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ORIGINAL RESEARCH

Serological and molecular detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle of dairy herds in Colombia

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Abstract The objective of this study is the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by serum enzyme-linked immunosorbent assay (ELISA), fecal polymerase chain reaction (PCR), and fecal culture in Colombian dairy herds. Serum and fecal samples from asymptomatic cows (n=307) of 14 dairy herds were tested for MAP by an unabsorbed ELISA test (ELISA-A). Serum and fecal samples from positive ELISA-A animals (n=31) were further tested by an absorbed ELISA test (ELISA-B) and PCR. Fecal samples from animals of herds positive by ELISA-A and PCR (n=105) were inoculated onto three different culture media. ELISA-A produced positive results in 10% of the serum samples and 71% of the herds. ELISA-B and PCR results were positive in two and six serum and fecal samples from positive ELISA-A animals, respectively. Fecal samples were negative for MAP on all culture media. The results of this study confirmed the presence of MAP in local dairy herds and the difficulties of MAP detection in asymptomatic animals by ELISA, PCR, and fecal culture.

Keywords *Mycobacterium avium* subsp. *paratuberculosis* · ELISA · PCR · Culture · Colombia

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causal agent of paratuberculosis or Johne's disease, a slow-developing disease of ruminants characterized by chronic granulomatous enterocolitis and regional lymphangitis and lymphadenitis (Clarke 1997). Economic losses due to reduced milk production, increased cow replacement, lower cull–cow revenue, and greater cow mortality are higher in paratuberculosis-positive herds compared with paratuberculosis-negative herds (Ott et al. 1999). On the other hand, it has been suggested that MAP could be part of the causal structure or an opportunist in Crohn's disease of humans, which is still being discussed (Waddell et al. 2008).

The enzyme-linked immunosorbent assay (ELISA) is the test most widely used to establish paratuberculosis status of herds, but the isolation of MAP from an animal by culture is still considered the golden standard of Johne's disease diagnosis (Chiodini et al. 1984; Collins 1996). On the other hand, the polymerase chain reaction (PCR) technique is speed and specific, and in contrast to culture-based diagnostic, no additional tests are required to confirm the identity of the organism detected.

Colombian cattle population was estimated in 26.8 million of heads for 2008. However, no official control or eradication program for paratuberculosis is executed. Because of this, the currently epidemiological situation of this disease in Colombia is practically unknown. Although some research on diagnosis of MAP in Colombia has been conducted in cattle since more than 60 years, information about its presence and distribution in cattle is still scarce. The objective of this study was the detection of MAP in asymptomatic cattle of 14 dairy

herds of a region in Colombia, using ELISA, fecal culture, and fecal PCR.

Materials and methods

Selection of herds and animals

Fourteen dairy herds of nine districts located in and around a municipality of a dairy region in Colombia were sampled for detection of MAP in November 2007 (Table 1). The municipality is situated in the Andean region of Colombia; it has an area of 296 km^2, an altitude of 2,500 m, a mean annual temperature of 14°C, and a cattle population of approximately 21,500 animals. The herds were selected attempting a representation of all productive districts of the municipality. The calculation of the number of animals per herd to be sampled was carried out based on a procedure previously reported (Johnson-Ifearulundu and Kaneene 1998). Of these 14 herds, only one herd had presented sporadic clinical cases compatible with paratuberculosis confirmed by PCR and histopathology (Zapata et al. 2010). The number of animals estimated to be tested in every herd was randomly sampled.

Collection of samples and information

Blood and fecal samples were taken from each animal. Serum and feces were frozen at −20°C until analysis at the Institute of Veterinary Food Science (Institute für Tierärztliche Nahrungsmittelkunde) of the Justus–Liebig–University Giessen (Germany) in January 2008. Information about age and some features of the animals, as well as herd management practices were collected.

Serum ELISA

An indirect ELISA test based on detection of lipoarabinomannan (LAM) (Svanovir Para–TB Ab ELISA Kit, Svanova Biotech AB, Uppsala, Sweden; ELISA-A) was used to test all serum samples (n=307). A second indirect ELISA test based on detection of antibodies to protoplasmic MAP antigens including a pre-absorption step with *Mycobacterium phlei* (ELISA paratuberculosis antibody verification, Institut Pourquier, Montpellier, France; ELISA-B) was applied to serum samples positive and doubtful by ELISA-A. A herd was considered positive if at least one animal tested positive by ELISA-A.

