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Short communication

Development and validation of a triplex real-time PCR for rapid detection and specific identification of *M. avium* sub sp. *paratuberculosis* in faecal samples

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ABSTRACT

A triplex real-time (TRT-PCR) assay was developed to ensure a rapid and reliable detection of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) in faecal samples and to allow routine detection of *Map* in farmed livestock and wildlife species. The TRT-PCR assay was designed using IS900, ISMAP02 and f57 molecular targets. Specificity of TRT-PCR was first confirmed on a panel of control mycobacterial *Map* and non-*Map* strains and on faecal samples from *Map*-negative cows ($n = 35$) and from *Map*-positive cows ($n = 20$). The TRT-PCR assay was compared to direct examination after Ziehl-Neelsen (ZN) staining and to culture on 197 faecal samples collected serially from five calves experimentally exposed to *Map* over a 3-year period during the sub-clinical phase of the disease. The data showed a good agreement between culture and TRT-PCR (κ score = 0.63), with the TRT-PCR limit of detection of 2.5×10^2 microorganisms/g of faeces spiked with *Map*. ZN agreement with TRT-PCR was not good (κ = 0.02). Sequence analysis of IS900 amplicons from three single IS900 positive samples confirmed the true *Map* positivity of the samples. Highly specific IS900 amplification suggests therefore that each single IS900 positive sample from experimentally exposed animals was a true *Map*-positive specimen. In this controlled experimental setting, the TRT-PCT was rapid, specific and displayed a very high sensitivity for *Map* detection in faecal samples compared to conventional methods.

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1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the etiological agent of paratuberculosis or Johne's disease (JD), a chronic debilitating enteritis with worldwide

distribution in ruminants and a significant impact on the world economy (Sweeney, 1996; Chacon et al., 2004). Clinical signs of the disease are mostly absent until 2 or more years after the initial infection, which usually occurs shortly after birth. Despite controversies, the assumed implication of *Map* as one of the causative agents of Crohn's disease in humans (Chacon et al., 2004) further imposes consideration of precautionary control measures in livestock and food sectors.

Although crucial, diagnosis of JD remains difficult. Definitive diagnosis is often made post-mortem on

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relevant biological samples (Godfroid et al., 2005). Specific and sensitive diagnostic methods allowing rapid ante-mortem detection of *Map* are critically needed. The detection of *Map* in faecal samples is among the few direct methods that can be implemented both at the sub-clinical and clinical stages of disease. Accordingly, direct detection of acid-fast rod bacilli, and culture isolation, currently remain the most common methods for detecting *Map* in faecal samples, the latter being considered as gold standard for the identification of *Map* in cattle (Collins et al., 2005). However, faecal culture is slow and poorly sensitive (Bogli-Stubber et al., 2005; Harris and Barletta, 2001) whereas direct examination after Ziehl-Neelsen (ZN) staining lacks both specificity and sensitivity (Salem et al., 2005). Such limitations hamper a rapid identification of *Map* in faeces, delay management decisions to cull infected animals, and allow the pathogen to circulate in herds. Alternatives are given by immunological diagnostic tests. However, their specificity is compromised by close antigenic similarity between *Map* and other mycobacteria (Collins, 1996; Manning and Collins, 2001). PCR methods have made the rapid identification of *Map* in clinical samples possible, by reducing detection time (Englund et al., 1999; Fang et al., 2002; Ikonomopoulos et al., 2004; Motiwala et al., 2003, 2004; Rajeev et al., 2005). In most studies, the main DNA target has been the insertion sequence IS900, initially considered to be a *Map*-specific marker (Green et al., 1989). However, recent reports have challenged the specificity of IS900-based assays due to the discovery of IS900-like sequences in non-*Map* mycobacteria (Englund et al., 2002; Cousins et al., 1999; Godfroid et al., 2005; Motiwala et al., 2004; Tasara et al., 2005). In order to improve PCR identification of *Map*, a triplex real-time PCR (TRT-PCR) co-amplifying in the same tube the multi-copy IS900, the single-copy *Map*-restricted f57 target (Coetsier et al., 2000; Godfroid et al., 2005; Poupart et al., 1993) and the new multi-copy *Map*-restricted ISMAP02 genetic target (Paustian et al., 2004) was designed. TRT-PCR results were compared on the same faecal samples with methods routinely used for *Map* identification (i.e. microscopic examination of acid-fast rods after ZN staining and culture).

