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1 DIAGNOSTIC PERFORMANCE OF THE POURQUIER ELISA FOR DETECTION OF
2 ANTIBODIES AGAINST MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN
3 INDIVIDUAL MILK AND BULK MILK SAMPLES OF DAIRY HERDS

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25 **Abstract**

26 The objective of the study was to determine the diagnostic performance of the Pourquier ELISA for
27 detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (Map) in individual
28 milk samples and in bulk milk samples. For individual milk samples the specificity of the Pourquier
29 ELISA was estimated by testing a panel of individual milk samples from certified Map-free herds. The
30 relative sensitivity of the assay in individual milk samples and agreement of the results with those of
31 serum samples was estimated by testing panels of paired serum-milk samples from seropositive cattle,
32 whole-herd investigations, and moderate or heavy shedders.

33 The specificity of the ELISA for individual milk samples was still 99.8% at a cut-off of 20% sample to
34 positive (S/P) value, clearly lower than the cut-off defined by the manufacturer (30% S/P). The relative
35 sensitivity for individual milk samples as compared with positive serum samples was 87% for a cut-off
36 of 20% S/P, and 80% for a cut-off of 30% S/P. The sensitivity of this ELISA for detection of high
37 shedders was > 90% both for individual milk and serum samples, also agreement was very good
38 ($\kappa = 0.91$ for all paired samples).

39 The specificity of the Pourquier ELISA in bulk milk samples was investigated by testing bulk milk
40 samples from certified Map-free herds. Feasibility of bulk milk testing was investigated by titrating
41 ELISA positive individual milk samples in negative milk. In addition, 383 bulk milk samples from
42 herds with a known within-herd seroprevalence were tested.

43 The specificity of the ELISA for bulk milk samples was 100% at a cut-off of 12.5% S/P. At the cut-off
44 recommended by the manufacturer (30% S/P) performance of the bulk milk ELISA related to herd
45 status (≥ 2 seropositive cows) was rather poor, corresponding with a sensitivity of 24% and a
46 specificity of 99% relative to serology. However, at the revised cut-off for bulk milk of 12.5% S/P and
47 a within-herd seroprevalence of $\geq 3\%$, sensitivity and specificity relative to serology were 85% and
48 96%, respectively. Given the current herd-level seroprevalence in the Netherlands, these test
49 characteristics corresponded with positive and negative predictive values for bulk milk of 67% and
50 94%, respectively. In conclusion, the diagnostic performance of the Pourquier ELISA for individual

51 milk samples creates opportunities for a cheaper and more feasible testing scheme, while the diagnostic
52 performance for bulk milk samples warrants further consideration.

53

54 Keywords: M. avium subsp. paratuberculosis; ELISA; antibodies; diagnosis; serum; individual
55 milk; bulk milk; sensitivity; specificity; agreement; diagnostic performance; titration

56

57 1. Introduction

58 Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), is a
59 frequently present infectious disease in dairy cattle herds in many developed countries (Collins et al.,
60 2005). In the Netherlands, as in other countries, much effort has been invested in the implementation of
61 control programs, certification of herds and evaluation of these programs (Muskens et al., 2000);
62 (Groenendaal et al., 2003; Weber et al., 2004; Weber et al., 2006).

63 Detection of antibodies against Map by ELISA technology is an important tool in many regional and
64 national control schemes for paratuberculosis. Although the relative sensitivity of ELISA as compared
65 with faecal culture is rather low, especially for light shedders (van Schaik et al., 2003; Collins et al.,
66 2005), ELISA technology has gained an important place in herd-based testing schemes because of its
67 low cost and high-throughput potential. However, to obtain an acceptable herd sensitivity many cattle
68 have to be tested, with sample size being negatively correlated with herd specificity. Therefore, in
69 1999 a large validation study with commercially available ELISAs was performed (van Maanen et al.,
70 *Mycobacterium paratuberculosis* Antibody detecting ELISAs, internal validation report Animal Health
71 Service). Subsequently, an ELISA (Institut Pourquier, ELISA Paratuberculosis Antibody screening)
72 was selected and implemented with a specificity of 99.8% and an overall relative sensitivity (as
73 compared with faecal culture) of 40.8%. The test characteristics of the Pourquier ELISA also appeared
74 to be quite satisfactory for small ruminants (Gumber et al., 2006).

75 Recently, a thorough evaluation of five ELISAs for diagnosis of bovine paratuberculosis was
76 published. (Collins et al., 2005). In this study, the Pourquier ELISA also demonstrated an excellent
77 specificity of $\geq 99.8\%$. The only milk ELISA described in this study, however, was not sold as a
78 diagnostic kit but offered as a diagnostic service by a Michigan laboratory.

