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1 ***Mycobacterium porcinum* strains isolated from bovine bulk milk: implications for**
2 ***Mycobacterium avium* subsp. *paratuberculosis* detection by PCR and culture**

3
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20 **Abstract**

21

22 In this study, the isolation of 52 mycobactin-independent fast growing mycobacteria from 631
23 bulk milk samples (8.2%), is reported. These strains, isolated during a bulk milk survey for
24 *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), strongly affected *Map* detection both by
25 PCR and by culture, as they gave a positive IS900 PCR signal and resulted to totally inhibit the
26 growth of *Map* when spotted on HEYM slants already inoculated with 200 µl of 10 fold dilutions
27 containing from 5×10^1 to 5×10^3 *Map* cells/ml. 16S rRNA gene sequencing, using the MicroSeq
28 500 16S rDNA Bacterial Sequencing Kit (Applied Biosystems), was performed on a subset of 6
29 strains, identifying *Mycobacterium porcinum* with 100% homology in all 6 cases. The 52 strains
30 were characterized by PCR-restriction fragment length polymorphism (RFLP) analysis of the
31 *hsp65* gene, which confirmed the identification of *M. porcinum* for all the isolates. Using specific
32 primers designed on the *Map* IS900 sequence and on the *M. porcinum* sequence determined in
33 this study, a 1385 bp sequence from the *M. porcinum* genome was characterized. This IS900-
34 like sequence showed 82% homology with *Map* IS900. From our findings the following results
35 emerged: a) any culture showing one or more *M. porcinum* colonies represents a potential
36 “false negative” result and should therefore be considered as contaminated; b) IS900-like
37 elements could be more widespread than was previously thought; c) IS900 PCR positive results
38 should be interpreted cautiously, as confirmed by the evidence that the primer pair used in this
39 study resulted not to be specific.

40

41 **Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*, diagnosis, PCR, IS900,
42 *Mycobacterium porcinum*.

43 **Introduction**

44

45 *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the etiologic agent of paratuberculosis,
46 commonly known as Johne's Disease (JD), a chronic, progressive, granulomatous enteritis of
47 ruminants.

48 At present, the best diagnostic method is the isolation of the organism by culture. However, *Map*
49 detection by conventional culturing procedure requires a long incubation time (up to 16 weeks)
50 and the isolation is often hampered by the overgrowth of faster growing bacteria, moulds or
51 fungi, which can survive after decontamination treatments. The in vitro inhibition of *Map* by
52 contaminants growing on culture media, has been previously reported for acid-fast bacilli
53 (Richards, 1988).

54 *Map* detection by PCR represents a rapid, alternative diagnostic tool. This technique has been
55 routinely used both to directly determine the presence of *Map* from different sources (faeces,
56 milk, tissues, semen), and to confirm the identification of isolates obtained by cultural methods.

57 Since its identification in the *Map* genome (Collins et al., 1989; Green et al., 1989), the IS900
58 insertion element has been largely used as a diagnostic PCR target. IS900 is a 1451 bp
59 segment belonging to the IS110 family, whose main characteristic is that all family members
60 have no or very small inverted terminal repeats and do not generate direct repeats in target
61 DNA.

62 IS900 provides an ideal diagnostic PCR-target since it has been considered for a long time
63 highly specific for *Map*, where it is present in multiple copies (14 to 20), thereby making IS900
64 PCR a very sensitive detection assay. Most of the IS900 PCR primers are located within the first
65 450 base pairs of IS900 sequence since the greatest variability among insertion sequences
66 belonging to IS110 family is observed at 5' end of the nucleotide sequence. Among these
67 primers, the primer pair p90/p91 (Millar et al., 1995) and 150/921 (Vary et al., 1990) have been
68 widely used for *Map* detection or characterization of isolates in routine diagnosis. Nevertheless,
69 the specificity of IS900 diagnostic PCR has been questioned since some reports, mostly using
70 these established PCR systems, indicated the presence of sequences highly homologous to
71 IS900, in mycobacterial species other than *Map* (Cousin et al., 1999; Naser et al., 1999;
72 Motiwala et al., 2004).

73 Cousins et al. (1999), reporting the amplification of IS900-like elements from environmental
74 mycobacteria closely related to *M. scrofulaceum*, demonstrated for the first time that false
75 positive results could be obtained from strains other than *Map* and thus recommended the
76 confirmation of the internal sequence of the IS900 amplified product by restriction endonuclease
77 analysis. Following this, Naser et al. (1999) found an IS900-like element in *M. avium* subsp.
78 *avium* strains isolated from HIV infected patients, while Englund et al. (2002) isolated from
79 bovine faeces a mycobacterium named *Mycobacterium* sp. 2333, related to *M. cookii*,
80 harbouring one copy of a sequence with 94% identity to IS900 at the nucleic acid level. In this
81 case, restriction endonuclease analysis did not solve the problem since the restriction sites
82 were identical to the restriction sites in amplified DNA from *Map*. Consequently, Englund et al.
83 (2002) recommended that a positive IS900 PCR should be confirmed by DNA sequencing of the
84 amplified product or by a PCR assay targeting genes other than IS900. During the last three
85 years, Motiwala et al. (2004) reported the presence of IS900-like elements in six isolates from
86 wild animal faeces and tissues, and Rajeev et al. (2005) reported the isolation of one non-*Map*
87 mycobacterium strain positive by IS900 PCR, which has not been typed. Nevertheless, since
88 IS900-like elements have been described in mycobacteria very rarely encountered, the
89 detection of a portion of IS900 sequence by PCR is still routinely used to determine the
90 presence of *Map* in both field and clinical samples.

91 In this study, the isolation and characterization of IS900 PCR positive fast growing mycobacteria,
92 which inhibited the growth of *Map* in vitro, is described. The genotypic features of these isolates
93 were investigated and sequencing of the IS900-like element was performed.

