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1 **Comparative evaluation of eight serological assays for**
2 **diagnosing *Chlamydophila abortus* infection in sheep**

3

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10

11 **Abstract**

12 *Chlamydophila abortus* is one of the principal causes of late-term abortion (Enzootic
13 Abortion of Ewes or EAE) in sheep across Europe. Serological diagnosis of EAE is
14 routinely carried out by the complement fixation test, although the interpretation of
15 results can often be difficult because of cross reaction with *Chlamydophila pecorum*,
16 which also commonly infects sheep. The purpose of this study was to evaluate and
17 compare four ELISAs developed at Moredun Research Institute and based on whole
18 *C. abortus* elementary bodies (EB), an outer membrane preparation of the whole
19 organism (SolPr) and two recombinant polymorphic outer membrane protein
20 fragments (rOMP90-3 and rOMP90-4), with 3 commercial tests, the CHEKIT
21 *Chlamydophila Abortus*, Pourquoi ELISA *Chlamydophila abortus* and ImmunoComb
22 *Ovine Chlamydophila* Antibody tests. The tests were evaluated using a panel of 202
23 sera from experimentally and naturally infected animals, as well as from EAE-free
24 flocks. The EB, SolPr and CHEKIT ELISAs performed similarly to the CFT, all
25 lacking in specificity by cross reacting with sera from *C. pecorum* infected animals.

26 The ImmunoComb also lacked specificity with *C. pecorum* sera, but also badly cross
27 reacted with sera from EAE-free flocks. The rOMP90-3, rOMP90-4 and Pourquier
28 ELISAs were the most specific, although the Pourquier test appeared less sensitive
29 with sera from naturally infected animals. Overall, the rOMP90-3 ELISA performed
30 the best, with high sensitivity (96.8%) and no cross reaction with sera from *C.*
31 *pecorum* infected animals or from EAE-free flocks (100% specificity) and so would
32 be a suitable alternative to the CFT for the serological diagnosis of EAE.

33

34 **Keywords:** Ovine enzootic abortion; *Chlamydophila abortus*; *Chlamydophila*
35 *pecorum*; polymorphic outer membrane proteins; serological diagnosis; indirect
36 ELISA

37

38

39 **1. Introduction**

40 Enzootic abortion of ewes (EAE) or Ovine Enzootic Abortion (OEA), caused
41 by the obligate intracellular bacterium *Chlamydophila abortus* (*C. abortus*), affects
42 many sheep-rearing countries throughout the world, resulting in late-gestation lamb
43 loss or the birth of weak lambs that fail to survive beyond 48 hrs. *Chlamydophila*
44 *abortus* is the most common infectious cause of abortion in sheep and goats in the UK
45 and Northern Europe, having a major economic impact on agricultural industries
46 (Longbottom and Coulter, 2003). The pathogen can also cause reproductive failure in
47 cattle, horses, pigs and deer, however the prevalence of such infections is unknown
48 due to the lack of epidemiological data. Importantly, *C. abortus* is also a zoonotic
49 pathogen and can present a serious health risk to pregnant women (Longbottom and
50 Coulter, 2003).

51 The organism targets the placenta where it causes pathological damage
52 affecting pregnancy outcome in the final 2-3 weeks of gestation. A major feature of
53 this disease is the apparent lack of any clinical signs prior to abortion occurring,
54 highlighting the importance of being able to detect infection in animals earlier in
55 pregnancy. Therefore, it is important to have sensitive and specific diagnostic tools to
56 allow the identification of infected animals, so that appropriate control strategies can
57 be implemented to reduce transmission of the disease.

58 Diagnosis and screening of sheep for *C. abortus* infection relies heavily on
59 antibody detection in serum samples. The most commonly used serological assay is
60 the complement fixation test (CFT), which is based on chlamydial lipopolysaccharide
61 (LPS). However, there are inherent problems associated with the use of LPS and
62 other genus-specific antigens due to the lack of specificity, resulting from cross-
63 reaction with *Chlamydophila pecorum* (*C. pecorum*), which also infects sheep causing
64 a wide variety of conditions such as inapparent enteric infections, polyarthritis,
65 conjunctivitis and pneumonia (Fukushi and Hirai, 1993; Anderson et al., 1996). A
66 number of indirect enzyme-linked immunosorbent assays (iELISAs) based on various
67 chlamydial antigen preparations (Anderson et al., 1995), LPS (Griffiths et al., 1996),
68 the major outer membrane protein (MOMP) (Kaltenboeck et al., 1997; Salti-
69 Montesanto et al., 1997; Hoelzle et al., 2004) and the polymorphic outer membrane
70 proteins (POMPs) (Buendia et al., 2001; Longbottom et al., 2001, 2002), as well as an
71 indirect immunofluorescence assay (Markey et al., 1993a) have been developed in an
72 attempt to improve on the detection of *C. abortus* antibodies, with varying degrees of
73 sensitivity and specificity.