2. Materials and methods

2.1. Faeces from calves exposed to *Map*

In December 2000, five animals (2–3-week-old calves) were experimentally exposed to *Map* by the oral route with 10 mg (10^8 CFU) of living *Map* (ATCC 19698) as previously described (Rosseels et al., 2006a). All experiments were approved by the Veterinary and Agrochemical Research Center (VAR) Ethics Committee. Animals were maintained at VAR experimental facilities at Machelen, in accordance with an approved animal care and use protocol. The serological-induced responses and the bacterial excretion in faeces were both monitored during 120 weeks (every week during the first 6 months of the experiment, every 2 weeks after) and *Map*-specific T-cell immune responses were also analysed during this period (Rosseels et al., 2006a). Between December 2000 and October 2003, 197

faecal samples from these calves were collected and shipped to laboratory for detection and identification of *Map*. Each sample from the *Map* exposed calves ($n = 197$) was sonicated during 2 min at full power (100 W) using a W-380 sonicator (Heat Systems, Farmingdale, NY) fitted with a cup horn for ice water cooling (Schleig et al., 2005). The sample was subsequently divided into three aliquots, and subjected to microscopic examination after ZN staining, culture, and TRT-PCR. As positive and negative controls for TRT-PCR specificity, additional faecal samples ($n = 35$) were also obtained from seven calves (3–6-month-old) born from *Map* culture-negative cows without any history of paratuberculosis and 20 *Map* culture-positive faecal samples from *Map* infected cows.

2.2. Microscopic direct examination

Faecal smears were fixed on glass slides in an oven at 65 °C, stained by the ZN method and examined by light microscopy as previously described (Murray et al., 1999).

2.3. Culture of *Map*

Faecal samples (1 g) were decontaminated in hexadecylpyridium 0.75% overnight and were centrifuged at $3500 \times g$ for 20 min. Pellets were resuspended in 0.85% saline and incubated onto four mycobactin J-supplemented HEYM slants (Biomérieux, France). Cultures slopes were incubated at 37 °C for 2–3 months.

2.4. Extraction of *Mycobacterium* DNA

Faecal samples (1 g) were subjected to DNA extraction according to a modified version of a previously described method (Garrido et al., 2000). In brief, 1 g of faeces was mixed with 20 mL of SDS 5% and allowed to sediment for 15 min. The upper aqueous phase was transferred into 15 mL tubes and washed in PBS three times. The pellet was resuspended in 2.0 mL of PBS and transferred into a 2 mL screw cap tube. The suspension was centrifuged at $9600 \times g$ and the supernatant discarded. Bacteria from the pellet were disrupted physically by boiling and freezing, and genomic DNA was extracted with a guanidine isothiocyanate and ammonium acetate solution. The aqueous phase was recovered and extracted twice with an equal volume of chloroform:octanol (24:1). DNA was precipitated with isopropanol and washed with 70% ethanol. The pellet was air-dried, and DNA was resuspended in 50 μ L water and quantified by spectroscopy (Nanodrop ND-1000 spectrophotometer, NanoDrop Technologies, Inc., Montchanin, USA).

2.5. IS900, f57 and ISMAP02 control plasmid

A plasmid harbouring the three targets was engineered by directed mutagenesis and subsequently used as a control. Briefly, *Map* DNA was first amplified in two separate PCR reactions using two specific primer pairs, to produce IS900 and f57 overlapping fragments at the 3' end. The IS900 reverse primer was indeed designed to be complementary to the f57 forward primer. Next, the pooled amplicons were

amplified using the IS900 forward and f57 reverse primers, to generate a unique recombinant IS900–f57 target (R1). In a third step, ISMAP02 amplicons were generated to overlap R1 amplicons. A final PCR reaction on pooled R1 and ISMAP02 amplicons was carried out with IS900 forward and ISMAP02 reverse primers, to produce the final IS900–f57–ISMAP02 recombinant target (R2). R2 amplicons were then cloned in a TOPO-XL vector according to the manufacturer's instructions (Invitrogen, Merelbeke, Belgium).