94

95 **Materials and Methods**

96

97 **Bacterial isolates and culture method**

98 52 mycobacteria strains other than *Map* were isolated from 631 bovine bulk milk samples
99 (8.2%) collected from 631 dairy herds in Northern Italy, during a *Map* survey carried out in
100 2004/2005.

101 Milk samples were subjected to the following culture procedure (Dundee et al., 2001):

102 a 50-ml aliquot of each sample was centrifuged (15 min at 2500 g), and the pellet resuspended
103 in 25 ml 0.75% (w/v) hexadecylpyridinium chloride (HPC). Following incubation at room
104 temperature (21°C) for 5 hours and a further centrifugation (15 min at 2500 g), the pellet was
105 resuspended in 0.8 ml of PBS-Tween20 (0.05%). Four slopes of Herrold's egg yolk medium
106 (HEYM) containing 2 µg of mycobactin J/ml, two supplemented with Chloramphenicol (30 µg/l)
107 and two with Nalidixic acid (50 µg/l) and Vancomycin (50 µg/l), were each inoculated with 200 µl
108 of the resuspended pellet. The media were incubated at 37°C and weekly examined for up to 16
109 weeks. When the presence of fast growing mycobacteria was observed in the first weeks of
110 incubation, media were reincubated and, at the end of the incubation period (16th week), IS900
111 PCR on pools of colonies collected from each tube was carried out, in order to detect the
112 possible presence of *Map* colonies hidden by contaminants. The suspected colonies were
113 subjected to microscopic examination after Ziehl-Neelsen staining, and subcultured in HEYM
114 with and without mycobactin to confirm slow growth rate and mycobactin dependence.

115

116 **IS900 PCR**

117 The PCR test using IS900 specific primers described by Millar et al. (1995), was carried out as
118 follows:

119 DNA extraction: one colony (or a pool of different colonies from one tube) was resuspended in
120 100 µl distilled water and boiled (100°C) for 20 min.

121 DNA amplifications: the PCR mixture consisted of 1X *Taq* Buffer (Eppendorf), 1.0 mM MgCl₂,
122 200 µM of each of the four dNTPs, 0.3 µM each of oligonucleotide primers p90 and p91 (Table
123 1), and 0.5 U of *Taq* DNA polymerase (Eppendorf) and 5 µl of DNA template (25 µl total
124 volume). Amplification parameters consisted of an initial denaturation step at 96°C for 5 min,
125 followed by 30 cycles of primer annealing for 1 min at 62°C, elongation for 1 min at 72°C and
126 denaturation for 1 min at 95°C, and finally 10 min extension at 72°C in Mastercycler gradient
127 (Eppendorf). PCR products were separated by electrophoresis in 2% (w/v) agarose gel in 1X
128 TBE buffer. Gels were stained for 60 min in ethidium bromide (0.5 µg ml⁻¹), rinsed in distilled
129 water and visualised by UV light transillumination.

130

131 **IS900 RFLP**

132 IS900 amplified products consistent with the *Map* amplicon were subjected to restriction
133 endonuclease analysis with *Afl* and *Mse*I (Cousins et al.,1999). Aliquots (10 µl) of PCR
134 products were digested in a final volume of 20 µl with 2 U of one of the restriction enzymes
135 (New England Biolabs, UK) according to the supplier's instructions. The PCR product obtained
136 from *Map* ATCC19698 was included as a positive control. Digested products were analysed by
137 electrophoresis on a 1.5% (w/v) agarose gel in 1X TBE buffer. The gel was stained with
138 ethidium bromide and the size of the digested DNA fragments was compared using the
139 appropriate size marker (marker VIII, Roche, Mannheim, Germany).

140

141 **In vitro inhibition of *Map* growth**

142 The hypothesis that contaminating-mycobacteria could inhibit the growth of *Map* in vitro was
143 tested as previously described by Richards, 1988. *Map* ATCC19698 strain was grown in
144 continuously stirred Middlebrook 7H9 broth containing 10% (vol/vol) Middlebrook OADC (oleic
145 acid-dextrose-catalase; Difco, Detroit, Mich.), 0.5% (vol/vol) Tween 80 (Sigma, St. Louis, Mo.),
146 and 0.0002% (wt/vol) mycobactin J (Allied Monitor Inc., Fayette, Mo.) for 2 months. The number
147 of viable mycobacterial cells, as determined by standard plate counting on HEYM plates
148 containing 2 µg of mycobactin J/ml, resulted 5×10^6 *Map* cells/ml.

149 *Map* ATCC19698 suspension was serially diluted in 10-fold steps from 5×10^3 to 5×10 *Map*
150 cells/ml in 10 mM PBS (pH 7.2). Two hundreds microliters from each dilution were inoculated on
151 53 HEYM slopes and incubated at 37°C, in a horizontal position, for four days with loose caps.
152 After four days incubation, the contaminant mycobacteria were "spotted" on inoculated slants.
153 For each dilution, the 52 contaminant mycobacteria strains were applied to 52 slopes (target
154 slant) by touching a sterile inoculation needle to the contaminant mycobacteria colony and then
155 touching the same needle tip to the centre of the target slant. The 53rd slope was not inoculated
156 with contaminant mycobacteria (control slope). The caps were then tightened and all the slopes
157 were incubated at 37°C in upright position. The spotted (target) slopes were incubated until the
158 unspotted (control) slope showed visible colonies of *Map*. The caps were then tightened and all
159 the slopes were incubated at 37°C in upright position. The spotted (target) slopes were
160 incubated until the unspotted (control) slope showed visible colonies of *Map*.

161 The caps were then tightened and all the slopes were incubated at 37°C in upright position. The
162 spotted (target) slopes were incubated until the unspotted (control) slope showed visible
163 colonies of *Map*.