DNA isolation

DNA isolation from fecal samples of ELISA-A-positive animals was carried out in duplicate by using a commercial DNA preparation kit (High Pure PCR Template Preparation Kit, Roche, Mannheim, Germany). DNA from bacteria isolated on fecal culture was extracted using a commercial preparation kit including a pretreatment protocol for Gram-positive bacteria (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany).

Fecal PCR

DNA from fecal samples and from bacteria were tested in duplicate for MAP with a real-time PCR method (F57–IS*Mav2*–real-time PCR) using primers and PCR conditions according to Schönenbrücher et al. (2008). The samples were also tested in duplicate with a nested PCR (nested–IS*900*–PCR) method, using primers and PCR conditions as reported before (Bull et al. 2003). Additional to the

Table 1 Herds of a dairy region in Colombia tested for detection of MAP

District	Herd	Herd cattle population	Number of samples	Current tuberculosis status
Monterredondo	1[a]	102	20	Free
Playas	2	75	19	Free[b]
Zona Urbana	3	128	21	Undet
Labores	4	300	29	Undet
	5	100	19	Undet
	6	176	25	Undet
	7	102	23	Undet
El Yuyal	8	140	20	Free
	9	74	22	Undet
Santo Domingo	10	181	23	Reactive
Amoladora	11	83	20	Undet
	12	75	20	Undet
Zafra	13	67	21	Undet
Zancudito	14	96	25	Undet
Total		1699	307	

Free free status expired in October 2010, *Undet* undetermined

[a] Herd with history of diagnosis and clinical cases of paratuberculosis

[b] Free status for this herd expired in October 2010

samples, a positive and a negative preparation control as well as a blank control were included. In the PCR system, a positive MAP control (DNA of a positive MAP strain); a non-MAP negative control, this means DNA of a non-MAP mycobacteria; and a master–mix blank control were also included.

Fecal culture

Fecal samples from all animals of herds positive by ELISA-A and by PCR (n=105), regardless of individual result, were decontaminated and cultured using three different methods. The first method included the decontamination of samples with 0.75% (w/v) Hexadecylpyridinium Chloride (Merck, Darmstadt, Germany) solution (0.75% HPC) and the inoculation of samples in duplicate onto Herrold's yolk agar medium (HEYM) slants, supplemented with mycobactin J and amphotericin B, nalidixic acid, and vancomycin mix (Becton Dickinson, Heidelberg, Germany) following standard procedures (Anonymous 2007).

The second method included the decontamination of fecal samples by addition of a solution of 4% NaOH (w/v) and a solution of 5% oxalic acid (w/v). The decontaminated sample suspension were inoculated in duplicate onto slants of Lowenstein–Jensen medium (LJ) containing mycobactin J and a mix of polymyxin B, amphotericin B, carbenicilin, and trimetoprim (Bioservice, Waldenburg, Germany) (Glanemann et al. 2004).

The third method consisted on the decontamination of samples with 0.75% HPC and the inoculation in duplicate onto modified Middlebrook 7 H11 medium (MB 7 H11) supplemented with mycobactin J (Bioservice, Waldenburg, Germany) (Greig et al. 1997). All culture media were incubated for 6 months and were checked weekly for mycobacterial growth or contamination with undesirable germs.

Colonies with compatible mycobacterial morphology were tested for acid fastness by the Ziehl–Neelsen stain of smears method (Becton Dickinson, Heidelberg, Germany). The mycobacterial isolates were tested for MAP confirmation by the PCR methods described above. Acid-fast mycobacteria that tested negative for MAP by PCR were further examined to determine their identity by PCR amplification of the 16S rRNA gen as described previously (Kuhnert et al. 1996). Sequences obtained by PCR were compared for similarity-based species identification using the databases Ribosomal Differentiation of Medical Microorganisms (RIDOM) from the University of Würzburg, Würzburg, Germany (available at http://www.RIDOM.de) (Harmsen et al. 1999), and the basic local aligment search tool of the National Center for Biotechnology Information (NCBI) (available at http://www.ncbi.nlm.nih.gov). Similar isolates sharing the distinct phenotypical characteristics of growth and pigmentation of acid-fast mycobacteria already identified were not further tested for 16S rRNA similarity-based species identification due to economical reasons, and because the specific species determination of all mycobacterial isolates different from MAP was beyond the scope of the study.