79 Adaptation of ELISA technology for milk samples for testing dairy cattle and herds would be very
80 cost-effective for several reasons. In Denmark, much experience has been obtained with testing of
81 individual milk samples in an in-house ELISA (Nielsen, 2002; Nielsen et al., 2002a; Nielsen et al.,
82 2002b; Kudahl et al., 2004). However, only scarce literature is available about the feasibility of bulk

83 milk testing for diagnosis of bovine paratuberculosis (Nielsen et al., 2000).

84 According to the manufacturer the Pourquier ELISA can also be used for testing milk samples. The

85 manufacturer, however, could only supply limited validation data. Therefore, in this study we

86 evaluated the diagnostic performance and feasibility of testing individual milk samples and bulk milk

87 samples in a commercially available ELISA (Institut Pourquier, ELISA Paratuberculosis Antibody

88 screening) at optimised cut-off values.

89

90 **2. Materials and Methods**

91 *2.1 Individual milk samples*

92 Panel A consisted of individual milk samples (n=435), obtained from ten different certified Map-free
93 herds (Groenendaal et al., 2003; Weber et al., 2004; Ezanno et al., 2005; Weber et al., 2006) with a 5
94 to 8 years history of negative annual whole-herd faecal culture and no introduction of cattle from other
95 herds during this period to determine specificity and optimise the cut-off of the ELISA kit.

96 Panel B consisted of individual milk samples (n=52) from different seropositive cattle originating from
97 infected herds to determine relative sensitivity of antibody detection in milk versus serum.

98 Panel C consisted of individual milk samples (n=30) from cattle tested seropositive in the previous 2-3
99 months in a study with twice yearly serological monitoring of a cohort of Map-infected dairy herds.

100 Panel D consisted of individual milk samples (n=300) from six different infected herds, samples were
101 obtained cross-sectionally 1-3 months after the last serological investigation from all cattle in milk.

102 Panel E consisted of individual milk samples (n=36) from cattle recently diagnosed as moderate or
103 heavy shedders either by faecal culture or by direct acid fast stain on faecal samples. Simultaneously
104 also faecal samples and serum samples were taken to determine relative sensitivity of antibody
105 detection in milk and serum for moderate and heavy shedders (resp. 10-100 or > 100 CFU on four
106 Löwenstein-Jenssen slants after 8 weeks culture period, respectively, (Kalis et al., 2000). To prevent
107 premature removal of faecal shedders from the herd, farmers were asked for their consent to take
108 individual milk samples, faecal samples and blood samples by the local practitioner before reporting
109 the faecal culture results.

110

111 *2.2. Titrations of individual milk samples*

112 To determine feasibility of pooling or bulk milk testing, the majority of ELISA positive individual milk
113 samples (either from seropositive cattle, n=64, or from moderate and heavy shedders, n=32) were
114 serially diluted in two-fold dilutions in negative milk from a certified Map-free herd and titres were
115 calculated.

116

117 *2.3 Bulk milk samples*

118 Bulk milk samples (n=110) were obtained from 110 certified Map-free herds to determine specificity
119 and optimise the cut-off.

120 In a randomized seroprevalence study in the Netherlands in 2004, bulk milk samples were obtained
121 simultaneously with serum samples from 383 dairy herds (21,411 individual serum samples with a
122 mean number of 53 sera per herd). Bulk milk samples were defatted by manual removal of the cream
123 layer after storage overnight at 4-8 °C and subsequently stored in 1 mL aliquots at -20 °C. These bulk
124 milk samples (n=383) were tested to determine the relationship between bulk milk ELISA result and
125 within-herd seroprevalence.

126

127 *2.4 Between-test variability*

128 A subset of 64 ELISA positive individual milk samples were retested with a week interval and the
129 correlation coefficient between results of both tests was determined. These sera were randomly
130 selected, and represented the whole range of low-positive to high positive results (Fig. 4).

131

132 *2.5 Absorbed ELISA*

133 All samples were tested in a commercially available ELISA kit (Institut Pourquier, ELISA
134 Paratuberculosis Antibody screening) according to the instructions of the manufacturer. As a first step
135 in the test protocol serum samples were diluted to 1/20 and milk samples to 1/2 in dilution buffer
136 containing *Mycobacterium phlei* extract, also according to the instructions of the manufacturer. Results
137 were expressed as percentage S/P, calculated by $100 \times (\text{OD value of the sample} - \text{the OD value of the negative control}) / (\text{OD value of the positive control} - \text{the OD value of the negative control})$. Cut-offs as
138 recommended by the manufacturer are for serum samples < 60% S/P negative, 60-70% S/P ambiguous,
139 and >70% S/P positive, and for milk samples < 30% S/P negative, 30-40% S/P ambiguous, and >40%
140 S/P positive.
141