164

165 **16S rRNA gene sequencing**

166 The 500 bp informative portion of 16S rRNA gene of a subset of 6 mycobacterial isolates was
167 amplified and sequenced using a MicroSeq 500 16S rDNA Bacterial Sequencing Kit (Applied
168 Biosystems Division, Foster City, Calif.), following the manufacturers guidelines. Sequence
169 analysis was carried out using the MicroSeq database system (MicroSeq ID Microbial
170 Identification Software V1.0, Applied Biosystems Division, Foster City, Calif.) and the BLAST
171 search programs (Altschul et al., 1990).

172

173 ***hsp65* RFLP**

174 Genomic DNA from the 52 strains was subjected to amplification of the 441-bp Telenti
175 sequence of the *hsp65* gene (Telenti et al., 1993; Brunello et al., 2001) using 1 U *Taq* DNA
176 Polymerase (Eppendorf), 1X *Taq* Buffer (Eppendorf), 1.5 mM MgCl₂, 200 µM of each of the four
177 dNTPs, 0.6 µM of each primer, i.e. TB11 and TB12 (Table 1) and 5 µl of the boiled lysate as
178 DNA template (50 µl total volume). Amplification parameters consisted of an initial denaturation
179 step at 96°C for 5 min followed by 45 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and
180 a 7 min extension at 72°C.

181 PCR products were digested with *Bst*EII, *Hae*III and *Bsa*HI (New England Biolabs, UK),
182 following the manufacturer's recommendations. The DNA restriction fragments obtained from *M.*
183 *porcinum* ATCC33776 were also included as a positive control. The DNA restriction fragments
184 were resolved on a 4% (w/v) agarose gel, the size of the digested DNA fragments was
185 evaluated using an appropriate size marker (marker VIII, Roche, Mannheim, Germany).

186

187 **DNA sequencing of the IS900-like element and sequence analysis**

188 The IS900 false positive PCR amplicon, obtained with primers p90/p91 and Phusion™ High
189 Fidelity DNA Polymerase (New England Biolabs, UK) from one *M. porcinum* selected strain,
190 was purified using the MinElute Gel Extraction Kit (Qiagen, GmbH, Germany) and sequenced

191 using the ABI Prism Big Dye Terminator Cycle sequencing Ready Reaction Kit (Applied
192 Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Reactions were
193 analysed using ABI Prism 310 DNA genetic analyser (Applied Biosystems, Foster City, Calif.).
194 Attempts were made to sequence the entire IS900-like element, using additional primers
195 specific to the *Map* IS900 sequence. The primer that proved successful in covering nearly the
196 entire length of the IS900-like sequence, when used in combination with p90, was the primer
197 END (Cousins et al., 1999) (Table 1). The 1385-bp amplicon was completely sequenced using
198 two additional primers, F448 and R1261 (Table 1), designed on the basis of the *M. porcinum*
199 sequence obtained in the first round of sequencing. The IS900-like sequence of *M. porcinum*
200 has been deposited in the GenBank under accession No. EU126150.
201 The BLAST search programs (Altschul et al., 1990) were used to compare the DNA sequence
202 with database entries at the server of the National Center of Biotechnology Information. The
203 DNA sequence was aligned with the CLUSTAL_X program (Chenna et al., 2003).
204 Sequence comparisons were made in reference to the IS900 element of the sequenced strain
205 *Map* k10 (GenBank accession no. NC002944), as recommended by Semret et al. (2006).

206

207 **Duplex PCR to differentiate *Map* IS900 and *M. porcinum* IS900-like sequence**

208 A duplex PCR assay was developed on the basis of the *Map* IS900 and the *M. porcinum* IS900-
209 like element, in order to differentiate the two sequences.

210 The forward primer, DF, targets a common region of *Map* IS900 and *M. porcinum* IS900-like
211 sequence, the reverse *Map* specific primer, DR1, resulted in a 99-bp product while the reverse
212 primer specific for *M. porcinum*, DR2, amplified a 259-bp gene sequence (Table 1).

213 The PCR reaction was performed in 25 µl using 0.5 U of *Taq* DNA polymerase, 200 nM of each
214 of the three primers and 0.2 mM dNTPs. The amplification consisted of an initial denaturation
215 step at 96°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at
216 58°C for 1 min and extension at 72°C for 3 min. The PCR products were separated by
217 electrophoresis in a 2% (w/v) agarose gel.

218 Specificity of the primers was evaluated on genomic DNA extracted from bacterial cultures of 30
219 *Map* isolates, 52 *M. porcinum* strains isolated in this study and various species of mycobacteria:
220 *Map* ATCC19698, *M. porcinum* ATCC33776, *M. avium* subsp. *avium* ATCC25291, *M.*

221 *scrofulaceum* ATCC19981, *M. gordonae* ATCC14470, *M. bovis* ATCC19210, *M. fortuitum* subsp.
222 *fortuitum* ATCC6841, *M. peregrinum* CIP105382, *M. kansasii* ATCC12487, *M. smegmatis* and
223 *M. phlei* belonging to the collection of Istituto Zooprofilattico Sperimentale della Lombardia e
224 dell'Emilia Romagna.

225

226 **Results**

227

228 **Bacteriological analysis of IS900 PCR positive mycobacteria**

229 During a *Map* survey, we observed, in the first weeks of culture incubation, the presence of fast
230 growing mycobacteria in 72 out of 631 milk samples. However, in order to detect the possible
231 presence of *Map* colonies hidden by contaminants, the tubes were re-incubated and, at the end
232 of the incubation period (16 weeks), IS900 PCR on pools of colonies collected from each tube
233 was carried out: 52 out of 72 resulted positive.

