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

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28

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35 work.

36

37 **ABSTRACT**

38

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

56 **INTRODUCTION**

57 *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the etiological agent of
58 paratuberculosis or Johne's disease (JD), a chronic debilitating enteritis with worldwide
59 distribution in ruminants and a significant impact on the world economy (Sweeney, 1996,
60 Chacon et al., 2004). Clinical signs of the disease are mostly absent until two or more years
61 after the initial infection, which usually occurs shortly after birth. Despite controversies, the
62 assumed implication of *Map* as one of the causative agents of Crohn's disease in humans
63 (Chacon et al., 2004) further imposes consideration of precautionary control measures in
64 livestock and food sectors.

65 Although crucial, diagnosis of JD remains difficult. Definitive diagnosis is often made
66 post-mortem on relevant biological samples (Godfroid et al., 2005). Specific and sensitive
67 diagnostic methods allowing rapid ante-mortem detection of *Map* are critically needed. The
68 detection of *Map* in faecal samples is among the few direct methods that can be implemented
69 both at the sub-clinical and clinical stages of disease. Accordingly, direct detection of acid-
70 fast rod bacilli, and culture isolation, currently remain the most common methods for
71 detecting *Map* in faecal samples, the latter being considered as gold standard for the
72 identification of *Map* in cattle (Collins et al., 2005). However, faecal culture is slow and
73 poorly sensitive (Bogli-Stuber et al., 2005; Harris and Barletta, 2001) whereas direct
74 examination after Ziehl-Neelsen (ZN) staining lacks both specificity and sensitivity (Salem et
75 al., 2005). Such limitations hamper a rapid identification of *Map* in faeces, delay management
76 decisions to cull infected animals, and allow the pathogen to circulate in herds. Alternatives
77 are given by immunological diagnostic tests. However, their specificity is compromised by
78 close antigenic similarity between *Map* and other mycobacteria (Collins, 1996; Manning and
79 Collins, 2001). PCR methods have made the rapid identification of *Map* in clinical samples
80 possible, by reducing detection time (Englund et al., 1999; Fang et al., 2002; Ikonopoulou
81 et al., 2004; Motiwala et al., 2003; Motiwala et al., 2004; Rajeev et al., 2005). In most
82 studies, the main DNA target has been the insertion sequence IS900, initially considered to be
83 a *Map*-specific marker (Green et al., 1989). However, recent reports have challenged the
84 specificity of IS900-based assays due to the discovery of IS900-like sequences in non-*Map*
85 mycobacteria (Englund et al., 2002, Cousins et al., 1999; Godfroid et al., 2005; Motiwala et
86 al., 2004, Tasara et al., 2005). In order to improve PCR identification of *Map*, a triplex real-
87 time PCR (TRT-PCR) co-amplifying in the same tube the multi-copy IS900, the single-copy
88 *Map*-restricted f57 target (Coetsier et al., 2000; Godfroid et al., 2005; Poupart et al., 1993)
89 and the new multicopy *Map*-restricted ISMAP02 genetic target (Paustian et al., 2004) was

90 designed. TRT-PCR results were compared on the same faecal samples with methods
91 routinely used for *Map* identification (*i.e.* microscopic examination of acid-fast rods after ZN
92 staining and culture)

93

94 **MATERIALS AND METHODS**

95

96 **Faeces from calves exposed to *Map*.** In December 2000, 5 animals (2- to 3-week-old calves)
97 were experimentally exposed to *Map* by the oral route with 10 mg (10^8 CFU) of living *Map*
98 (ATCC 19698) as previously described (Rosseels et al., 2006a). All experiments were
99 approved by the Veterinary and Agrochemical Research Center (VAR) Ethics Committee.
100 Animals were maintained at VAR experimental facilities at Machelen, in accordance with an
101 approved animal care and use protocol. The serological induced responses and the bacterial
102 excretion in faeces were both monitored during 120 weeks (every week during the first 6
103 months of the experiment, every two weeks after) and *Map* specific T-cell immune responses
104 were also analysed during this period (Rosseels et al., 2006a). Between December 2000 and
105 October 2003, 197 faecal samples from these calves were collected and shipped to laboratory
106 for detection and identification of *Map*. Each sample from the *Map* exposed calves (n= 197)
107 was sonicated during 2 minutes at full power (100 W) using a W-380 sonicator (Heat
108 Systems, Farmingdale, NY) fitted with a cup horn for ice water cooling. The sample was
109 subsequently divided into three aliquots, and subjected to microscopic examination after ZN
110 staining, culture, and TRT-PCR. As positive and negative controls for TRT-PCR specificity,
111 additional faecal samples (n=35) were also obtained from 7 calves (3- to 6 month-old) born
112 from *Map* culture-negative cows without any history of paratuberculosis and 20 *Map* culture
113 positive faecal samples from *Map* infected cows.