Statistical analysis

The descriptive analysis of age, estimation of standard deviation (SD), and determination of confidence intervals 95% (95% CI) were carried out by using the program packages BMPD for XP, release 8.1, and BIAS for Windows, release 8.2.

Results

ELISA-A

The ELISA-A test produced 31 out of 307 (10.1%) positive (95% CI; 7.0–14.0%), 268 (87.3%) negative, and eight (2.6%) doubtful results. Ten out of 14 (70%) of the herds were considered as positive (Table 2). Information could not be collected from 53 animals sampled, which were removed from the descriptive analysis of age to avoid bias. Age of animals sampled ranged between 2.7 and 13 years (mean 5.7, SD 2.4), age mean in ELISA-A-positive was 6.4 (range 3–10.6, SD 2.13) and in ELISA-A-negative was 5.6 (2.7–13.9, SD 2.45). Analysis of age of animals vs. type of ELISA-A result revealed that group of 8.1–10.9 years was the group in which the highest proportion of ELISA-A-positive results (14.9%) were produced (Table 3). In the group of 5.1–8 and 3–5 years, 13.2% and 6.8% of the samples produced positive results, respectively. In the older group (>11 years of age), no positive result by ELISA-A was produced. The highest proportion of doubtful results was found in the group of 5.1–8 years.

ELISA-B

From 39 serum samples (31 positive and eight doubtful by ELISA-A), only two (5.1%; 5% C.I.; 0.6–17.3%), from two different herds were positive by ELISA-B, 37 (94.9%) were negative, and no sample was doubtful (Table 2). All serum samples that produced doubtful results by ELISA-A (n=8) produced negative results by ELISA-B. ELISA-B-positive serum samples (n=2) were from animals that were 6 and 4.2 years old at the moment of sampling and had never shown clinical signs of paratuberculosis. These ELISA-B-positive animals were from two different herds that belong to the same farmer. Between both herds cattle exchange was reported as a common practice.

Table 2 Results of tests for detection of MAP in cattle from 14 dairy herds

Test result		ELISA-A	ELISA-B[a]	Nested-IS*900*-PCR[b]	Real-time PCR[b]		Culture[d]
					F57	IS*Mav2*	
Positive	Animals	31 (10.1%)	2 (5.1%)	6 (19.4%)	2 (6.5%)	1[c] (3.2%)	0
	Herds	10 (70%)	2 (14.3%)	4 (40%)	2 (20%)	1 (10%)	0
Negative	Animals	268	37	25	29	30	105
	Herds	4	8	6	8	9	4
Doubtful	Animals	8	0	0	0	0	0
Total	Animals	307	39	31	31	31	105
	Herds	14	10	10	10	10	4

[a] Performed only to serum samples from animals positive and doubtful by ELISA-A

[b] Performed only to fecal samples from animals positive by ELISA-A

[c] Corresponds to a sample simultaneously positive by nested-PCR and weakly positive by real-time PCR

[d] Performed to all fecal samples of animals belonging to ELISA-A/PCR positive or real-time PCR weakly positive herds, regardless of individual ELISA result

Fecal PCR

The F57–IS*Mav2*–real-time PCR produced 3 (9.7%) weakly positive results out of 31 fecal samples from ELISA-A-positive animals belonging to 10 herds examined, while the IS*900*-nested PCR produced 6 (19.4%) positive results (Fig. 1). From the three F57–IS*Mav2*–real-time PCR weakly positive samples, two (6.5%) were positive only in the F57, and one (3.2%) only in the IS*Mav2* molecular target (Table 2). All PCR positive results were produced in fecal samples from animals belonging to herds 1, 2, 8, 9, and 10. Fecal samples from animals of remaining herds did not produce positive results by PCR. The two ELISA-B-positive animals produced negative results in both fecal PCR tests. Only in one fecal sample, both PCR tests produced a positive and a weakly positive result simultaneously (Table 2). All fecal samples, except for one, were only positive (IS*900*–nested PCR) or only weakly–positive (F57–IS*MaV2*–real-time PCR) in one sample of the duplicate.