2.6. TRT-PCR

Primers and probes were designed from f57 sequence (accession number no. X70277) and ISMAP02 (accession number no. AF445436), using the Primer Express software (version 1.5, PE Applied Biosystems, Foster City, CA, USA). The resulting primer pair candidates and the probe were tested *in silico* using the GenBank to confirm *Map* specificity.

Primers and probes from IS900 sequence (accession no. AJ250018) were designed manually in the *Map*-specific part of IS900, after alignment with all known IS900-like sequences. IS900 primers and probes were selected in *Map*-specific parts of the IS900 (Table 1). Primers and probes were purchased from Eurogentec (Ougrée, Belgium). TRT-PCR assay was performed using 2.5 μ L of DNA solution, 12.5 μ L Universal PCR Master Mix (Applied Biosystems, Foster City, USA), 300 nM of each primer, and 100 nM of each probe, in a total reaction volume of 25 μ L. The reaction was initiated at 50 °C for 2 min, and 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each sample was tested in triplicate and data were recorded as cycle threshold (C_t) on a TaqMan 7700 Sequence Detection System (Applied Biosystems), using the analytical software from the same manufacturer. The threshold for a positive fluorescent signal was set arbitrarily at 1 C_t value < C_t of negative controls.

The TRT-PCR was first tested on *Map* reference strains ($n = 3$) and *Map* field isolates ($n = 22$) (Supplemental Table S1). Amplicons obtained from these samples were sequenced as previously described (Lecouvet et al., 2004). Sequences obtained were compared with public available sequence databases for similarity-based species identification, using the BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The specificity of the test was further investigated by performing TRT-PCR analysis on DNA samples from a panel of 48 non-*Map* mycobacteria, as well as 112 non-

mycobacteria species (Supplemental Table S1). TRT-PCR was subsequently performed on DNA extracted from 197 faecal specimens. For each negative sample, PCR inhibition was assessed as previously described (Lecouvet et al., 2004).

In order to compare culture, ZN staining and DNA-based results, a TRT-PCR assay targeting IS900, f57 and ISMAP02 genetic markers was considered unambiguously positive when giving at least two out of three positive signals. Samples with single IS900 positive signal were a priori considered as doubtful until further investigated. For this purpose, the TRT-PCR product of three samples producing a IS900 single positive TRT-PCR signal was cloned in a TOPO-XL vector according to the manufacturer's instructions (Invitrogen, Merelbeke, Belgium) and sequenced as previously described (Lecouvet et al., 2004). Sequences obtained were compared to public available sequence databases for species identification.

2.6.1. Limit of detection of TRT-PCR

- Serial dilution of control IS900–f57–ISMAP2-plasmid*: The control plasmid was serially diluted 1 in 10 to construct a standard curve.
- Serial dilution of Map in faecal material*: Luminescent *Map* (ATCC 19698 harbouring plasmid pSMT1 encoding luxAB genes from *Vibrio harveyi*) was used to spike faeces (Rosseels et al., 2006b). Briefly, *Map* was grown on 7H9 Middelbrook medium supplemented with OADC, mycobactin J and hygromycin to an O.D. between 0.6 and 0.8. Bacteria were centrifuged and resuspended in PBS. The number of colony forming units (CFU) of *Map* was checked by plating serial dilutions in PBS on Middlebrook 7H11-OADC agar supplemented with mycobactin J and hygromycin. Petri dishes were incubated at 39 °C for 8 weeks, before counting colonies visually. Stock concentration was determined to be 1.58×10^7 CFU/mL. The stock solution was sonicated as described above and serially diluted. Faecal samples were prepared by spiking serial dilutions of *Map* (10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10 and 1 CFU) in 4 g of faeces from a *Map*-free donor calf. After overnight stirring, samples were subjected to DNA extraction and subsequent TRT-PCR for *Map* detection.

2.7. Statistical analysis

Statistical analyses were performed using with the NCSS 2004 statistical package release (Kaysville, UT, USA).

Table 1

Primers and probes selected for the triplex PCR identification of *M. avium* subsp. *paratuberculosis*.