114

115 **Microscopic direct examination.** Faecal smears were fixed on glass slides in an oven at
116 65°C, stained by the ZN method and examined by light microscopy as previously described
117 (Murray PR et al., 1999).

118

119 **Culture of *Map*.** Faecal samples (1g) were decontaminated in hexadecylpyridium 0.75%
120 overnight and were centrifuged at 3500 g for 20 minutes. Pellets were resuspended in 0.85%
121 saline and incubated onto four mycobactin J-supplemented HEYM slants (Biomérieux,
122 France). Cultures slopes were incubated at 37°C for 2-3 months

123

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

136

137 **IS900, f57 and ISMAP02 control plasmid.** A plasmid harbouring the three targets was
138 engineered by directed mutagenesis and subsequently used as a control. Briefly, *Map* DNA
139 was first amplified in two separate PCR reactions using two specific primer pairs, to produce
140 IS900 and f57 overlapping fragments at the 3'end. The IS900 reverse primer was indeed
141 designed to be complementary to the f57 forward primer. Next, the pooled amplicons were
142 amplified using the IS900 forward and f57 reverse primers, to generate a unique recombinant
143 IS900-f57 target (R1). In a third step, ISMAP02 amplicons were generated to overlap R1
144 amplicons. A final PCR reaction on pooled R1 and ISMAP02 amplicons was carried out with
145 IS900 forward and ISMAP02 reverse primers, to produce the final IS900-f57-ISMAP02
146 recombinant target (R2). R2 amplicons were then cloned in a TOPO-XL vector according to
147 the manufacturer's instructions (Invitrogen, Merelbeke, Belgium).

148

149 **TRT-PCR.** Primers and probes were designed from f57 sequence (accession number n°
150 X70277) and ISMAP02 (accession number n° AF445436), using the Primer Express software
151 (version 1.5, PE Applied Biosystems, Foster City, CA, USA). The resulting primer pair
152 candidates and the probe were tested *in silico* using the GenBank to confirm *Map* specificity.
153 Primers and probes from IS900 sequence (accession n° AJ250018) were designed manually in
154 the *Map*-specific part of IS900, after alignment with all known IS900-like sequences. IS900
155 primers and probes were selected in *Map*-specific parts of the IS900 (Table 1). Primers and
156 probes were purchased from Eurogentec (Ougrée, Belgium). TRT-PCR assay was performed
157 using 2.5 μ L of DNA solution, 12.5 μ L Universal PCR Master Mix (Applied Biosystems,

158 Foster City, USA), 300 nM of each primer, and 100 nM of each probe, in a total reaction
159 volume of 25 μ L. The reaction was initiated at 50°C for 2 min, and 95°C for 10 min followed
160 by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Each
161 sample was tested in triplicate and data were recorded as Cycle threshold (Ct) on a TaqMan
162 7700 Sequence Detection System (Applied Biosystems), using the analytical software from
163 the same manufacturer. The threshold for a positive fluorescent signal was set arbitrarily at 1
164 Ct value < Ct of negative controls.

165 The TRT-PCR was first tested on *Map* reference strains (n = 3) and *Map* field isolates (n =
166 22) (Supplemental Table S1). Amplicons obtained from these samples were sequenced as
167 previously described (Lecouvet et al., 2004). Sequences obtained were compared with public
168 available sequence databases for similarity-based species identification, using the BLAST
169 (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The specificity of the test was further
170 investigated by performing TRT-PCR analysis on DNA samples from a panel of 48 non-*Map*
171 mycobacteria, as well as 112 non-mycobacteria species (supplemental Table S1). TRT-PCR
172 was subsequently performed on DNA extracted from 197 faecal specimens. For each negative
173 sample, PCR inhibition was assessed as previously described (Lecouvet et al., 2004).

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

183

184 **Limit of detection of TRT-PCR.**

185

186 (a) **Serial dilution of control IS900-*f57*-ISMAP2-plasmid.** The control plasmid was
187 serially diluted 1 in 10 to construct a standard curve.