142

143 *2.6 Statistical analysis*

144 Test agreement, sensitivity, specificity and 95% confidence intervals were calculated using

145 WinEpiscope 2.0 (N. de Blas, C. Ortega, K. Frankena, J. Noordhuizen, M. Thrusfield:

146 <http://www.clive.ed.ac.uk/winepiscopes/>). Scatter plots and correlation analyses were performed using

147 Microsoft EXCEL 2000. ROC analysis was performed in SPSS 10.0.

148 3. Results

149 3.1 Specificity of ELISA for individual milk samples

150 To determine the specificity of the milk ELISA the milk samples indicated as panel A (n=435) were
151 used. Fig. 1 shows the frequency distribution and the cumulative distribution for S/P values of
152 individual milk samples. Using the cut-off of 30% S/P value as defined by the manufacturer the
153 specificity was 100%. To achieve a similar specificity for individual milk samples as achieved for
154 serum samples, a cut-off of 20% S/P was selected, yielding a specificity of 99.8% (95% CI: 99.3-
155 100%) in the individual milk sample set investigated.

156

157 3.2 Relative sensitivity and agreement of ELISA for individual milk samples as compared with 158 serology

159 To determine the relative sensitivity of the milk ELISA in relation to serology the milk samples of
160 panel B and C (n= 82) were used. At a cut-off of 20% S/P for individual milk and a cut-off of 60%
161 S/P – as defined by the manufacturer - for serum 71 out of 82 seropositive cattle scored positive in
162 individual milk samples (relative sensitivity of 87% (95% CI: 79-94%)). At a cut-off of 30% S/P and
163 60% S/P for individual milk and serum samples, respectively (both as defined by the manufacturer) the
164 relative sensitivity for individual milk samples was 80% (95% CI: 72-89%). When for serum samples
165 a cut-off of 90% S/P was used – previously defined by the manufacturer and still used in our
166 laboratory for specificity considerations – 71 out of 74 seropositive cattle scored positive in individual
167 milk samples at a cut-off of 20% S/P (relative sensitivity of 96% (95% CI: 92-100%)).

168 Agreement beyond chance, expressed by kappa-values between individual milk results on the one hand
169 and serum results on the other hand was high with a kappa-value of 0.91 (95% CI: 0.87-0.95) when all
170 samples of panels B-E were included (n=417). Also, in panel D, a cross-sectional sample (n=300) in
171 six different infected herds of milk samples 1-3 months after the serological investigations the
172 agreement between serum and individual milk results was high (kappa=0.83 (95% CI: 0.72-0.94)).

173 Furthermore, S/P values of serum samples and individual milk samples were clearly correlated

174 (R=0.92) as demonstrated in Fig. 2, although not in a strictly linear way and with generally lower S/P
175 values in milk than in serum.

176

177 *3.3 Relative sensitivity of ELISA for individual milk and serum samples as compared with faecal*
178 *shedding*

179 To determine the relative sensitivity of the milk ELISA in relation to faecal shedding the samples
180 indicated as panel E (n=36) were used. The agreement between ELISA results of individual milk and
181 serum samples was very good with a relative sensitivity as compared to faecal shedding of 89% (95%
182 CI: 79-99%). When only high shedders were included (based on the previous and/or current culture
183 results, n=30) the relative sensitivity of this ELISA was 97% (90-100%) for both individual milk and
184 serum samples.

185

186 *3.4 Titre distribution of individual ELISA positive milk samples*

187 In total, 97 ELISA positive individual milk samples from panel B-E were titrated in negative milk. The
188 relationship between S/P value of undiluted milk samples and the \log_{10} titres of the same samples is
189 presented in Fig. 3. S/P values and titres were clearly correlated (R=0.90).

190 S/P values were arbitrarily categorized in four S/P classes and mean \log_{10} titres were calculated
191 (Table 1). The overall geometric mean titre of all ELISA positive individual milk samples investigated
192 was 1.0 \log_{10} or 1:10. For a category of moderate and high shedders the geometric mean titre of ELISA
193 positive individual milk samples was 1.36 \log_{10} or 1:23.

194

195 *3.5 Between-test variability of ELISA results for individual milk samples*

196 To get an impression of the within-laboratory reproducibility of the Pourquier ELISA for milk samples
197 a set of ELISA positive individual milk samples from panel B-E (n=64) was tested twice with eight
198 days interval in the same laboratory. Results are presented in Fig. 4. All samples scored positive again
199 in the second test and S/P values correlated very well between the two tests with correlation and

200 regression coefficients close to 1 ($r = 0.97$; regression coefficient = 1.06).