74 In this study, we have evaluated three commercially available serological
75 assays: the ImmunoComb® Ovine Toxo & *Chlamydophila* Antibody Test Kit, which

76 is based on a solid phase immunoassay using purified *Chlamydomphila* antigens; the
77 CHEKIT-*Chlamydomphila* Abortus Antibody Test Kit, which uses inactivated *C.*
78 *abortus* antigen; and the POURQUIER® ELISA CHLAMYDOPHILA abortus, which
79 is based on a recombinant 80-90kDa protein fragment from *C. abortus*. The
80 performances of the commercial assays were compared to the standard reference CFT
81 (Aitken and Longbottom, 2004) and four “in-house” ELISAs: the SolPr ELISA
82 (Anderson et al., 1995), which is based on a solubilized outer membrane preparation
83 of *C. abortus*; an EB ELISA, which uses purified *C. abortus* elementary bodies (EBs);
84 and the rOMP90-3 and rOMP90-4 iELISAs, which are based on recombinant protein
85 fragments of POMP90 of *C. abortus* (Longbottom et al., 2002). The tests were
86 assessed for their suitability for detecting animals infected with *Chlamydomphila* spp.
87 and also for specifically detecting *Chlamydomphila abortus*-infected animals, using a
88 panel of defined ovine experimental sera and flock sera.

89

90 **2. Materials and methods**

91 *2.1 Antibody assays*

92 (i) **Complement Fixation Test.** The CFT was performed as described previously
93 (Stamp et al., 1952) using *C. abortus* strain S26/3 as antigen. Samples were tested at
94 two-fold dilutions from 1/32 to 1/512. CFT titres were expressed as the highest serum
95 dilution giving 50% or less haemolysis, where 50% haemolysis was graded 2+, and
96 0% was graded 4+. A titre of 4+ at a dilution of 1/32 or greater was assumed to be
97 positive, whereas a titre of 2+ at a dilution of 1/32 was assumed to be equivocal.

98 (ii) **ImmunoComb® Ovine Toxo & *Chlamydomphila* Antibody Test Kit** (Biogal,
99 Galed Kibbutz, Israel.). This test, which determines sheep serum IgG titres for
100 *Chlamydomphila* species, is based on a solid phase immunoassay with purified

101 *Chlamydophila* antigen attached to a plastic comb. The assay was performed
102 according to manufacturer's instructions. Determination of positivity for this test is
103 subjective and based on staining intensity: test samples are compared to a positive
104 control using a calibrated scale.

105 (iii) **Soluble protein (SolPr) ELISA.** The SolPr ELISA was performed as described
106 previously (Anderson et al., 1995) with modifications. Sodium periodate and sodium
107 deoxycholate EDTA treatments were omitted. Sheep sera were diluted 1:800 in 5%
108 rabbit serum (Sigma, Poole, Dorset, UK) in 2.5% non fat dried milk (NFDM) in
109 0.05% Tween 20 / phosphate-buffered saline (PBST). Bound antibody was detected
110 with tetramethylbenzidine (TMB) (KPL, Gaithersburg, Maryland, USA) and the
111 reaction was stopped after 5 minutes by the addition of 0.18 M sulphuric acid.

112 (iv) **Elementary body ELISA.** *C. abortus* EBs were prepared and purified as
113 described previously (McClenaghan et al., 1984). 96 well microtitre plates (Greiner
114 Bio-one GmbH, Frickenhausen, Germany) were coated overnight at 4°C with
115 sonicated EBs in 0.1M sodium carbonate/bicarbonate buffer, pH9.6. Following
116 washes with PBST, the plates were blocked with 10% horse serum in 5%
117 NFDM/PBST for 60 minutes at 37°C. After washing, sheep sera (diluted 1:400 with
118 5% horse serum in 2.5% NFDM/PBST) were added to appropriate wells and the
119 plates incubated for 60 minutes at 37°C. After further washing, horseradish peroxidase
120 conjugated anti-sheep IgG (Sigma) at a 1:2000 dilution, was added and incubated for
121 60 minutes at 37°C. Bound antibody was detected as for the Solpr ELISA.

122 (v) **CHEKIT® Chlamydophila Abortus Antibody Test Kit** (IDEXX Europe B.V.,
123 Koolhovenlaan, The Netherlands): an enzyme immunoassay for detecting antibodies
124 to *C. abortus* in ruminant serum. The assay was performed according to
125 manufacturer's instructions.

126 (vi) **Pourquier® ELISA CHLAMYDOPHILA abortus –Serum-B** version:

127 P00700/04 -18/02/05 (Institut Pourquier, Montpellier, France). The assay was

128 performed according to manufacturer's instructions.