234 Primary isolation slopes showed 1-50 colonies of the IS900 PCR positive strains. The colonies
235 grew both on culture medium supplemented with Chloramphenicol and on medium
236 supplemented with Nalidixic acid and Vancomycin and were both rough and smooth, all were
237 negative for early and late pigmentation. From each PCR positive tube, IS900 PCR positive
238 strains were examined for mycobactin dependence, growth rate and Ziehl-Neelsen staining.
239 They all resulted as acid-fast rod bacteria when stained by the Ziehl-Neelsen method.
240 Microscopic examination revealed a bacterial shape comparatively longer and slenderer than
241 *Map*. When sub-cultured, all the strains grew within a 5-7 day period, on HEYM, with and
242 without mycobactin J.

243 None of these characteristics matched the *Map* phenotype, indicating that the IS900 PCR
244 results were not specific.

245

246 **IS900 RFLP**

247 The restriction enzymes *A1wI* and *MseI* were used to assess the IS900 PCR products generated
248 by Millar primers, as recommended by Cousins et al. (1999). In all the 52 cases the amplified
249 products remained undigested with both enzymes (predicted size after restriction digestion:

250 *AluI*: 258, 155 bp, *MseI*: 283, 130 bp), confirming the IS900 PCR products were non-specific
251 (Fig. 1).

252

253 **In vitro inhibition of *Map* growth**

254 During this survey, *Map* was never isolated from the culture slopes on which 52 IS900 PCR
255 false positive strains had grown. This observation prompted us to evaluate if the IS900 PCR
256 false positive strains may inhibit the *Map* growth.

257 Slopes initially inoculated with *Map* ATCC19698 10 fold dilutions containing from 5×10^3 to 5×10^4
258 *Map* cells/ml, were subsequently spotted with the 52 isolates. *Map* growth was evaluated after
259 16 weeks incubation: in all target slopes containing the 52 isolates, no *Map* growth was
260 detected, while in control unspotted slopes inoculated only with *Map* ATCC19698 10 fold
261 dilutions, the expected number of *Map* colonies was observed (Table 2). This finding clearly
262 indicated that the isolates were able to cause total inhibition of *Map* growth in vitro.

263

264 **Molecular typing of isolated strains**

265 In order to identify the IS900 false positive strain, a subset of 6 selected strains underwent 16S
266 rRNA gene sequencing over the first 500 bp. The MicroSeq 500 16S ribosomal DNA (rDNA)-
267 based bacterial identification system (Applied Biosystems Division, Foster City, Calif.) was
268 applied, and sequence analysis by MicroSeq database system was able to identify
269 *Mycobacterium porcinum* with 100% homology in all six cases. Moreover, since Blast analysis
270 revealed also 100% homology with the recently described *M. neworleansense* (Schinsky et al.,
271 2004), a species belonging to *Mycobacterium fortuitum* group and not included in the MicroSeq
272 500 Database, the 52 strains were subjected to further investigation. PCR-restriction fragment
273 length polymorphism analysis (RFLP) of the *hsp65* gene by REs *BstEII*, *HaeIII* and *BsaHI* was
274 performed and compared to the restriction map described by Wallace et al. (2004), which
275 clearly differentiates *M. porcinum* from *M. neworleansense* (Table 3). In all 52 cases the
276 products obtained after restriction digestion were consistent with the *hsp65* RFLP pattern of *M.*
277 *porcinum* (Fig. 2).

278

279 **DNA sequencing of the IS900-like element and sequence analysis**

280 From one selected strain of *M. porcinum* isolated in this study, the nucleotide sequence
281 homologue to a genomic portion of *Map* IS900 was determined. The sequence spanning
282 nucleotide positions 43 to 1432 was obtained using *Map* p90 and END primers and two novel
283 primers (F448, R1261) designed on the *M. porcinum* IS900-like sequence obtained in the first
284 round of sequencing.

285 Sequence alignment with the *Map* IS900 element (GenBank accession NO AE016958.1)
286 showed an homology of 82%. In particular, within the first 450 bases, where most IS900 PCR
287 primers are positioned, the identity at the nucleic acid level was reduced to 77%, which
288 correlates with other findings that in other IS900-related sequences the greatest variability is
289 observed at the 5' end of the element. Five mismatches, detected in the p91 IS900-specific
290 primer, didn't prevent priming and amplification, while one base difference in the *A/wl* restriction
291 site and two in the *MseI* restriction site explained the absence of the IS900-like PCR product
292 digestion.

293

294 **Duplex PCR to differentiate *Map* IS900 and *M. porcinum* IS900-like sequence**

295 To simultaneously differentiate the *Map* IS900 from the *M. porcinum* IS900-like sequences, we
296 developed a duplex PCR assay. A common forward primer was combined with two reverse
297 primers, which specifically recognise either the *Map* IS900 or the *M.porcinum* IS900-like
298 sequence.

299 Results show that IS900-specific and IS900-like-specific primers amplify DNA from the two
300 sequences: when *Map* and *M. porcinum* isolates were tested, the resulted amplicons were of
301 the expected sizes, according to the published sequence and that obtained in this work, and no
302 secondary bands have been detected. No amplification was observed for *M. porcinum*
303 ATCC33776. PCR products of the predicted lengths were not observed for any of the other
304 mycobacterial strains tested (Fig 3).

305

306 **Discussion**

307

308 The isolation and characterization of the 52 mycobactin independent fast growing mycobacteria
309 strains clearly demonstrated that *Map* detection from milk, both by traditional cultural method
310 and by IS900-PCR, is inherently complicated.