201

202 3.6 Specificity of ELISA for bulk milk samples

203 Fig. 5 shows the frequency distribution and the cumulative distribution for S/P values of bulk milk
204 samples (certified Map-free herds, a subset of the herds mentioned in paragraph 2.3, $n=110$). Using the
205 cut-off of 30% S/P value as defined by the manufacturer the specificity was 100%. A cut-off of 12.5%
206 S/P, however, still yielded a specificity of 100% in the bulk milk sample set investigated.

207

208 3.7 Diagnostic performance of ELISA for bulk milk samples related to within-herd seroprevalence

209 Bulk milk ELISA results were initially interpreted as described by the manufacturer for individual milk
210 samples ($< 30\%$ S/P negative, $\geq 30\%$ S/P suspect/positive). Diagnostic performance of the Pourquier
211 ELISA for bulk milk samples related to within-herd seroprevalence is summarised in Table 2. From the
212 383 herds participating in a randomized seroprevalence study with a bulk milk sample, 267 herds were
213 completely seronegative, 62 herds had one seropositive and/or suspect animal, and 54 herds had two or
214 more seropositive and/or suspect animals. There was a fair correlation between S/P values of bulk
215 milk samples and within-herd seroprevalence ($r = 0.70$). Values for sensitivity, specificity and overall
216 diagnostic potential as indicated by the Area Under the Curve (AUC) are presented in Table 2 for
217 different herd status criteria based on the interpretation of test results in practise (absolute # of
218 positives) and for different prevalences ($\geq 2\%$ to $\geq 5\%$). Test characteristics are presented at a cut-off
219 of 30% S/P as indicated by the manufacturer and at a cut-off of 12.5% S/P. The latter cut-off was
220 chosen because it corresponded with the cut-off resulting in 100% specificity for bulk milk samples for
221 certified Map-free herds, and this cut-off also yielded a high specificity for the bulk milk samples from
222 the seronegative herds in the seroprevalence study.

223 Because bulk milk samples originated from a randomised prevalence study in the Netherlands, also
224 positive and negative predictive values (PVP and PVN) could be calculated. For example, for a herd
225 with $\geq 3\%$ seroprevalence which is the most common seroprevalence in the Netherlands (Muskens et

226 al., 2000) and a cut-off for bulk milk of 12.5% S/P, sensitivity and specificity were 85% and 96%,
227 respectively, and the PVP and PVN were 67% and 94%, respectively. Experiences with the Dutch
228 paratuberculosis programmes since 1998 demonstrated that a level of $\geq 3\%$ seroprevalence (all present
229 cattle ≥ 3 years of age tested) also indicate an infection level of the herd where one or more heavy
230 shedders are present. It should be noted that in almost all herds (12 out of 14) that had a prevalence
231 $< 3\%$ and tested positive in bulk milk one or more seropositive animals were detected.

232 4. Discussion

233 One of the advantages of many years of paratuberculosis research and control in the Netherlands is the
234 presence of a large pool of certified Map-free herds with a long history of negative ELISA and faecal
235 culture test results. From these herds individual milk samples and bulk milk samples were taken and
236 investigated in the Pourquier ELISA. As shown in Figs. 1 and 5, cut-offs corresponding with a
237 specificity of nearly 100% for both individual and bulk milk samples could be (much) lower than the
238 cut-off of 30% S/P defined by the manufacturer. For individual milk samples the revised cut-off was
239 higher (20% S/P) than for bulk milk samples (12.5% S/P), but much lower than for serum samples
240 (60% S/P). Apparently, un-specific reactions are more predominant in serum samples than in milk
241 samples, allowing lower cut-offs and lower pre-dilutions for milk samples, as described as well for
242 several other infectious diseases (Bjorkman et al., 1997; Kramps et al., 1999; Beaudreau et al., 2001;
243 Nielsen et al., 2002a; Bartels et al., 2005; Schares et al., 2005).

244 The relative sensitivity of testing individual milk samples (at revised cut-off) as compared with serum
245 samples was high, and varied between 87 and 96%, depending on the cut-off used for serum samples.
246 In recent years, the manufacturer has decreased the cut-off for serum samples to 60% S/P, but because
247 of concerns of losing specificity we maintained the formerly prescribed cut-off of 90% S/P. The
248 agreement beyond chance, as expressed by kappa-values, between individual milk and serum results
249 was high, also for the unbiased cross-sectional sample panel D.