129 (vii & viii) **rOMP90-3 & rOMP90-4 iELISAs**. The assays were performed as

130 described previously, using 3,3',5,5'-tetramethylbenzidine (TMB) as substrate

131 (Longbottom et al., 2001, 2002) .

132 For the six ELISA based tests (iii to viii) absorbance readings (Optical

133 Densities (ODs)) were measured at 450nm using a Labsystems iEMS MF microplate

134 reader (Thermo Life Science, Basingstoke, UK). Results were normalised using the

135 positive and negative control sera supplied with the commercial kits or derived from

136 experimentally infected post-abortion sheep and EAE-free sheep, respectively, and

137 expressed as a percentage of the positive control according to the following formula:

138 $[(\text{OD sample} - \text{OD negative control}) / (\text{OD positive control} - \text{OD negative control})] \times$

139 100. For tests (iii), (iv) and (v), sera with values less than 30% were considered

140 negative, sera with values between 30% and 40% were ambiguous, and sera with

141 values greater than 40% were positive. For tests (vi), (vii) and (viii), sera with values

142 less than 50% were considered negative, sera with values between 50% and 60% were

143 doubtful, while sera with values greater than 60% were considered positive. Equivocal

144 (test (i)), ambiguous (tests (iii) to (v)) and doubtful (tests (vi) to (viii)) results were

145 considered positive for calculation purposes and for determining concordance

146 between tests.

147

148 2.2 Statistical analysis

149 The appropriate cut-off boundaries for each test were determined using the

150 estimated 1%, 5%, 95% and 99% quantiles of the distributions for the negative and

151 positive animals respectively (data not shown). For each test, the aim was to ensure
152 very few false positives whilst maintaining greater than 95% sensitivity. All statistical
153 analyses were carried out using Genstat 10th edition.

154

155 2.2 Animal Sera

156 A total of 202 ovine and caprine experimental and field serum samples were
157 tested in each of the assays. These sera have been previously described (Longbottom
158 et al., 2002). Group 1 consisted of sera from 62 ewes that were experimentally
159 infected at 70 to 75 days gestation with *C. abortus* isolate S26/3 and yielded heavily
160 infected placentas and/or aborted (n=52; Group 1A), or which lambled normally and
161 yielded lightly infected placentas or had no placental lesions but were positive by cell
162 culture or modified Ziehl Neelsen (n=10; Group 1B). Group 2 consisted of sera from
163 52 ewes that were known through their participation in the UK Sheep and Goat
164 Premium Health Scheme for EAE to be clinically free of disease and which were used
165 as negative controls in experimental trials. Group 3 comprised 9 samples from
166 specific-pathogen-free (SPF) lambs that had been experimentally infected with
167 arthritogenic (strain P787; n=3), conjunctival (strain 84-796; n=2) or enteric (strain
168 W73; n=4) subtypes of *C. pecorum*. Group 4 comprised 38 field samples from a flock
169 of ewes from Scotland in which there was clinical evidence of EAE. Group 5
170 comprised 30 field serum samples from a herd of goats from New Zealand from
171 which an arthritogenic subtype of *C. pecorum* had been isolated. Group 6 consisted of
172 11 serum samples from a flock of ewes from the Republic of Ireland from which an
173 enteric subtype of *C. pecorum* (subtype W73) (Markey et al., 1993b) had been
174 isolated.

175

176 **3. Results**177 *3.1 Performance of the serological tests with experimental sera*

178 The sensitivities and specificities of the eight serological assays were
179 evaluated using a panel of sera derived from ewes experimentally infected with *C.*
180 *abortus* (Groups 1A and 1B), from ewes clinically free of EAE (Group 2) and from
181 SPF lambs that had been experimentally infected with different subtypes of *C.*
182 *pecorum* (Group 3). The results are shown in Table 1, while the distribution of sero-
183 positive and –negative data is illustrated in Figure 1.

184 The Pourquier, rOMP90-3 and rOMP90-4 iELISAs were the most specific
185 with the Group 3 SPF *C. pecorum* sera, with none identified as positive. However, of
186 these three tests, the rOMP90-3 iELISA was the only one that did not produce any
187 false positives with the Group 2 sera (100% specificity), while the Pourquier test
188 produced one high false positive (test result of 95%) and rOMP90-4 test produced two
189 false positives, one of which was borderline (test results of 56 & 72%). The EB
190 iELISA also had a specificity of 100% with the Group 2 sera, but cross reacted with 5
191 of the 9 Group 3 SPF sera. The CFT, ImmunoComb, SolPr and CHEKIT tests also
192 identified between 3 and 5 of the Group 3 serum samples as positive, although the
193 CHEKIT and CFT reacted with sera from animals infected with the arthritogenic and
194 conjunctival but not enteric subtypes. Indeed, three of the Group 3 serum samples that
195 were negative with all the tests were from SPF lambs immunized with an enteric
196 subtype of *C. pecorum*. All the tests other than the CFT (titre of 2+ at 1/32) and
197 ImmunoComb were also negative with sera from a lamb infected with the S45 strain
198 of *Chlamydia suis* (results not shown). The SolPr iELISA performed similarly to the
199 EB iELISA, but with one extremely borderline false positive (test result of 30%) with
200 the Group 2 sera (98.1% specificity). The one CFT positive serum sample with the