Fecal culture

Fecal culture of 105 fecal samples was negative for MAP on HEYM, LJ, and MB7H11 media (Table 2). Fecal samples decontaminated with HPC and cultured on HEYM and on MB7H11 presented less contamination and less growth of atypical mycobacteria (AM) compared to fecal samples decontaminated with NaOH–oxalic acid solution and cultured on LJ after 16 weeks of incubation. AM were isolated on approximately 50% of the LJ slants inoculated with fecal samples decontaminated with NaOH–oxalic acid. AM were rarely isolated on HEYM. AM isolates were confirmed as acid-fast rod-shape bacteria in the Ziehl–Neelsen stain of smears. They produced negative results by IS*900*-nested PCR and F57–IS*MaV2*–real-time PCR. These isolates were identified as *Mycobacterium engbackii* by sequencing of a fragment of the 16S rRNA gen. The comparison of the sequences of the 16S rRNA gene amplified showed 99.8% of similarity to the sequences on the RIDOM and NCBI databases used for species identification.

Discussion

In this study, a combination of direct and indirect diagnostic methods was applied to detect MAP infection in cattle of dairy hers in Colombia. We considered that a combination of ELISA, fecal culture, and PCR was necessary and sufficient to obtain an accurate detection of paratubercu-

Table 3 ELISA-A results according to group of age

Group of age (years)	ELISA-A result			
	Positive (%)	Negative (%)	Doubtful (%)	Total (%)
3–5	6 (6.8)	81 (92.0)	1 (1.1)	88 (35.0)
5.1–8	14 (13.2)	89 (84.0)	3 (2.8)	106 (42.0)
8.1–10.9	7 (14.9)	39 (83.0)	1 (2.1)	47 (18.0)
>11	0 (0.0)	13 (100.0)	0 (0.0)	13 (5.0)

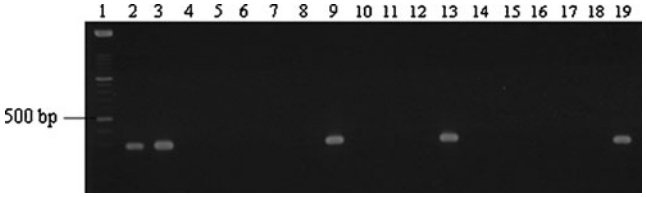

Fig. 1 Representative IS*900*-nested PCR positive results (294 bp) of fecal samples. Molecular size marker (100 bp DNA ladder; *lane 1*), animal 1 of herd 1 (*lanes 2* and *3*), animal 12 of herd 2 (*lane 9*), animal 19 of herd 2 (*lane 13*), positive control (*lane 19*), negative results (*lanes 4–8*, *10–12*, and *14–18*)

losis. However, we expected to find a higher proportion of MAP-positive animals in all herds examined based on the observation of some inappropriate herd management practices known to be associated with an increment of the risk of paratuberculosis transmission.

The proportion of ELISA-A-positive results obtained (10.1%) falls in the range of the animal level apparent prevalences reported for cattle in the world. In the same way, the proportion of ELISA-A-positive herds (70%) agrees with the herd level apparent prevalences from other countries using both unabsorbed and absorbed ELISA tests. Results of the distribution of age according to ELISA-A-positive results disagrees with the finding of a highest probability of testing positive with an ELISA test between 2.5 and 5.5 years in infected animals (Nielsen and Ersboll 2006).

The ELISA-B results were surprising because we expected a higher concordance to ELISA-A results. Studies with similar results by ELISA-A and ELISA-B have shown that the difference in antigens (LAM vs. protoplasmic antigen) is responsible for the poor concordance between both tests. In addition, the pre-absorption step with *M. phlei* applied in ELISA-B reduces the number of false positives, but could also reduce the sensitivity to detect true positives making an unabsorbed test more sensitive but less specific (Köhler et al. 2008). In addition, the animals evaluated in our study, except from one herd, were animals from herds without previous diagnosis of MAP and were asymptomatic for Johne's disease. This subclinical status could be responsible for a diminished sensitivity and specificity of tests compared with the sensitivity and specificity if they have been applied to affected or to infectious animals (Köhler et al. 2008; Nielsen and Toft 2008).