250 High relative sensitivities for individual milk samples and good agreement with serum results for
251 diagnosis of bovine paratuberculosis have also been described by others (Sweeney et al., 1994;
252 Winterhoff et al., 2002; Nielsen et al., 2002a; Collins et al., 2005).

253 On the other hand, several authors have reported poor sensitivities and/or poor agreements and
254 correlations for individual milk relative to serum (Hardin and Thorne, 1996; Hendrick et al., 2005a;
255 Hendrick et al., 2005b). Several factors may play a role, such as the different commercial or in-house
256 ELISAs that were evaluated in the publications. Moreover, the stage of lactation in which the paired
257 samples were taken may have played a role. (Nielsen et al., 2002a) demonstrated that in the beginning

258 of the lactation the probability of being positive was highest in the milk ELISA, while in the serum
259 ELISA the probability of being positive was highest at the end of lactation. Because of practical
260 considerations for implementation of individual milk testing into our control program we did not take
261 the stage of lactation into account.

262 We also evaluated the diagnostic performance of individual milk and serum samples for moderate to
263 heavy shedders. Heavy shedders in particular play an important role in the within-herd transmission of
264 paratuberculosis, with moderate shedding as a preceding transitional stage (van Schaik et al., 2003;
265 Collins et al., 2005). For a set of 36 predominantly high shedders, the relative sensitivity of the
266 Pourquier ELISA using faecal culture as a gold standard was 89% both for serum and for milk.
267 Sensitivities increased to 97% when the moderate shedders were excluded. High sensitivities of
268 ELISAs for detection of heavy shedders were also reported by others (van Schaik et al., 2003; Collins
269 et al., 2005).

270 Contrary to some other authors (Hardin and Thorne, 1996; Hendrick et al., 2005b) we found a clear,
271 although not linear, correlation between result of individual milk and serum samples (Fig. 2). However,
272 S/P values in individual milk samples were generally lower than in serum samples, as reported by
273 others (Winterhoff et al., 2002). Also the reproducibility of S/P values of positive milk samples
274 appeared to be good, which is important for consistent results in certification schemes, control
275 programs and longitudinal studies.

276 Because bulk milk is essentially a pooled sample of individual cows, we determined the titre
277 distribution of a panel of ELISA positive individual milk samples. Titres varied between 1/1 and
278 1/1024 with a clear correlation between the S/P value of the undiluted sample and the titre. Although
279 this phenomenon matched our expectations, titres were in general rather low with a geometric mean titre
280 of 1/10 of a milk panel obtained from seropositive cattle, and a geometric mean titre of 1/23 for a milk
281 panel derived from moderate to high shedders. These results matched those of Arrigoni et al. (Institut
282 Pourquier, personal communication) and would imply restricted possibilities for pooling of samples. In
283 other words: for detection of infected herds (with a pooled milk sample) in general a 10%

284 seroprevalence would be the mean limit of detection and for detection of infected herds that contain at
285 least one moderate to heavy shedders, a 4-5% seroprevalence would be the mean limit of detection.
286 Subsequently, we analysed bulk milk samples of a large number of herds with known seroprevalence.
287 Although many examples exist for bulk milk testing for other diseases, for paratuberculosis hardly any
288 literature is available (Nielsen et al., 2000; Beyerbach et al., 2004). Nielsen et al. (2000) concluded
289 that the technical performance of the ELISA was not sufficient to provide a tool for surveillance.
290 Beyerbach et al. (2004) used a modification of a non-absorbed LAM-ELISA for bulk milk testing, and
291 related the test results to within-herd test prevalence for individual milk samples. However, only 28
292 herds were involved in the study, and in our validation study and that of others (Collins et al., 2005)
293 the LAM-ELISA lacked specificity, at least for serum samples.
294 Indeed, diagnostic performance of the bulk milk ELISA was rather poor at a low prevalence (≥ 1
295 seropositives in a herd; $\geq 2\%$ seroprevalence in a herd) as demonstrated by low sensitivities, even at a
296 much lower cut-off than defined by the manufacturer, and rather low AUC values in a ROC analysis.
297 However, at prevalence levels of $\geq 3\%$ and with the revised cut-off the bulk milk ELISA appeared to
298 have diagnostic potential with a sensitivity and specificity of 85 and 96%, respectively. This would
299 imply a detection level of 1 seropositive out of 30 cattle or 3 out of 100 cattle. (Beyerbach et al.,
300 2004) reported for their bulk milk ELISA a sensitivity and specificity of 75 and 84%, respectively, at a
301 within-herd prevalence level of 5% . The even lower cut-off for bulk milk samples may have
302 contributed to more favourable results than we expected from the titration experiments. When bulk
303 milk testing would be used as a first screening test for regional or national programs the negative
304 predictive value would be particularly important. At a 3% seroprevalence level in test-positive herds,
305 the negative predictive value of bulk milk would be 94%, which seemed quite acceptable. The positive
306 predictive value was only 67% using the $\geq 3\%$ seroprevalence criterium. On the other hand, in almost
307 all “false-positive” herds one or more seropositive animals were detected.
308 In conclusion, the Pourquier ELISA can be used for testing individual milk samples as an alternative
309 for individual serum samples, and currently individual milk samples are already routinely submitted to

310 our laboratory. Bulk milk testing warrants further consideration, and needs further evaluation.