201 Group 2 sera had a high titre of 4+ at 1/128, whereas the two false positives with the
202 CHEKIT were well above the test cut-off of 40% (test results of 52 and 79%). The
203 ImmunoComb was the least specific test, identifying 18 of the Group 2 serum samples
204 as positive (65.4% specificity), and also identifying the most Group 3 sera (6 of 9) as
205 positive.

206 In terms of sensitivity, the ImmunoComb appeared to be the most sensitive
207 test (98.4% sensitivity), identifying all but one of the Group 1 samples as being
208 positive (Table 1). The EB and rOMP90-3 iELISAs were the next most sensitive tests
209 (both 96.8%), each producing two false negative results, one of which, for both
210 assays, was from the Group 1B sera, and originated from an ewe that lambed
211 normally with low grade placental lesions. This particular serum sample was also
212 negative by the other 6 tests. The CFT and Pourquier test both had 4 false negative
213 samples (93.5% sensitivity), 3 of which were from Group 1B. Similarly, 4 of the 5
214 false negatives with the SolPr test were from Group 1B. Two of the 3 false negative
215 Group 1A samples with the rOMP90-4 iELISA were just below the 50% cut-off for
216 doubtful samples at 47% and 49.1%. The least sensitive test was the CHEKIT, failing
217 to detect chlamydial antibodies in 9 of the 62 Group 1 serum samples (85.5%
218 sensitivity), 6 of which came from Group 1A animals that either aborted or lambed
219 but had heavily infected placentas.

220

221 *3.2 Performance of the serological tests with field sera*

222 All of the serological assays were evaluated further using a panel of 79 field
223 sera (Groups 4-6; Table 2 & Fig. 1). Group 4 comprised 38 samples originating from
224 a Scottish flock of ewes in which clinical evidence of EAE had been proven. The
225 ImmunoComb assay identified 84.2% of samples (32 sera) as being seropositive, the

226 highest number of positives detected for all the assays. This was followed by the EB
227 ELISA which detected 73.7% (28 sera) as positive, with the SolPr, CFT, CHEKIT,
228 rOMP90-4 and rOMP90-3 tests detecting similar numbers of positive samples
229 (ranging from 25 to 21 seropositives). These results contrasted with those of the
230 Pourquier ELISA, which only identified 39.5% of serum samples (15 sera) as
231 seropositive. A total of 11 (28.9%) and 5 (13.2%) of the 38 samples were identified as
232 positive or negative, respectively, with all the tests. Of the 22 samples for which there
233 was no complete agreement (57.9% discordant), 8 samples were positive by all but
234 one of the tests (5 of these were specifically negative with the Pourquier test) and a
235 further 5 were positive by all but 2-3 tests. The remaining 9 discordant results were
236 negative with all but 1-2 (6 samples) or 3-4 (3 samples) of the tests.

237 The results obtained with the Group 5 sera, which consisted of 30 serum
238 samples from a herd of goats infected with an arthritogenic subtype of *C. pecorum*,
239 were particularly interesting. Both rOMP90 iELISAs performed well, with the
240 rOMP90-3 assay producing no false positive results and the rOMP90-4 assay only
241 incorrectly identifying 1 of the 30 samples as positive. The Pourquier, EB and SolPr
242 ELISAs each identified 2 samples as positive, although between 1 and 6 further
243 samples were considered doubtful or ambiguous (Table 2). CHEKIT unambiguously
244 identified 7 of the 30 samples as positive. However, the results obtained by CFT and
245 ImmunoComb were distinct from the other assays as both identified a large number of
246 samples, 14 and 19 respectively, as being positive. Overall, 8 of the 30 samples
247 (26.7%) were negative by all 8 tests, although there were a further 18 samples that
248 were only positive with up to 3 of the tests. The remaining 4 samples were considered
249 positive by four or more of the tests.

250 Group 6 consisted of 11 serum samples obtained from a flock of sheep
251 infected with an enteric subtype of *C. pecorum*. On the whole, the tests showed little
252 reactivity with these sera, except for the CHEKIT and EB ELISAs where both tests
253 identified 1 sample as ambiguous, and the ImmunoComb assay which identified 3
254 seropositives.