On the other hand, it is possible that other mycobacteria could have played an important role in the positive results of unabsorbed ELISA-A, according to previous reports (Osterstock et al. 2007). In our study, AM were isolated by fecal culture of feces of ELISA-A-positive herds. Therefore, some of our ELISA-A-positive results could be false positive, especially in those animals in which ELISA-B (absorbed test) and PCR produced negative results. Likewise, it is possible that some of the positive ELISA results have been produced due to the interference with tuberculin from intradermal test applied occasionally to some animals, in order to declare herds as free from bovine tuberculosis (Varges et al. 2009).

Our PCR results confirmed the results of Zapata et al. (2010) who found three out of 15 positive fecal samples from the herd number 1 of our study by IS*900*–real-time PCR. Nevertheless, our results suggest that some asymptomatic animals of ELISA-A-positive herds were probably light shedders, which could not be easily detected by the PCRs used and probably by any other currently PCR system available, even in herds with previous history of paratuberculosis (Diéguez et al. 2009). In contrast, some studies have found a higher sensitivity by IS*900* PCR when this method is applied to fecal samples from clinically suspected cases of Johne's disease (Soumya et al. 2009).

Low concordance between ELISA and PCR results seem to be explained by the fact that different target regions with different characteristics have been chosen to develop the PCR systems used. The molecular target IS*900* has been implicated since more than a decade in producing false positive results in MAP diagnosis (Cousins et al. 1999). Moreover, the IS*Mav2* marker and the nested-PCR procedure have been implicated in producing false-positive results and disturbance due to contamination compared to other markers and to single-round PCRs (Möbius et al. 2008). However, the PCR systems used in our study are very reliable. F57–IS*MaV2*–real-time PCR has been strength tested for specificity and includes an internal amplification control, which makes the system very improbable to produced false-positive results. IS*900*-nested PCR is carried out using multiple reaction controls, which avoid the misinterpretations of results due to disturbed contamination (Schönenbrücher et al. 2008).

Based on the lack of information of which MAP strains could be found in Colombian fecal samples, we used three different culture media and two different decontamination procedures in order to increase sensitivity by meeting growth requirements of diverse MAP strains. However, no isolation of MAP was obtained even in herds with history of paratuberculosis. A possible explanation of this is that the sampling plan did not consider the collection of fecal samples of all adult cattle in every herd or a serial testing, which could have lead to some MAP shedders not to be included in the sample, while non-infectious animals could have been randomly selected. It is also possible that conservation of our serum and fecal samples at −20°C for several weeks or months could have affected our ELISA-A and the culture results, as previously reported (Alinovi et al. 2009; Khare et al. 2008).

On the other hand, the isolation of AM and the higher proportion of contaminated slants obtained with a NaOH–oxalic acid solution and cultivation on LJ agrees with

previous studies (Glanemann et al. 2004; Nielsen et al. 2004). Characteristics of *M. engbaekii* on LJ slants inoculated with fecal samples decontaminated with NaOH and oxalic acid agree with the description of mycobacteria with pink-colored colonies isolated from cattle feces, as previously described (Korsak and Boisvert 1972). This mycobacterium has been also isolated from water samples collected from the drinking troughs of buffaloes in Africa (Michel et al. 2007). Acid-fast Mycobacteria testing negative for MAP–PCR and sharing the distinct phenotypical characteristics of *M. engbaekii* were not further tested for similarity-based species identification and were assumed to be an isolate of this mycobacteria.

Finally, the low concordance between ELISA and fecal culture results has been also reported before (Muskens et al. 2003; Glanemann et al. 2004; Dreier et al. 2006) and could be explained in 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 (Sweeney et al. 2006).

Conclusion

The results confirm the presence of MAP in dairy herds in Colombia, and the limitations of serum ELISA, fecal PCR, and fecal culture for the detection of this microorganism in asymptomatic dairy cattle from herds with and without history of Johne's disease. Further microbiological and epidemiological studies have to be carried out in Colombia in order to increase the knowledge about bovine paratuberculosis.

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