188

189 (b) **Serial dilution of *Map* in faecal material : Luminescent *Map* (ATCC 19698**
190 **harbouring plasmid pSMT1 encoding luxAB genes from *Vibrio harveyi*)** was used to spike
191 faeces (Rosseels et al., 2006b). Briefly, *Map* was grown on 7H9 Middelbrook medium

192 supplemented with OADC, mycobactin J and hygromycin to an O.D between 0.6 and 0.8.
193 Bacteria were centrifuged and resuspended in PBS. The number of colony forming units
194 (CFU) of *Map* was checked by plating serial dilutions in PBS on Middlebrook 7H11-OADC
195 agar supplemented with mycobactin J and hygromycin. Petri dishes were incubated at 39°C
196 for 8 weeks, before counting colonies visually. Stock concentration was determined to be
197 1.58×10^7 CFU/ml. The stock solution was sonicated as described above and serially diluted.
198 Faecal samples were prepared by spiking serial dilutions of *Map* (10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10
199 and 1 CFU) in 4g of faeces from a *Map*-free donor calf. After overnight stirring, samples were
200 subjected to DNA extraction and subsequent TRT-PCR for *Map* detection.

201

202 **Statistical analysis**

203

204 Statistical analyses were performed using with the NCSS 2004 statistical package release
205 (Kaysville, UT, USA). Agreement between TRT-PCR and culture and between TRT-PCR and
206 direct examination was assessed using the Kappa statistics of Cohen.

207

208 **RESULTS**

209

210 **(i) Assessment of *Map*-specificity of the TRT-PCR**

211

212 The combined use of IS900, ISMAP02 and f57 in a TRT-PCR assay produced three
213 concordant positive signals with each of the 25 *Map* strains tested (3 reference strains and 22
214 field isolates). Further, TRT-PCR results remained negative (Ct 40) with all non-*Map*
215 mycobacteria strains and all other bacteria species. Sequence analysis of each amplified target
216 confirmed a 100% identity with the corresponding target in the *Map* genome. A series of *Map*
217 positive and –negative faecal field samples were also assessed as control of specificity. TRT-
218 PCR results were consistently negative with *Map* culture-negative samples (n=35) from
219 calves and positive in each sample (n=20) from proved *Map* culture-positive cows. Positive
220 results included IS900 / ISMAP02 positive (n=6) samples or all three positive markers (n=
221 14) (Table 2).

222

223 **(ii) Limit of detection : serial dilution of control IS900-f57-ISMAP2-plasmid and** 224 ***Map* spiked in faecal material**

225

226 The lowest reproducible level of detection in triplicate was 10 plasmid copies. At 10
227 copies, three exponential curves, one for each insert, were consistently obtained (mean Ct:
228 38.1 ± 0.47), giving a limit of detection of 10 plasmid copies per 25 μL -reaction for the TRT-
229 PCR assay. The single copy gene *f57* being the limiting factor for the sensitivity of detection
230 of *Map* genomic DNA, the detection limit was set at 10 *Map* per PCR reaction. The dynamic
231 range of amplification was identical for each cloned genetic target, ranging from 10^2 to 10^7
232 copies. Considering that DNA of *Map* is extracted from 1 g of faeces and suspended in a final
233 volume of 50 μL from which 2.5 μL is used in the TRT-PCR assay, a limit of detection of
234 ~ 10 *Map* gDNA corresponds theoretically, at most, to 200 *f57* positive-*Map*/g of stool. This
235 estimation is made assuming a DNA extraction efficiency of 100%.
236 In our study, data from faecal samples spiked with *Map* showed a limit of detection
237 equivalent to 12.5 CFU per PCR which corresponds to 250 *Map* cells / g of faeces. This result
238 corresponds to a *Map* DNA extraction efficiency of 80%.

239

240 **(iii) Comparison between TRT-PCR assay, culture and Ziehl-Neelsen staining on**
241 **faecal samples (Table 2 and 3)**

242

243 Comparison of ZN staining, culture and TRT-PCR assay in calves experimentally
244 exposed to *Map* is shown in Table 2. There was no agreement between TRT-PCR and ZN
245 (kappa value: 0.02). The agreement between culture and TRT-PCR was good (kappa value:
246 0.63). For each of the five calves, positive and negative culture and PCR results were
247 alternatively observed over time, the interval between two samples testing positive being
248 extremely variable (Table 3). None of the negative control samples were positive for any of
249 the 3 molecular targets.