255

256 3.3 Concordance between serological tests

257 The concordances between the 8 tests based on the results obtained with the
258 individual experimental serum samples and the field serum samples are presented in
259 Tables 3 and 4, respectively. Overall, the concordance of the assay results was greater
260 and less variable for the experimental than the field sera. Agreement was particularly
261 good between the CFT, SolPr and EB assays with the experimental sera (96.7%), but
262 considerably lower with the field sera (77.2 – 88.6%), mainly as a consequence of the
263 CFT identifying more samples as positive. The same appears true when comparing the
264 CFT with the field sera using the other tests (67.1-78.5%; Table 4), with the exception
265 of the ImmunoComb, which did not agree well with any of the tests with either the
266 experimental (75.6-83.7%) or the field (49.4-74.7%) sera, largely due to the detection
267 of the greatest number of samples as positive across all groups. The CHEKIT also
268 performed better with the experimental (concordances of 89.4-91.1%) than with the
269 field (72.2-82.3%) sera, when compared to the other tests (not including the
270 ImmunoComb results). The best agreement between tests with the experimental sera
271 was obtained for the Pourquier, rOMP90-3 and rOMP90-4 iELISAs (95.1-95.9%),
272 although this again dropped with the field sera (84.8-93.7%), mainly as a result of the
273 Pourquier test producing more false negative results. With the field sera, the best
274 agreement was obtained for the rOMP90-3 and rOMP90-4 tests (93.7%).

275

276 **4. Discussion**

277 Over the last 20 years there have been a number of serological assays
278 developed in an attempt to improve on the specific detection of antibodies to *C.*
279 *abortus* and thus the diagnosis of EAE infected sheep flocks. Most of these tests are
280 based on reactivity to the three main immunodominant antigens on the surface of the
281 chlamydial EB, namely LPS, MOMP and POMP, using whole EBs, solubilised EB
282 extracts, peptides and recombinant antigen preparations (Anderson et al., 1995;
283 Griffiths et al., 1996; Kaltenboeck et al., 1997; Salti-Montesanto et al., 1997;
284 Longbottom et al., 2001, 2002; Hoelzle et al., 2004). All of these antigens contain
285 epitopes shared between the two principal chlamydial species that infect livestock, *C.*
286 *abortus* and *C. pecorum*, thus making it difficult to differentiate between resultant
287 infections. In an attempt to address this situation, more specific tests based on specific
288 regions of MOMP and POMP have been developed and shown to have improved
289 specificity (Salti-Montesanto et al., 1997; Longbottom et al., 2001, 2002; Livingstone
290 et al., 2005).

291 During the course of this study we have compared the performance of two
292 POMP iELISAs (rOMP90-3 and rOMP90-4 (Longbottom et al., 2002)) with two
293 commercial *C. abortus*-specific ELISAs, the CHEKIT-Chlamydomphila Abortus and
294 the POURQUIER CHLAMYDOPHILA abortus tests, and one commercial
295 *Chlamydomphila*-specific test, the ImmunoComb Ovine *Chlamydomphila* Antibody test.
296 Furthermore, we have also compared the sensitivities and specificities of these tests
297 with two 'in-house' ELISAs based on whole EBs and a detergent solubilised
298 preparation of EBs (SolPr test) to determine whether any of these tests are an
299 improvement on the CFT, which is the only test currently recognised by the World

300 Organisation for Animal Health (OIE) for diagnosing EAE (Aitken and Longbottom,
301 2004).

302 The results of the test comparisons show that the rOMP90-3 iELISA
303 performed the best in terms of both sensitivity and specificity. It was the most specific
304 of all the tests since it did not produce any false positive results with the standard
305 reference sera from EAE-free flocks, and did not cross react with the sera from SPF
306 lambs that had been experimentally infected with different *C. pecorum* subtypes, or
307 with field sera from animals naturally infected with arthritogenic or enteric strains of
308 *C. pecorum*. Of the other tests the rOMP90-4 and Pourquoi ELISAs were the only
309 ones that also did not react with the experimental SPF *C. pecorum* sera, although both
310 reacted with a small number of field sera from goats infected with the arthritogenic
311 subtype of *C. pecorum*. The rOMP90-3 test was also one of the most sensitive of these
312 tests, as shown by the results obtained with both the experimental (Group 1) and field
313 (Group 4) sera from flocks infected with *C. abortus*. The Pourquoi test however was
314 less sensitive than the other tests with the field sera from the aborted ewes (Group 4),
315 as also recently observed by Vretou et al. (2007). These authors suggest that this is
316 likely to be due to the choice of recombinant POMP fragment used in the test or is a
317 consequence of the relatively high cut-off value of 60%. We believe that it is most
318 likely due to the former rather than the latter, as we have previously demonstrated
319 wide variation in sensitivity and specificity when assessing overlapping recombinant
320 fragments of POMP90 (Longbottom et al., 2002). Furthermore, 8 of the Group 4
321 Pourquoi negative samples that are positive with at least 5 of the other tests have
322 values of only 32.2-16%, which are considerably less than the 60% cut-off and are
323 comparable to the values observed in the negative groups.