311 Isolation of *Map* by culture relies on efficient decontamination of the sample. Chemical
312 decontamination causes selective suppression of rapid-growing non mycobacterial
313 contaminants, but it is less effective in suppressing mycobacteria. Consequently, the presence
314 in milk of fast growing mycobacteria resistant to decontamination, which can overgrow culture
315 media interfere with culture results. Moreover, since it has already been reported that fast
316 growing mycobacteria can inhibit *Map* growth (Richards, 1988), we tested the hypothesis of the
317 52 mycobacteria strains isolated in the study being inhibiting contaminants. The strains resulted
318 to totally inhibit the growth of *Map* when spotted on HEYM slants already inoculated with two
319 hundred microliters of 10 fold dilutions containing from 5×10^1 to 5×10^3 *Map* cells/ml. The relative
320 inefficiency of *Map* in acquiring iron may explain this growth inhibition by fast growing
321 contaminants, responsible for iron deprivation of the media.

322 Moreover, the presence of these strains may affect *Map* detection by PCR; in fact when DNA
323 obtained from these strains is amplified with the Millar primers for the 5' end of the insertion
324 sequence IS900, amplicons of the same size as PCR products from *Map* are obtained. In the
325 past, these primers gave false positive results when used both for single-round PCR and for
326 nested PCR (Cousin et al., 1999; Naser et al., 1999; Motiwala et al., 2004, Tasara et al., 2004).
327 Since false positive products which have the same size as the specific amplicon are obtained
328 also when the DNA from the 52 fast growing mycobacteria is amplified at the stringent
329 annealing temperature of 68°C (data not shown), our results confirm that primers pair p90/p91
330 should not be used as diagnostic tool for *Map* detection in routine analysis, as recently
331 recommended also by Möbius et al., 2008. However, other IS900 primer pairs providing more
332 specificity for *Map* have been recently developed and evaluated (Vansnick et al., 2004,
333 Ikononopoulos et al., 2004, Möbius et al., 2008), showing that the stringent selection of IS900-
334 specific primers ensures that IS900 remains a favourite target sequence for amplification of *Map*
335 specific loci (Möbius et al., 2008).

336 The digestion profile obtained by IS900-RFLP analysis of the false positive amplicons, was not
337 consistent with that of IS900. This data further substantiates that reported by Cousins et al.

338 (1999) that milk samples contaminated by these mycobacteria would be considered *Map*
339 positive by IS900-PCR alone, whereas subsequent analysis of the amplicons by restriction
340 enzyme digestion or amplification of alternative *Map* genomic targets, would prevent false
341 positive results.

342 All the 52 mycobacteria strains, subjected to molecular typing, were identified as
343 *Mycobacterium porcinum*. This rapidly growing mycobacterial species, described by Tsukamura
344 et al. (1993) as a causative agent of submandibular lymphadenitis in swine, was also recently
345 isolated from human clinical samples (respiratory infections, wound infections, catheter related
346 infections) (Schinsky et al., 2004; Wallace et al., 2004). Although its pathogenic role in post-
347 traumatic and post-surgical wound infections, osteomyelitis, and catheter-related infections is
348 well documented, its role in causing chronic lung infection has so far not been established
349 (Wallace et al., 2004).

350 The high rate of *M. porcinum* isolation from bovine bulk milk reported in this paper, suggests
351 that the micro-organism could be widely spread in dairy herds of Northern Italy. Indeed, *M.*
352 *porcinum* was recently isolated from tap water samples (Wallace et al., 2004), indicating that
353 this could be a source of the organism.

354 In this study, we determined that the 1385 bp fragment from *M. porcinum* shares a high degree
355 of sequence homology with the IS900 from *Map*.

356 Similarities between the nucleotide sequence of the IS900 and the sequences of other
357 organisms have been reported. In particular, IS900 is known to share sequence similarity with
358 some members of the IS110 family to which it belongs; IS901 (*M. avium* subsp. *avium*), IS902
359 (*M. avium* subsp. *sylvaticum*), IS1110 (*M. avium* subsp. *avium*), IS110 (*Streptomyces coelicolor*),
360 IS116 (*Streptomyces clavuligerus*), show 47-60% identity with IS900, while IS1626 (*M. avium*
361 subsp. *avium*) which is the insertion sequence most closely related to *Map* IS900, shows 82%
362 homology. The sequence similarity between IS900 and unnamed IS900-like sequences
363 reported by other authors, evaluated over the 232-1219 bp region, was from 77.2-82.6%
364 (Cousins et al., 1999) to 94% (Englund et al., 2002).

365 In our report, the sequence similarity between IS900 and the *M. porcinum* IS900-like sequence
366 was 82%, similar to the homology between IS900 and IS1626, the insertion sequence most
367 closely related to *Map* IS900.

368 The present paper also describes the application of a duplex PCR, which identifies the *M.*
369 *porcinum* IS900-like sequence and distinguishes it from *Map* IS900 using redesigned primers
370 for 5' regions. This assay could be successfully used to detect the presence of *Map* directly
371 from milk samples; nevertheless, even if these primers appear to be more specific than the
372 p90/p91 set, IS900-PCR positive results should prompt further investigation. No amplicon of the
373 predicted size was generated when the *M. porcinum* ATCC33776 was tested with the primers
374 designed in this study to specifically detect the IS900-like sequence: this finding indicates that
375 the IS900-like element may be present only in some isolates of *M. porcinum*. Similarly, the
376 presence of other insertion sequences belonging to IS110 family, such as IS901 and ISMpa1,
377 has been previously reported only in some isolates of *M. avium* subsp. *avium* (Pavlik et al.,
378 2000; Olsen et al., 2004).

379 In conclusion, the isolated *M. porcinum* strains strongly affected *Map* detection from milk,
380 leading to false negative results by traditional cultural method and false positive results by
381 IS900-PCR.