311

312

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317 **References**

- 318 Bartels, C. J., van Maanen, C., van der Meulen, A. M., Dijkstra, T., Wouda, W., 2005. Evaluation of
319 three enzyme-linked immunosorbent assays for detection of antibodies to *Neospora caninum* in bulk
320 milk. *Vet. Parasitol.*, 131: 235-246.
- 321 Beaudeau, F., Belloc, C., Seegers, H., Assie, S., Sellal, E., Joly, A., 2001. Evaluation of a blocking
322 ELISA for the detection of bovine viral diarrhoea virus (BVDV) antibodies in serum and milk. *Vet.*
323 *Microbiol.*, 80: 329-337.
- 324 Beyerbach, M., Ortmann, G., Gerlach, G. F., Homuth, M., Strutzberg, K., Kreienbrock, L., 2004.
325 [Considerations concerning diagnostic certainties and cut-off values for a bulk milk ELISA for
326 *Mycobacterium avium* ssp. *paratuberculosis*]. *Dtsch. Tierarztl. Wochenschr.*, 111: 220-225.
- 327 Bjorkman, C., Holmdahl, O. J., Ugglå, A., 1997. An indirect enzyme-linked immunoassay (ELISA) for
328 demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. *Vet. Parasitol.*, 68: 251-
329 260.
- 330 Collins, M. T., Wells, S. J., Petrini, K. R., Collins, J. E., Schultz, R. D., Whitlock, R. H., 2005.
331 Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. *Clin. Diagn. Lab*
332 *Immunol.*, 12: 685-692.
- 333 Ezanno, P., van Schaik, G., Weber, M. F., Heesterbeek, J. A., 2005. A modeling study on the
334 sustainability of a certification-and-monitoring program for paratuberculosis in cattle. *Vet. Res.*, 36:
335 811-826.
- 336 Groenendaal, H., Nielen, M., Hesselink, J. W., 2003. Development of the Dutch Johne's disease
337 control program supported by a simulation model. *Prev. Vet. Med.*, 60: 69-90.
- 338 Gumber, S., Eamens, G., Whittington, R. J., 2006. Evaluation of a Pourquier ELISA kit in relation to
339 agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and

- 340 goats with and without *Mycobacterium paratuberculosis* infection. *Vet. Microbiol.*, 115: 91-101.
- 341 Hardin, L. E., Thorne, J. G., 1996. Comparison of milk with serum ELISA for the detection of
342 paratuberculosis in dairy cows. *J. Am. Vet. Med. Assoc.*, 209: 120-122.
- 343 Hendrick, S., Duffield, T., Leslie, K., Lissemore, K., Archambault, M., Kelton, D., 2005a. The
344 prevalence of milk and serum antibodies to *Mycobacterium avium* subspecies paratuberculosis in
345 dairy herds in Ontario. *Can. Vet. J.*, 46: 1126-1129.
- 346 Hendrick, S. H., Duffield, T. E., Kelton, D. E., Leslie, K. E., Lissemore, K. D., Archambault, M.,
347 2005b. Evaluation of enzyme-linked immunosorbent assays performed on milk and serum samples for
348 detection of paratuberculosis in lactating dairy cows. *J. Am. Vet. Med. Assoc.*, 226: 424-428.
- 349 Kalis, C. H., Hesselink, J. W., Barkema, H. W., Collins, M. T., 2000. Culture of strategically pooled
350 bovine fecal samples as a method to screen herds for paratuberculosis. *J. Vet. Diagn. Invest.*, 12: 547-
351 551.
- 352 Kramps, J. A., van Maanen, C., van de, W. G., Stienstra, G., Quak, S., Brinkhof, J., Ronsholt, L.,
353 Nylin, B., 1999. A simple, rapid and reliable enzyme-linked immunosorbent assay for the detection of
354 bovine virus diarrhoea virus (BVDV) specific antibodies in cattle serum, plasma and bulk milk. *Vet.*
355 *Microbiol.*, 64: 135-144.
- 356 Kudahl, A., Nielsen, S. S., Sorensen, J. T., 2004. Relationship between antibodies against
357 *Mycobacterium avium* subsp. paratuberculosis in milk and shape of lactation curves. *Prev. Vet. Med.*,
358 62: 119-134.
- 359 Muskens, J., Barkema, H. W., Russchen, E., Van Maanen, K., Schukken, Y. H., Bakker, D., 2000.
360 Prevalence and regional distribution of paratuberculosis in dairy herds in The Netherlands. *Vet.*
361 *Microbiol.*, 77: 253-261.