324 Comparing the rOMP90-3, rOMP90-4 and Pourquier ELISAs to the CFT, we
325 can see quite clearly that the CFT has performed similarly in terms of sensitivity but
326 not in terms of specificity, significantly reacting with sera from animals both
327 experimentally and naturally infected with arthritogenic and conjunctival subtypes of
328 *C. pecorum*. As previously noted (Jones et al., 1997; Longbottom et al., 2002), this
329 could suggest that the reported lack of specificity of this test in the field is due to these
330 arthritogenic/conjunctival subtypes rather than the more common enteric subtype.
331 These results contrast somewhat with another recently published study (McCauley et
332 al., 2007) where the authors concluded that the rOMP90-3, rOMP90-4 and Pourquier
333 ELISAs performed comparably to the CFT. Although it should be noted that this was
334 only when using an avian source of CFT antigen, as with an ovine abortion antigen
335 source the sensitivity of the CFT dropped from around 95% to 60%. It should also be
336 pointed out that although they achieved 100% specificity with their CFTs, this was
337 based only on field sera obtained from New Zealand, a country free of EAE, and no
338 analysis was performed using sera from *C. pecorum* infected flocks to assess cross
339 reaction with this species.

340 The CHEKIT, SolPr, and EB ELISAs performed comparably in terms of
341 specificity with the *C. pecorum* sera in Groups 3, 5 and 6, showing that the CHEKIT
342 test is not specific for *C. abortus*. The CHEKIT was also the least sensitive of all the
343 tests identifying fewer of the sera from ewes that aborted (Group 1A) as positive. The
344 EB and SolPr ELISAs produced similar results to the CFT and would be suitable
345 alternatives to this test for identifying both *C. abortus* and *C. pecorum* infections, with
346 the exception perhaps of enteric *C. pecorum* infections. The ImmunoComb was the
347 least accurate of all the tests, showing poor specificity with a large number of false
348 positives identified with the EAE-free (Group 2) samples. This resulted in an apparent

349 overestimation of positivity with the other sera, as reflected by the lower concordance
350 figures when compared to the other tests. This is probably largely due to the
351 subjective nature of the test and resultant difficulties in interpretation of results.

352 It is becoming increasingly evident from the limited number of studies
353 comparing the various serological assays that are available, whether commercially-
354 available or 'in-house', as well as from seroepidemiological studies, that there is an
355 urgent need for standardisation and harmonisation through inter-laboratory trials
356 across Europe and further afield. However, in order to ensure proper validation and
357 comparison of the tests, it would be important to identify suitable serum panels
358 derived from animals of known individual clinical status, whether resulting from
359 experimental or natural infection, to ensure accurate interpretation of the results.
360 Ultimately, the aim is to identify a suitable alternative to the CFT that is both more
361 sensitive and specific, but is also able to detect infection earlier during pregnancy so
362 that appropriate control measures can be implemented prior to abortion occurring
363 rather than act as a retrospective diagnostic tool. The results from this study show that
364 the rOMP90-3 iELISA meets these first requirements, while previous studies have
365 demonstrated the utility of POMP based ELISAs in early detection (Livingstone et al.,
366 2005). The rOMP90-3 ELISA is not only more specific than the CFT but is also more
367 sensitive and specific than the Pouquier ELISA, which currently appears to be the best
368 commercially available test for detecting EAE-infected flocks.

369

370 **Conflict of interest statement**

371 None of the authors (K. Wilson, M. Livingstone or D. Longbottom) has a
372 financial or personal relationship with other people or organisations that could

