoiding dissemination of JD into the herd and the corresponding economic loss related to milk yield reduction in subclinical or clinical-infected animals, and increased culling rates (O'Mahony y Hill, 2002; Chi *et al.* 2002; Zapata *et al.* 2008). Similarly, practices related to replacing culled cows, calving, and management schedules for infected calves or adult animals, as well as preventive veterinary medicine schedules, are of key importance in the establishment of an integrated program for identification of MAP-infected animals, prevention of new infections, and control of the disease (Collins y Stephens, 1993b).

Identification of infected animals. The first step is to identify each individual to know is sanitary status regarding MAP infection, the potential and real routes of animal-to-animal infection and environmental borne sources of the agent. MAP-infected animal must be obligatory identified by a combined strategy of at least two diagnosis methods: bacterial culture and PCR testing and/or ELISA test. The main advantage of PCR consist in its high specificity, sensitivity for detecting subclinical-infected animals, and the low time required to obtain the PCR result (Zapata *et al.* 2008). In a dairy herd, fecal samples should be taken from each group of animals at six month interval and processed by PCR. Pregnant cows

at the last trimester of pregnancy should also be sampled. In addition, only paratuberculosis-free animals should be introduced into the herd as a replacement strategy, for which at least two negative PCR tests with a month interval should be confirmed in the animal (Zapata *et al.*, 2008).

Prevention strategies. Identification of MAP-infected or MAP-free animals would allow to established preventive schedules for avoiding paratuberculosis infection or re-infection in a herd. In a paratuberculosis-free herd, management strategies must be directed to avoid introduction of the agent by keeping the herd in a close-schedule of replacing management. On the contrary, in an infected herd, management schedules should be directed to avoiding dissemination of the agent from infected animals to other individuals or group of non-infected animals into de herd.

Control strategies. In herds in which JD have been diagnosed, the most recommended procedure is to culling off animals that are diagnosed as positive for infection with MAP, based on the strategy of PCR testing. This measure must be accompanied by the implementation of measures directed to avoid new cases and dissemination of the agent to the rest of the animals (Collins y Stephens, 1993b). Some of these measures include: 1) fecal sampling of animals at 6 months interval for detecting MAP genome by PCR, 2) isolation of animal with clinical signs compatible with JD, and testing of fecal samples by PCR, 3) keeping utensils and milking devices clean and free of fecal contamination, 4) keeping away young from adult animals and specially from animal with clinical signs compatible with JD, and 5) culling of MAP positive cows. In addition, pregnant cows with a PCR positive result as well as their positive calves must be culled. For instance it is necessary to feed a calf born from an infected cow with callostrum taken from a negative cow or with milk replacement food. The calf must be sampled twice at its first and second months of life for PCR testing. Only two consecutive negative PCR results must be considered to keep the calf in the herd replacement stock (Sevilla, 2007; Zapata *et al.*, 2008). Finally, in some countries there are commercial vaccines available for MAP. But preventive schedules based on vaccination strategies are not recommended for

MAP free herds, because this may reduce the number of animals developing the clinical stages of the disease and the number of infected cows excreting detectable quantities of MAP in fecal samples, but it is rarely effective for prevention of Johne's disease in a herd (Sevilla, 2007).

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