- 362 Nielsen, S. S., 2002. Variance components of an enzyme-linked immunosorbent assay for detection of
363 IgG antibodies in milk samples to *Mycobacterium avium* subspecies paratuberculosis in dairy cattle. *J.*
364 *Vet. Med. B Infect. Dis. Vet. Public Health*, 49: 384-387.
- 365 Nielsen, S. S., Enevoldsen, C., Grohn, Y. T., 2002a. The *Mycobacterium avium* subsp.
366 paratuberculosis ELISA response by parity and stage of lactation. *Prev. Vet. Med.*, 54: 1-10.
- 367 Nielsen, S. S., Grohn, Y. T., Enevoldsen, C., 2002b. Variation of the milk antibody response to
368 paratuberculosis in naturally infected dairy cows. *J. Dairy Sci.*, 85: 2795-2802.
- 369 Nielsen, S. S., Thamsborg, S. M., Houe, H., Bitsch, V., 2000. Bulk-tank milk ELISA antibodies for
370 estimating the prevalence of paratuberculosis in Danish dairy herds. *Prev. Vet. Med.*, 44: 1-7.
- 371 Schares, G., Barwald, A., Conraths, F. J., 2005. Adaptation of a surface antigen-based ELISA for the
372 detection of antibodies against *Neospora caninum* in bovine milk. *J. Vet. Med. B Infect. Dis. Vet.*
373 *Public Health*, 52: 45-48.
- 374 Sweeney, R. W., Whitlock, R. H., Buckley, C. L., Spencer, P., Rosenberger, A. E., Hutchinson, L. J.,
375 1994. Diagnosis of paratuberculosis in dairy cattle, using enzyme-linked immunosorbent assay for
376 detection of antibodies against *Mycobacterium paratuberculosis* in milk. *Am. J. Vet. Res.*, 55: 905-
377 909.
- 378 van Schaik, G., Rossiter, C. R., Stehman, S. M., Shin, S. J., Schukken, Y. H., 2003. Longitudinal study
379 to investigate variation in results of repeated ELISA and culture of fecal samples for *Mycobacterium*
380 *avium* subsp paratuberculosis in commercial dairy herds. *Am. J. Vet. Res.*, 64: 479-484.
- 381 Weber, M. F., Groenendaal, H., van Roermund, H. J., Nielen, M., 2004. Simulation of alternatives for
382 the Dutch Johne's disease certification-and-monitoring program. *Prev. Vet. Med.*, 62: 1-17.
- 383 Weber, M. F., van Roermund, H. J., Vernooij, J. C., Kalis, C. H., Stegeman, J. A., 2006. Cattle

384 transfers between herds under paratuberculosis surveillance in The Netherlands are not random. Prev.
385 Vet. Med.

386 Winterhoff, C., Beyerbach, M., Homuth, M., Strutzberg, K., Gerlach, G. F., 2002. [Establishment and
387 evaluation of an ELISA for the detection of antibodies in milk against *Mycobacterium avium*
388 subspecies paratuberculosis]. Dtsch. Tierarztl. Wochenschr., 109: 230-234.

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391

392 *Table 1. Relationship between S/P values of ELISA positive undiluted individual milk samples and*
393 *end-point dilutions in negative milk, presented as geometric mean titres per S/P% class,*
394 *range of positive S/P values divided into four classes.*

Class S/P%	n	Mean log ₁₀ titre	Mean titre
20-90	30	0,39	2
90-160	29	0,92	8
160-230	27	1,46	29
230-300	11	1,94	87
All classes	97	1,00	10

395 Table 2. Sensitivity and specificity of the Pourquier ELISA for bulk milk samples (n=383, n=267
 396 bulk milk samples from seronegative herds) at two different cut-offs and six different
 397 herd criteria for seroprevalence (between brackets the number of herds fulfilling the
 398 criterion).. Per herd criterion the AUC is given as an indicator of diagnostic potential of
 399 the test.