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375

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381

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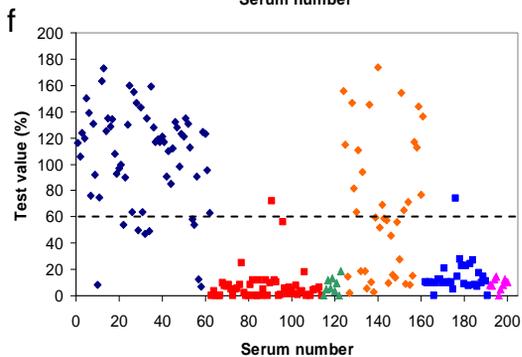
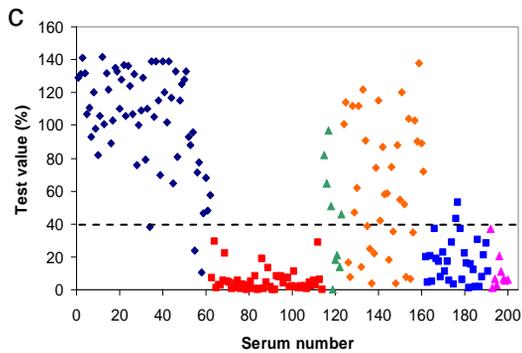
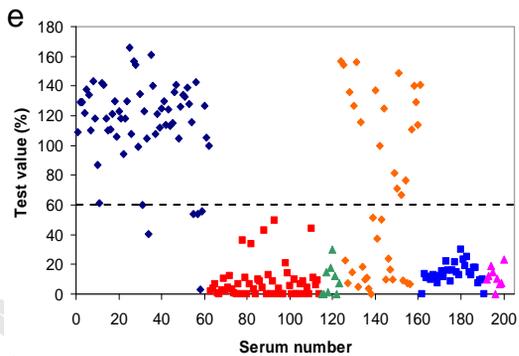
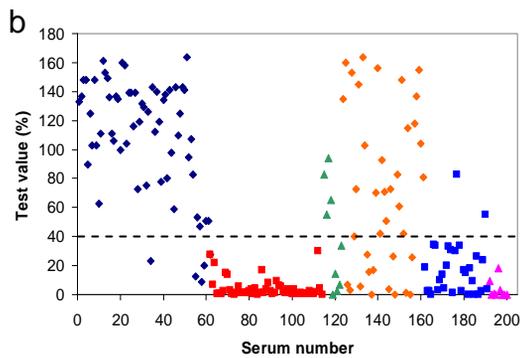
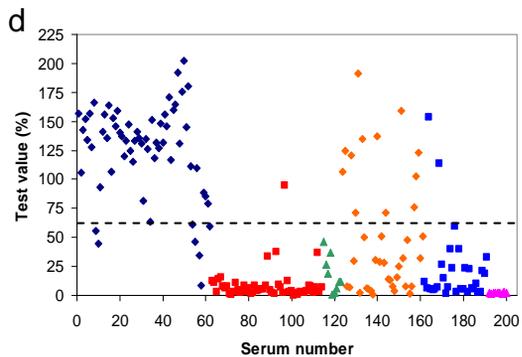
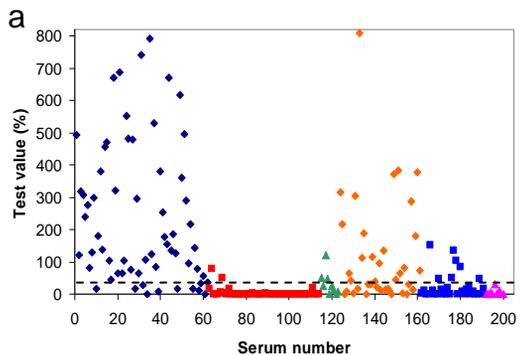
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456
457

457 Figure legend

458 Figure 1. Distribution of results obtained for each ELISA (CHEKIT (a), SolPr (b), EB
459 (c), Pourquier (d), rOMP90-3 (e) and rOMP90-4 (f)) with experimental and field sera.
460 Tests were evaluated with sera from sheep experimentally infected with *C. abortus*
461 (Group1A, serum samples 1-52; Group 1B, samples 53-62); from EAE-free flocks
462 (Group 2, samples 63-114); from SPF lambs immunised with different subtypes of *C.*
463 *pecorum* (Group 3, samples 115-123); from an EAE-infected flock (Group 4, samples
464 124-161); and from animals infected with arthritogenic (Group 5, samples 162-191)
465 or enteric (Group 6, samples 192-202) subtypes of *C. pecorum*. For further details see
466 Materials and Methods. The cut-off for each test is shown as a dashed horizontal line.



ACC

1 Table 1. Evaluation of the eight serological assays with experimental sera

2

Test	Group 1A	Group 1B	Group 1A + 1B		Group 2		Group 3 positives		
	^a Positive	Positive	Positive	^b Sensitivity (%)	Positive	^c Specificity (%)	Arthrit	Conjunc	Enteric
CFT	48 (+3)	7	55 (+3)	93.5	1	98.1	2	2	0
ImmunoComb	52	9	61	98.4	18	65.4	3	2	1
SolPr	51	6	57	91.9	0 (1)	98.1	2	2	0 (1)
EB	51 (+1)	8	59 (+1)	96.8	0	100	2	2	1
CHEKIT	46	5 (+2)	51 (+2)	85.5	2	96.2	2	1	0
Pourquier	50 (+1)	6 (+1)	56 (+2)	93.5	1	98.1	0	0	0
rOMP90-3	51	6 (+3)	57 (+3)	96.8	0	100	0	0	0
rOMP90-4	47 (+2)	6 (+2)	53 (+4)	91.9	1 (1)	96.2	0	0	0

3

4 Group 1A ($n = 52$) and 1B ($n = 10$), sera from ewes experimentally infected with *C. abortus*; group 2, sera from EAE-free flocks ($n = 52$); group
5 3, sera from SPF lambs vaccinated with arthritogenic ($n = 3$), conjunctival ($n = 2$) or enteric ($n = 4$) subtypes of *C. pecorum*. ^aNumber of positive
6 sera (number of doubtful/ambiguous/equivocal results are indicated in brackets). ^bSensitivity = [number of true positives/(number of true
7 positives + number of false negatives)] x 100. ^cSpecificity = [number of true negatives/(number of true negatives + number of false positives)] x
8 100.