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

254

255 **DISCUSSION**

256

257 The control of JD in farmed livestock and in other animal herds is a growing
258 challenge. Control programs are hampered by the lack of sensitive and specific tests able to
259 detect early *Map* infection. Although PCR-based screening methods performed on (pooled)

260 faecal samples are nowadays offered in some countries, a combination of direct examination
261 of ZN-stained smears, serological methods and culture is still mainly used routinely by
262 veterinary diagnostic laboratories for identifying infected farms (Harris and Barletta, 2001).
263 The rationale for using DNA-based *Map*-specific assays on faecal material stems from the
264 expected higher sensitivity and faster performance compared to the culture (Bogli-Stuber et
265 al., 2005; Fang et al., 2002; Huntley et al., 2005; Tasara et al., 2005; Tripathi et al., 2006).
266 Using this experimental *Map* infection model allowed us to monitor *Map* excretion over
267 almost three years time in the same *Map* exposed animals and to compare the performance of
268 conventional methods with our DNA-based detection assay on serial faecal samples.

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

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

294 specific PCR assay could be better used for the detection of these animals. Regarding single
295 IS900 PCR, many reports have highlighted the lack of specificity of IS900 for *Map* detection.
296 Previously published IS900 PCR protocols could indeed also amplify IS900-like sequences
297 (Kim et al., 2002; Rajeev et al., 2005; Tasara et al., 2005), which has prompted the use of
298 PCR assays excluding IS900 (Schönenbrücher et al., 2008). Nevertheless, IS900 primers
299 designed according to IS900-like sequences could specifically amplify *Map* (Kawaji et al.,
300 2007). Likewise, *Map*-specific IS900 probe and primers were designed in our study according
301 to all known IS900-like elements, giving a 100% IS900 concordant sequences in three single
302 IS900 PCR positive samples. Consequently, while each IS900 single positive sample is likely
303 to be true *Map*-positive, it would be time consuming, cumbersome and expensive to confirm
304 the *Map*-specificity of every IS900-only positive sample. ISMAP02 and/or f57 targets are
305 therefore an important adjunct for rapidly confirming the faecal presence of *Map*. Moreover,
306 the presence of ISMAP02 and/or f57 positive signals is indicative of a higher *Map* excretion,
307 compared to samples with IS900-only positive signal, as recently suggested (Meadus et al.,
308 2008). Opposingly, the IS900-only positive calves can therefore be considered as low *Map*
309 shedders. Considering that IS900 can still be found in soils many days after *Map* removal
310 from the field (Cook and Brilt, 2007), the epidemiological relevance of IS900-only positive
311 shedding requires further investigations. Accordingly, a prospective study focusing on *Map*
312 transmission in healthy animals exposed to IS900-only positive samples could help to assess
313 the epidemiological impact of such results with respect to the risk of *Map* transmission.

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

320

321

322

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

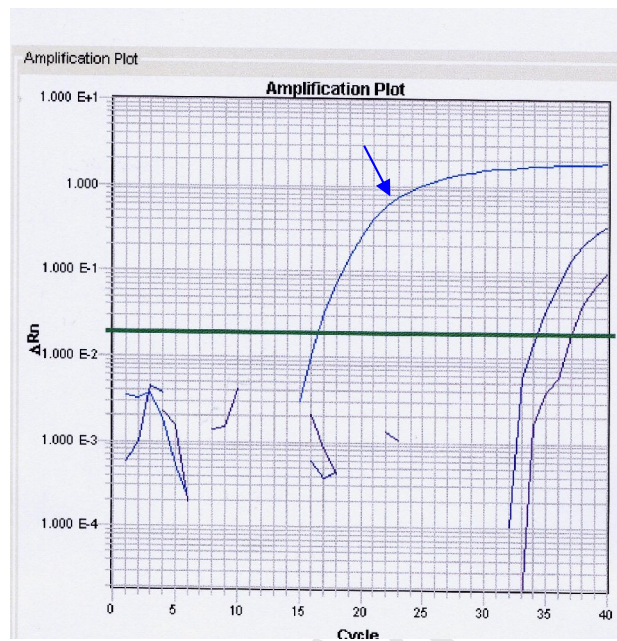


Table 1. Primers and probes selected for the triplex PCR identification of *M. avium* subsp. *paratuberculosis*.

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-Reverse	5'-GTTCGCCGCTTGAATGGT-3'	471-453
F57-probe	5'YAKIMA YELLOW 5'- TGCCAGCCGCCCACTCGTG -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-ACTCCGCATCCAACA ACTCACGCTG-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	+	+	+	+	+	+

^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).

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).