Primers	Sequence (5' → 3')	Location
IS900-for	5'-TGCTGATCGCCTTGCTCA-3'	1351–1369
IS900-rev	5'-GGGCCTGATCGGCGATGAT-3'	1488–1470
IS900-probe	5'-FAM-CCG GGC AGC GGC TGC TTT ATA TTC-3'-BHQ1	1375–1398
F57-for	5'-TTCATCGATACCCAAACTCAGAGA-3'	428–451
F57-rev	5'-GTTCCGCCCTTGAATGCT-3'	471–453
F57-probe	5'YAKIMA YELLOW 5'-TGCCAGCCGCCACTCGTG-3'-BHQ1	495–477
ISMAP02-for	5'-CGC CAG GAA CGC AAA CAT-3'	946–963
ISMAP02-rev	5'-GTG CAG GGT CGC TCT GAT G-3'	1023–1041
ISMAP02-probe	5'DragonflyOrange-ACTCCGCATCCAACAACTCAGCTG-3'-BHQ-2	946–963

Table 2

Identification of *Mycobacterium avium* subsp. *paratuberculosis* in faecal samples: comparison between ZN, culture and triplex real-time PCR.

Number of faecal samples	%	IS900	f57	ISMAP02	Final molecular result	Culture	Direct examination after ZN staining
130 ^a	51.59	–	–	–	–	–	–
14	5.56	+	–	–	–	–	–
3 ^b	1.19	+	–	–	+	–	–
43	17.06	+	–	–	–	–	+
3	1.19	+	–	+	+	–	–
3	1.19	+	–	+	+	–	+
9 ^c	3.57	+	–	+	+	+	–
7	2.78	+	+	–	+	–	–
2	0.79	+	+	–	+	–	+
8	3.17	+	+	+	+	–	–
4	1.59	+	+	+	+	–	+
19 ^c	7.54	+	+	+	+	+	–
7 ^c	2.78	+	+	+	+	+	+

Cohen's kappa correlation coefficient between TRT-PCR and culture on solid medium is 0.63 (good agreement) whereas the correlation between TRT-PCR and direct examination after ZN staining is 0.02 (no correlation at all).

^a This number includes the 35 negative control samples.

^b These samples were only IS900 positive and initially considered as negative. Confirmation of the amplification of *Map* IS900 was obtained by cloning and sequencing Taqman amplicons which displayed 100% identity with *Map* IS900.

^c This number includes the 20 positive control faecal samples (IS900/ISMAP02 positive ($n = 6$) and 14 IS900/ISMAP02/f57 positive ($n = 14$) samples).

Agreement between TRT-PCR and culture and between TRT-PCR and direct examination was assessed using the kappa statistics of Cohen.

3. Results

3.1. Assessment of *Map*-specificity of the TRT-PCR

The combined use of IS900, ISMAP02 and f57 in a TRT-PCR assay produced three concordant positive signals with each of the 25 *Map* strains tested (three reference strains and 22 field isolates). Further, TRT-PCR results remained negative (C_t 40) with all non-*Map* mycobacteria strains and all other bacteria species. Sequence analysis of each amplified target confirmed a 100% identity with the corresponding target in the *Map* genome. A series of

Map positive and -negative faecal field samples were also assessed as control of specificity. TRT-PCR results were consistently negative with *Map* culture-negative samples ($n = 35$) from calves and positive in each sample ($n = 20$) from proved *Map* culture-positive cows. Positive results included IS900/ISMAP02 positive ($n = 6$) samples or all three positive markers ($n = 14$) (Table 2).

3.2. Limit of detection: serial dilution of control IS900–f57–ISMAP2-plasmid and *Map* spiked in faecal material

The lowest reproducible level of detection in triplicate was 10 plasmid copies. At 10 copies, three exponential curves, one for each insert, were consistently obtained (mean C_t : 38.1 ± 0.47), giving a limit of detection of 10 plasmid copies per 25 μ L-reaction for the TRT-PCR assay. The

Table 3

Detection of *Mycobacterium avium* subsp. *paratuberculosis* by triplex real-time PCR in faecal samples collected from five calves from December 2000 to September 2003.