400						
Herd criterium		Cut-off 30% S/P		Cut-off 12.5% S/P		AUC
		Se (%)	Sp (%)	Se (%)	Sp (%)	
=1	(62)	14	100	35	99	0.78
≥2	(54)	24	99	52	96	0.87
≥2%	(82)	17	99	39	96	0.79
≥3%	(50)	28	99	85	96	0.88
≥4%	(32)	38	99	69	94	0.90
≥5%	(22)	50	99	72	92	0.90

401 LEGENDS TO FIGURES

402

403 Fig. 1. Frequency distribution and cumulative distribution of ELISA results for individual milk samples
404 from Map-certified herds

405

406 Fig. 2. Relationship between ELISA results of paired individual milk and serum samples, dotted lines
407 represent test cut-offs for individual milk and serum samples

408

409 Fig. 3. Relationship between ELISA results (S/P%) of undiluted individual milk samples and \log_{10}
410 titres of the same samples diluted in milk from a Map-certified herd

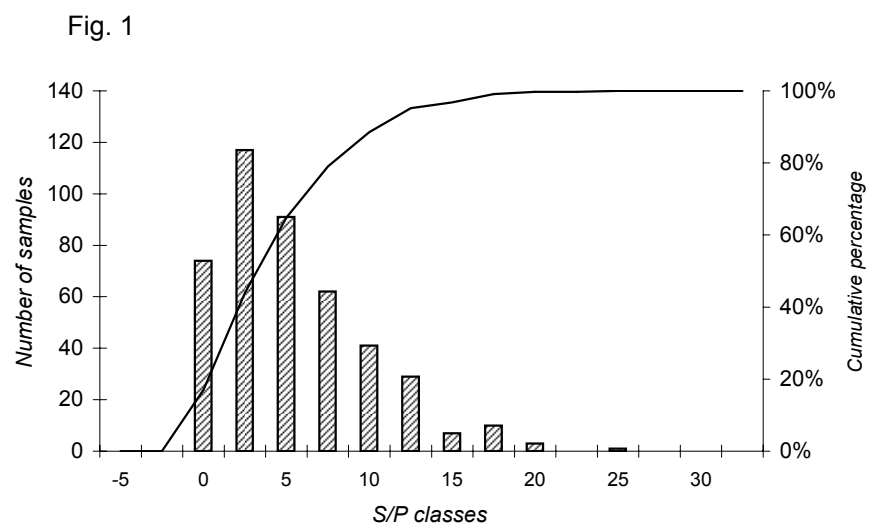
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412 Fig. 4. Between-test variability for individual milk samples in the Pourquier ELISA tested with an 8-day
413 interval

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415 Fig. 5. Frequency distribution and cumulative distribution of ELISA results for bulk milk samples from
416 Map-certified herds

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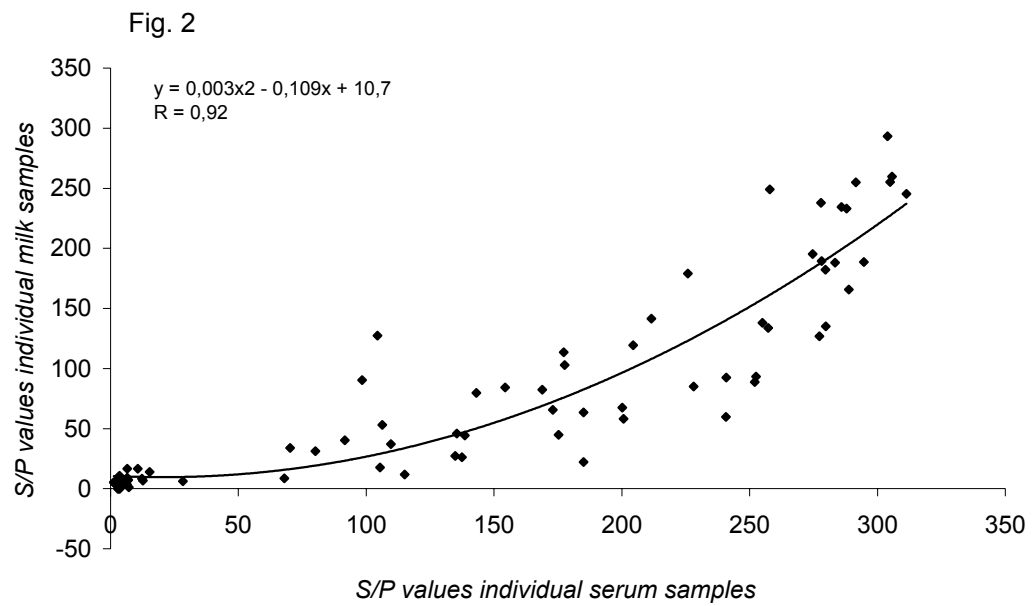


Fig. 3