1 Table 2. Evaluation of the eight serological assays with field sera
2

Test	Group 4		Group 5		Group 6	
	^a No. positive	^b Positive (%)	No. positive	Positive (%)	No. positive	Positive (%)
CFT	22 (+2)	63.2	10 (+4)	46.7	0	0
ImmunoComb	32	84.2	19	63.3	3	27.3
SolPr	24 (+1)	65.8	2 (+6)	26.7	0	0
EB	25 (+3)	73.7	2 (+3)	16.7	0 (+1)	9.1
CHEKIT	20 (+4)	63.2	7	23.3	0 (+1)	9.1
Pourquier	12 (+3)	39.5	2 (+1)	10.0	0	0
rOMP90-3	19 (+2)	55.3	0	0	0	0
rOMP90-4	18 (+5)	60.5	1	3.3	0	0

3
4 Group 4, sera from a flock of ewes with a documented history of EAE ($n = 38$); group 5, sera from a herd of goats infected with an arthritogenic
5 subtype of *C. pecorum* ($n = 30$); group 6, sera from a flock of ewes infected with an enteric subtype of *C. pecorum* ($n = 11$). ^aNumber of positive
6 sera (number of doubtful/ambiguous/equivocal results are indicated in brackets). ^bNumber of positive sera as a percentage of total number of
7 serum samples.

1 Table 3. Concordance between the results obtained for the eight assays with experimental sera

2

	ImmunoComb		SolPr		EB		CHEKIT		Pourquier		rOMP90-3		rOMP90-4	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
CFT														
Positive	62	1	61	2	62	1	55	8	56	7	58	5	55	8
Negative	23	37	2	58	3	57	3	57	3	57	2	58	4	56
% Concordance ^a		80.5		96.7		96.7		91.1		91.9		94.3		90.2
ImmunoComb														
Positive			62	23	65	20	58	27	59	26	61	25	57	28
Negative			1	37	0	38	0	38	0	38	0	37	2	36
% Concordance				80.5		83.7		78.0		78.9		79.7		75.6
SolPr														
Positive					62	1	54	9	55	8	57	6	54	9
Negative					3	57	4	56	4	56	3	57	5	55
% Concordance						96.7		89.4		90.2		92.7		88.6
EB														
Positive							56	9	58	7	59	6	56	9
Negative							2	56	1	57	1	57	3	55
% Concordance								91.1		93.5		94.3		90.2
CHEKIT														
Positive									52	6	53	5	52	6
Negative									7	58	7	58	7	58
% Concordance										89.4		90.2		89.4
Pourquier														
Positive											57	2	56	3
Negative											3	61	3	61
% Concordance												95.9		95.1
rOMP90-3														
Positive													57	3
Negative													2	61
% Concordance														95.9

3

4 ^a Concordance is the sum of positive-positive values and negative-negative values expressed as a percentage of the total number of serum samples.

1 Table 4. Concordance between the results obtained for the eight assays with field sera

2

	ImmunoComb		SolPr		EB		CHEKIT		Pourquier		rOMP90-3		rOMP90-4	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
CFT														
Positive	36	2	29	9	27	11	25	13	15	23	21	17	21	17
Negative	18	23	4	37	7	34	7	34	3	38	0	41	3	38
% Concordance ^a		74.7		83.5		77.2		74.7		67.1		78.5		74.7
ImmunoComb														
Positive			33	21	34	20	31	23	16	38	21	33	24	30
Negative			0	25	0	25	1	24	2	23	0	25	0	25
% Concordance				73.4		74.7		69.6		49.4		58.2		62.0
SolPr														
Positive					29	4	24	9	16	17	21	12	23	10
Negative					5	41	8	38	2	44	0	46	1	45
% Concordance						88.6		78.5		75.9		84.8		86.1
EB														
Positive							26	8	16	18	21	13	23	11
Negative							6	39	2	43	0	45	1	44
% Concordance								82.3		74.7		83.5		84.8
CHEKIT														
Positive									14	18	18	14	19	13
Negative									4	43	3	44	5	42
% Concordance										72.2		78.5		77.2
Pourquier														
Positive											14	4	15	3
Negative											7	54	9	52
% Concordance												86.1		84.8
rOMP90-3														
Positive													20	1
Negative													4	54
% Concordance														93.7

3

4 ^a Concordance is the sum of positive-positive values and negative-negative values expressed as a percentage of the total number of serum samples.