Year	2000			2001							2002							2003												
	Month	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12				
Calves	Methods																													
	6074	PCR	-	+	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	+	+	+					---	END		
	Culture	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	+	+	+					---	END			
6075	PCR	-	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-					---	END			
	Culture	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-					---	END			
7782	PCR	-	+++	-	-	+	-	-	-	-	+	+	-	-	+	-	+	+	-	-						---	END			
	Culture	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-						---	END			
7783	PCR	NA	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	+								---	END			
	Culture	NA	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	+								---	END			
7785	PCR	NA	+	+	-	-	-	+	-	-	-	-	+	-	-	+	+	+								---	NA	+	-	END
	Culture	NA	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	+								---	NA	+	-	END

Calves were 2–3 weeks old at time of infection: December 2000. Triplex test result (PCR) and the culture result obtained on a faecal sample collected at a specific day during the month and the year mentioned (+: positive result; -: negative result). Samples collected each month varied from one to maximum three. In the latter case, the samples were collected on different days over that period of time. END means the end of the study and of sample collection. NA: not available.

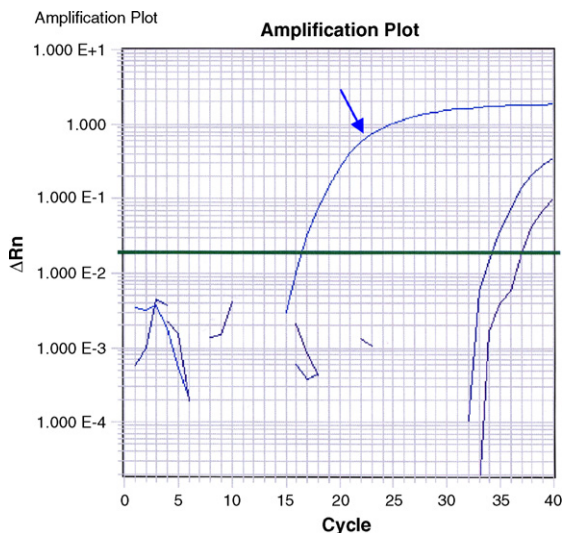


Fig. 1. TRT-PCR from a positive control (arrow) and two IS900-only TRT-PCR positive samples.

single-copy gene *f57* being the limiting factor for the sensitivity of detection of *Map* genomic DNA, the detection limit was set at 10 *Map* per PCR reaction. The dynamic range of amplification was identical for each cloned genetic target, ranging from 10^2 to 10^7 copies. Considering that DNA of *Map* is extracted from 1 g of faeces and suspended in a final volume of 50 μL from which 2.5 μL is used in the TRT-PCR assay, a limit of detection of ~ 10 *Map* gDNA corresponds theoretically, at most, to 200 *f57* positive-*Map*/g of stool. This estimation is made assuming a DNA extraction efficiency of 100%.

In our study, data from faecal samples spiked with *Map* showed a limit of detection equivalent to 12.5 CFU per PCR which corresponds to 250 *Map* cells/g of faeces. This result corresponds to a *Map* DNA extraction efficiency of 80%.

3.3. Comparison between TRT-PCR assay, culture and Ziehl-Neelsen staining on faecal samples (Tables 2 and 3)

Comparison of ZN staining, culture and TRT-PCR assay in calves experimentally exposed to *Map* is shown in Table 2. There was no agreement between TRT-PCR and ZN (kappa value: 0.02). The agreement between culture and TRT-PCR was good (kappa value: 0.63). For each of the five calves, positive and negative culture and PCR results were alternatively observed over time, the interval between two samples testing positive being extremely variable (Table 3). None of the negative control samples were positive for any of the three molecular targets.

Amplification of only IS900 was found in 60 of 197 of samples (30.5%), all culture-negative, and 43 of those (71.7%) were ZN-positive (Fig. 1). This is in agreement with other recently reported data (Meadeus et al., 2008). Of note, PCR fragments from three single IS900 positive samples were further cloned, sequenced and proved 100% identical to IS900.

4. Discussion

The control of JD in farmed livestock and in other animal herds is a growing challenge. Control programs are hampered by the lack of sensitive and specific tests able to detect early *Map* infection. Although PCR-based screening methods performed on (pooled) faecal samples are nowadays offered in some countries, a combination of direct examination of ZN-stained smears, serological methods and culture is still mainly used routinely by veterinary diagnostic laboratories for identifying infected farms (Harris and Barletta, 2001). The rationale for using DNA-based *Map*-specific assays on faecal material stems from the expected higher sensitivity and faster performance compared to the culture (Bogli-Stubler et al., 2005; Fang et al., 2002; Huntley et al., 2005; Tasara et al., 2005; Tripathi et al., 2006). Using this experimental *Map* infection model allowed us to monitor *Map* excretion over almost 3 years time in the same *Map* exposed animals and to compare the performance of conventional methods with our DNA-based detection assay on serial faecal samples.