382 From our findings the following results emerged:

- 383 a) any culture slope supporting one or more *M. porcinum* colonies represents a potential "false
384 negative" result and should therefore be considered as contaminated;
- 385 b) IS900-like elements could be more widespread than was previously thought;
- 386 c) IS900 PCR positive results should be interpreted cautiously, as confirmed by the evidence
387 that the Millar primers used in this study resulted not to be specific. Consequently, either the
388 confirmation of IS900 PCR positive results by amplification of other *Map* specific genomic target
389 or the strict selection of IS900 specific primers remain the molecular tools that can be reliably
390 applied to routine diagnosis of *Map*.

391

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395

396 **References**

397

- 398 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment
399 search tool. *J. Mol. Biol.* 215, 403-410.
- 400 Brunello, F., Ligozzi, M., Cristelli, E., Bonora, S., Tortoli, E., Fontana, R., 2001. Identification of
401 54 mycobacterial species by PCR-Restriction Fragment Length Polymorphism Analysis of
402 the *hsp65* gene. *J. Clin. Microbiol.* 39, 2799-2806.
- 403 Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., Thompson, J.D.,
404 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.*
405 31, 3479-500.
- 406 Collins, D.M., Gabric, D.M., De Lisle, G.W., 1989. Identification of a repetitive DNA sequence
407 specific to *Mycobacterium paratuberculosis*. *FEMS Microbiol. Lett.* 60, 175-178.
- 408 Cousins, D. V., Whittington, R., Marsh, I., Masters, A., Evans, R.J., Kluver, P., 1999.
409 *Mycobacteria* distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from
410 the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase
411 chain reaction: implications for diagnosis. *Mol. Cell. Probes* 13, 431-442.
- 412 Dundee, L., Grant, I.R., Ball, H.J., Rowe, M.T., 2001. Comparative evaluation of four
413 decontamination protocols for isolation of *Mycobacterium avium* subsp. *paratuberculosis*
414 from milk. *Lett. Appl. Microbiol.* 33, 173-177.
- 415 Englund, S., Bolske, G., Johansson, K.E., 2002. An IS900-like sequence found in
416 *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS*
417 *Microbiol. Lett.* 209, 267-271.
- 418 Green, E.P., Tizard, M.L., Moss, M.T., Thompson, J., Winterborne, D.J., McFadden, J.J.,
419 Hermon-Taylor, J., 1989. Sequence and characteristics of IS900, an insertion element
420 identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*.
421 *Nucleic Acid Res.* 17, 9063-9073.
- 422 Ikonomopoulos, J., Gazouli, M., Pavlik, I., Bartos, M., Zacharatos, P., Xylouri, E., Papalambros,
423 E., Gorgoulis, V., 2004. Comparative evaluation of PCR assays for the robust molecular
424 detection of *Mycobacterium avium* subsp. *paratuberculosis*. *J. Microbiol. Methods* 56,
425 315-321.
- 426 Millar, D.S., Withey, S.J., Tizard, M.L.V., Ford, J.G., Hermon-Taylor, J., 1995. Solid-phase
427 hybridization capture of low-abundance target DNA sequences: application to the

- 428 polymerase chain reaction detection of *Mycobacterium paratuberculosis* and
429 *Mycobacterium avium* subsp. *silvaticum*. Anal. Biochem. 226, 325-330.
- 430 Möbius, P., Hotzel, H., Raßbach, A., Köhler, H., 2008. Comparison of 13 single-round and
431 nested PCR assays targeting IS900, IsMav2, f57 and locus 255 for detection of
432 *Mycobacterium avium* subspecies *paratuberculosis*. Vet. Microbiol. 126, 323-333.
- 433 Motiwala, A.S., Amonsin, A., Strother, M., Manning, E.J., Kapur, V., Sreevatsan, S., 2004.
434 Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* isolates
435 recovered from wild animal species, J. Clin. Microbiol. 4, 1703-1712.
- 436 Naser, S.A., Felix, J., Liping, H., Romero, C., Naser, N., Walsh, A., Safranek, W., 1999.
437 Occurrence of the IS900 gene in *Mycobacterium avium* complex derived from HIV
438 patients. Mol. Cell. Probes 13, 367-372.
- 439 Olsen, I., Johansen, T.B., Billman-Jacobe, H., Nilsen S.F., Djonne B., 2004. A novel IS element,
440 ISMpa1, in *Mycobacterium avium* subsp. *paratuberculosis*. Vet Microbiol. 98, 297-306.
- 441 Pavlik, I., Svastova, P., Bartl, J., Dvorska, L., Rychlik, I., 2000. Relationship between IS901 in
442 *Mycobacterium avium* complex strains isolated from birds, animals, humans, and the
443 environment and virulence for poultry. Clin. Diagn. Lab. Immunol. 7, 212-217.
- 444 Rajeev, S., Zhang, Y., Sreevatsan, S., Motiwala, A.S., Byrum, B., 2005. Evaluation of multiple
445 genomic target for identification and confirmation of *Mycobacterium avium* subsp.
446 *paratuberculosis* isolates using real-time PCR. Vet. Microbiol. 105, 215-221.
- 447 Richards, W.D., 1988. Competing siderophores - a new concept for the inhibition of
448 *Mycobacterium paratuberculosis*. Acta 2nd Inter. Coll. Parat., Maison-Alfort Cedex, France.
449 pp. 318-330.
- 450 Semret, M., Turenne, C.H., Behr, M.A., 2006. Insertion sequence IS900 revisited. J. Clin.
451 Microbiol. 44, 1081-1083.
- 452 Schinsky, M.F., Morey, R.E., Steigerwalt, A.G., Douglas, M.P., Wilson, R.W., Floyd, M.M.,
453 Butler, W.R., Daneshvar, M.I., Brown-Elliott, B.A., Wallace, R.J., McNeil, M.M., Brenner,
454 D.J., Brown, J.M., 2004. Taxonomic variation in the *Mycobacterium fortuitum* third
455 biovariant complex: description of *Mycobacterium boenickei* sp. nov., *Mycobacterium*
456 *houstonense* sp. nov., *Mycobacterium neworleansense* sp. nov. and *Mycobacterium*