Previous reports have shown that direct examination of faecal smears after ZN staining is not a reliable tool (Salem et al., 2005). This study confirms the lack of sensitivity of the ZN staining. Moreover, when comparing ZN results to culture and molecular method, data obtained with this model suggest that ZN staining in *Map* infected animals may not be an acceptable surrogate of *Map* faecal shedding as 43 of the 53 (81%) ZN-positive results were associated with both a negative culture and a negative *Map*-specific molecular TRT-PCR. Preliminary investigations on these samples show that they contain *hsp65* DNA sequences highly conserved among mycobacterial species but with identity to *Map* < 100%. This is compatible with the presence of non-*Map* mycobacteria in faeces. Accordingly, ZN-positive samples displaying negative results both in TRT-PCR and culture probably contained non-*Map* mycobacteria (personal communication). This finding is likely to explain the low specificity of ZN in our study.

While culture is still considered by most authors as the gold standard for *Map* identification, it remains a time consuming method which requires up to 16 weeks incubation and displays a low sensitivity (Bogli-Stubler et al., 2005; Harris and Barletta, 2001; Tripathi et al., 2006). In this respect, it has been shown that 30% of moderately infected sheep with previous negative faecal cultures, disclose gross and microscopic lesions at slaughter (Whitlock et al., 2000). Accordingly, more sensitive methods as real-time PCR should be proposed as gold standards. This report highlights the main diagnostic challenges: the intermittent nature of *Map* shedding and/or the lack of sensitivity of culture. Moreover, *Map* dependency for mycobactin J should be interpreted with caution as some *Map* strains may grow without mycobactin J (Motiwala et al., 2004; Godfroid et al., 2005). Culture limitations are therefore of particular concern regarding specificity and sensitivity of *Map* detection in cattle (Sockett et al., 1992). In the absence of a rapid and sensitive assay for the detection of *Map* infected sub-clinically affected shedders, as suggested by the results of this study, highly specific PCR assay could be better used

for the detection of these animals. Regarding single IS900 PCR, many reports have highlighted the lack of specificity of IS900 for *Map* detection. Previously published IS900 PCR protocols could indeed also amplify IS900-like sequences (Kim et al., 2002; Rajeev et al., 2005; Tasara et al., 2005), which have prompted the use of PCR assays excluding IS900 (Schönenbrücher et al., 2008). Nevertheless, IS900 primers designed according to IS900-like sequences could specifically amplify *Map* (Kawaji et al., 2007). Likewise, *Map*-specific IS900 probe and primers were designed in our study according to all known IS900-like elements, giving a 100% IS900 concordant sequences in three single IS900 PCR positive samples. Consequently, while each IS900 single positive sample is likely to be true *Map*-positive, it would be time consuming, cumbersome and expensive to confirm the *Map*-specificity of every IS900-only positive sample. ISMAP02 and/or f57 targets are therefore an important adjunct for rapidly confirming the faecal presence of *Map*. Moreover, the presence of ISMAP02 and/or f57 positive signals is indicative of a higher *Map* excretion, compared to samples with IS900-only positive signal, as recently suggested (Meaudus et al., 2008). Oppositely, the IS900-only positive calves can therefore be considered as low *Map* shedders. Considering that IS900 can still be found in soils many days after *Map* removal from the field (Cook and Britl, 2007), the epidemiological relevance of IS900-only positive shedding requires further investigations. Accordingly, a prospective study focusing on *Map* transmission in healthy animals exposed to IS900-only positive samples could help to assess the epidemiological impact of such results with respect to the risk of *Map* transmission.

In conclusion, the TRT-PCR presented in this study shows a higher sensitivity compared to culture and to direct examination. Although further validation on a higher number of samples from different herds is mandatory before routine application of the TRT-PCR, results obtained here show that this assay is expected to improve the true detection of *Map* in faecal samples of sub-clinically infected animals, and consequently, to support management of JD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetmic.2008.09.087](https://doi.org/10.1016/j.vetmic.2008.09.087).

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