- 457 *brisbanense* sp. nov. and recognition of *Mycobacterium porcinum* from human clinical
458 isolates. Int. J. Syst. Evol. Microbiol. 54, 1653-1667.
- 459 Tasara, T., Hoelzle, L.E., Stephan, R., 2005. Development and evaluation of a *Mycobacterium*
460 *avium* subspecies *paratuberculosis* (MAP) specific multiplex PCR assay. Int. J. Food
461 Microbiol. 104, 279-287.
- 462 Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E.C., Bodmer, T., 1993. Rapid identification
463 of mycobacteria to the species level by polymerase chain reaction and restriction enzyme
464 analysis. J. Clin. Microbiol. 31, 175-178.
- 465 Tsukamura, M., Remoto, H., Yugi, H., 1983. *Mycobacterium porcinum* sp. nov., a porcine
466 pathogen. Int. J. Syst. Bacteriol. 33, 162-165.
- 467 Vansnick, E., De Rijk, P., Vercammen, F., Geysen, D., Rigouts, L., Portaels, F., 2004. Newly
468 developed primers for the detection of *Mycobacterium avium* subspecies *paratuberculosis*.
469 Vet. Microbiol. 100, 197-204.
- 470 Vary, P.H., Andersen, P.R., Green, E., Hermon-Taylor, J., McFadden, J.J., 1990. Use of highly
471 specific DNA probes and the polymerase chain reaction to detect *Mycobacterium*
472 *paratuberculosis* in Johne's disease. J. Clin. Microbiol. 28, 933-937.
- 473 Wallace, R.J., Brown-Elliott, B.A., Wilson, R.W., Mann, L., Hall, L., Zhang, Y., Jost, K.C., Brown,
474 J.M., Kabani, A., Schinsky, M.F., Steigerwalt, A.G., Crist, C.J., Roberts, G.D., Blacklock,
475 Z., Tsukamura, M., Silcox, V., Turenne, C., 2004. Clinical and laboratory features of
476 *Mycobacterium porcinum*. J. Clin. Microbiol. 42, 5689-5697.

477 **Table 1.** Sequence, target gene and position of primers used in this study.

478

Primer	Nucleotide sequence	Target gene	Annealing position
p90	5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'	IS900	15-41
p91	5'-GGCGTTGAGGTCGATCGCCACGTGA-3'	IS900	402-427
TB11	5'-ACCAACGATGGTGTGTCCAT-3'	<i>hsp65</i>	269-288
TB12	5'-TTGTGGAACCGCATACCCT-3'	<i>hsp65</i>	690-708
END	5'-ATTCTCAATGACGTTGTCAAGC-3'	IS900	1430-1451
F448	5'-CAGCGGCTGCTCTACATTC-3'	IS900 like sequence	470-488
R1261	5'-GGTCGGATCGAATGCTGTA-3'	IS900 like sequence	1262-1280
DF	5'-GACGACTCGACCGCTAATTG-3'	IS900/IS900 like sequence	129-148/85-104
DR1	5'-CCGTAACCGTCATTGTCCAG-3'	IS900	208-227
DR2	5'-CATGTTGCGACAGAGCTG-3'	IS900 like sequence	323-341

479

480 **Table 2.** In vitro inhibition of *Map* growth. In the table the number of *Map* colonies grown on
 481 control slant and on target slants is reported.
 482

	Serial dilution of <i>Map</i> ATCC19698 inoculated on HEYM slopes (200 μ l)		
	5x10 ¹ cfu/ml	5x10 ² cfu/ml	5x10 ³ cfu/ml
Number of <i>Map</i> colonies on control slant	13	123	Uncountable
Number of <i>Map</i> colonies on 52 target slants	0	0	0
Result	Total inhibition	Total inhibition	Total inhibition

483

484 **Table 3.** *hsp65* RFLP patterns for *M. porcinum* and *M. neworleansense*.

485

	Restriction fragments length (bp)		
	<i>Bst</i> EI	<i>Hae</i> III	<i>Bsa</i> HI
<i>M. porcinum</i>	235/210	140/125/100	260/100/80
<i>M. neworleansense</i>	235/115/85	140/125	/

486

487 **Fig. 1.** Restriction endonuclease analysis of IS900 PCR products: Lane 1-4: Undigested IS900
488 PCR products; Lane 5, 6: *Map* ATCC19698.

489

490 **Fig. 2.** *hsp65* RFLP patterns. DNAs extracted from reference strain *M. porcinum* ATCC33776
491 and 52 mycobacterial isolates were amplified with the TB11/TB12 primers of Telenti et al (1993)
492 and digested by REs *Bst*EII, *Hae*III and *Bsa*HI. The comparison of digestion profiles identified
493 the unknown mycobacteria as *M. porcinum*. Lane 1: *M. porcinum* ATCC33776, Lane 2-5:
494 example of 3 *M. porcinum* isolates.

495

496 **Fig. 3:** PCR duplex. Lane 1: *M. peregrinum*, Lane 2: *M. smegmatis*, Lane 3: *M. fortuitum*, Lane
497 4: *M. gordonae*, Lane 5: *M. kansasii*, Lane 6: *M. scrofulaceum*, Lane 7: *M. bovis*, Lane 8: *M.*
498 *phlei*, Lane 9: *M. avium* subsp. *avium*, Lane 10: *M. porcinum* ATCC33776, Lane 11: *Map*
499 ATCC19698, Lane 12: *M. porcinum* isolate, Lane 13: *M. porcinum* isolate + *Map* ATCC19698.

500

Fig. 1

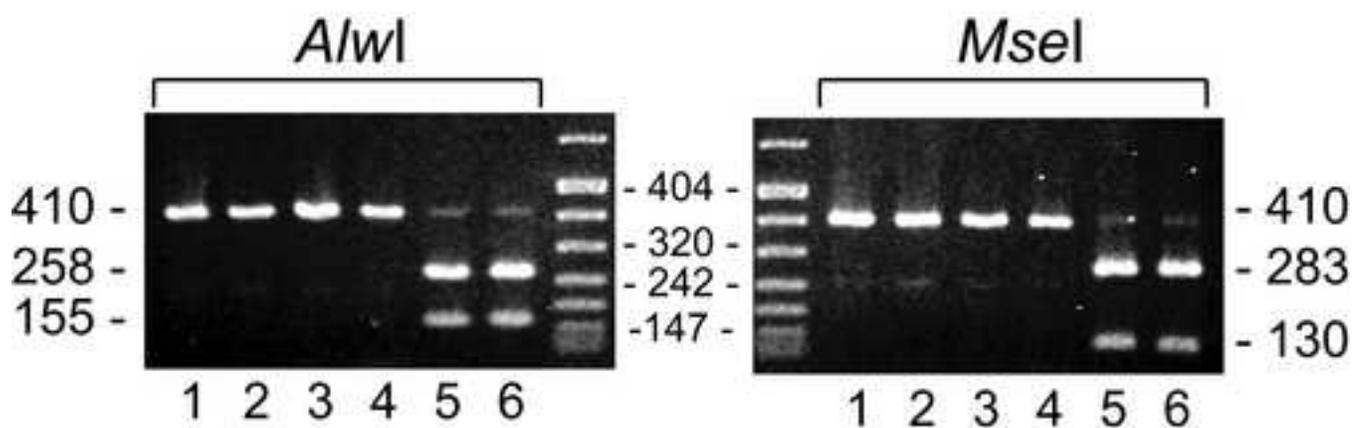
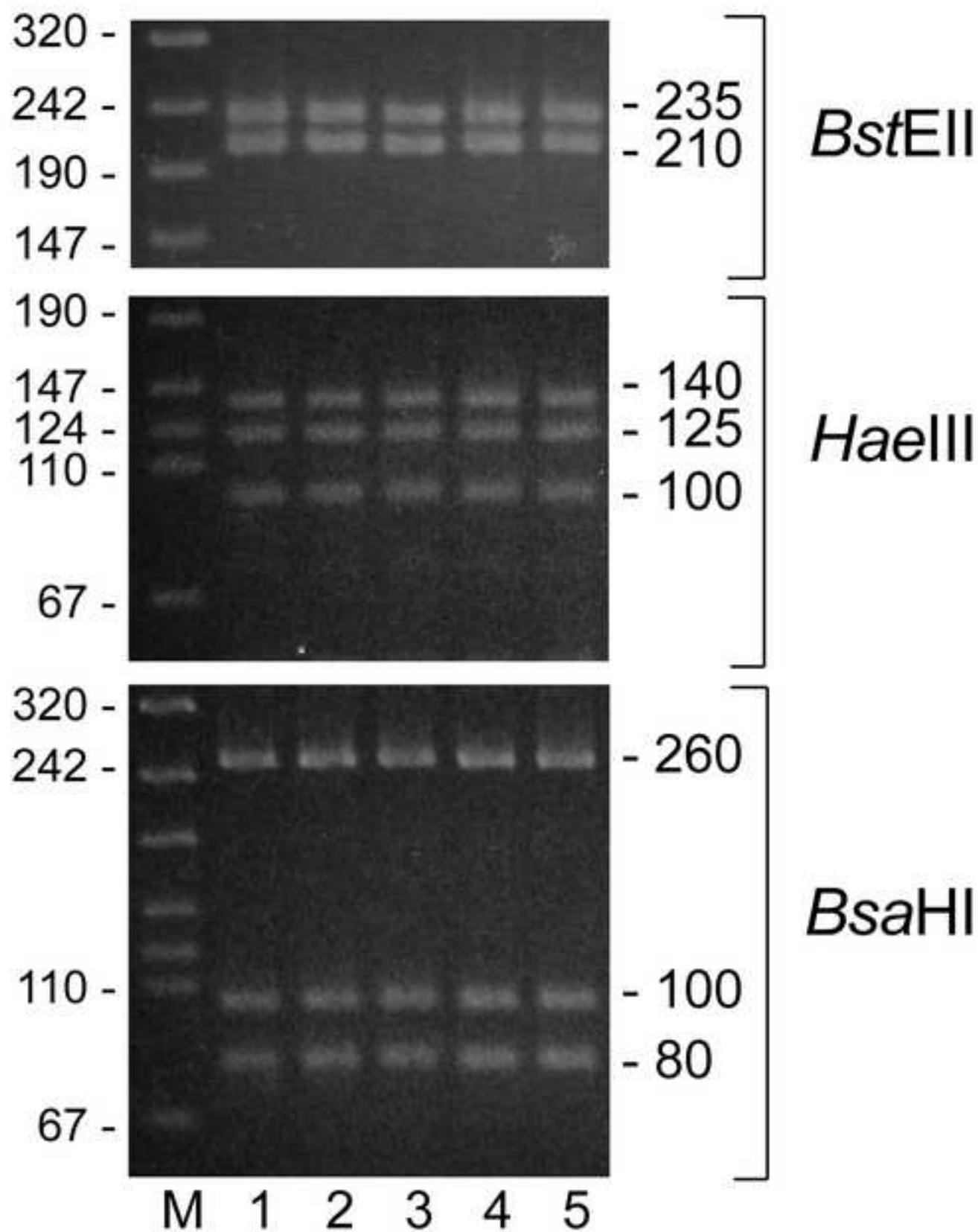


Fig. 2



Manuscript

Fig. 3

