



HAL
open science

Molecular detection of in post-abortion sheep at oestrus and subsequent lambing

Morag Livingstone, Nicholas Wheelhouse, Stephen W. Maley, David
Longbottom

► **To cite this version:**

Morag Livingstone, Nicholas Wheelhouse, Stephen W. Maley, David Longbottom. Molecular detection of in post-abortion sheep at oestrus and subsequent lambing. *Veterinary Microbiology*, 2009, 135 (1-2), pp.134. 10.1016/j.vetmic.2008.09.033 . hal-00532490

HAL Id: hal-00532490

<https://hal.science/hal-00532490>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Molecular detection of *Chlamydophila abortus* in post-abortion sheep at oestrus and subsequent lambing

Authors: Morag Livingstone, Nicholas Wheelhouse, Stephen W. Maley, David Longbottom



PII: S0378-1135(08)00400-8
DOI: doi:10.1016/j.vetmic.2008.09.033
Reference: VETMIC 4185

To appear in: *VETMIC*

Please cite this article as: Livingstone, M., Wheelhouse, N., Maley, S.W., Longbottom, D., Molecular detection of *Chlamydophila abortus* in post-abortion sheep at oestrus and subsequent lambing, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.033

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Molecular detection of *Chlamydophila abortus* in post-abortion sheep at oestrus**
2 **and subsequent lambing**

3

4 Morag Livingstone, Nicholas Wheelhouse, Stephen W. Maley, David Longbottom*

5

6 Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik,

7 Midlothian, EH26 OPZ, UK

8

9

10

11

12

13

14

15

16 *Corresponding author. Tel.: +44 131 445 5111; fax: +44 131 445 6235. E-mail:

17 david.longbottom@moredun.ac.uk.

18

18 **Abstract**

19 Enzootic abortion of ewes (EAE), resulting from infection with the bacterium
20 *Chlamydomphila abortus* (*C. abortus*), is a major cause of lamb loss in Europe. The
21 purpose of this study was to assess the potential impact of the shedding of organisms
22 in post-abortion ewes at oestrus and subsequent lambing on the epidemiology of EAE.
23 Using a newly developed *C. abortus* specific real-time PCR assay, few chlamydial
24 genomes could be detected in vaginal swabs taken from post-abortion ewes at oestrus.
25 At subsequent parturition, all ewes lambed normally with no macroscopic or
26 microbiological evidence of infection. Real-time PCR analysis of placental samples
27 identified very few or no chlamydial genomes, which contrasted significantly with
28 samples taken at the time of abortion, where an average of 2.7×10^7 chlamydial
29 genomes per microgram of total tissue DNA was detected. Few genomes could also
30 be detected from vaginal and cervical tissue samples and lymph nodes taken post
31 mortem. The results, although not discounting the possibility of a chronic low level
32 persistent infection in post-abortion ewes, suggest that the low levels of chlamydial
33 DNA detected during the periovulation period and at lambing do not significantly
34 impact on the epidemiology of EAE. In terms of flock management, the products of
35 abortion should be considered the major and principal source of infection for
36 transmission to naïve ewes.

37

38 **Keywords:** *Chlamydomphila abortus*; enzootic abortion of ewes; oestrus; real time
39 PCR; serological detection

40

41

41 1. Introduction

42 *Chlamydophila abortus*, the aetiological agent of enzootic abortion of ewes
43 (EAE) (also known as ovine enzootic abortion or OEA), is a major cause of lamb loss
44 in many sheep-rearing countries throughout the world. The disease also affects goats
45 and, to a lesser degree, cattle, horses, pigs and deer, although little is known about the
46 incidence of these infections due to a lack of epidemiological data (Longbottom and
47 Coulter, 2003).

48 Animals infected prior to pregnancy exhibit no clinical signs of infection, with
49 the organism entering into a latent phase. It is not until around day 90-95 of
50 pregnancy that *C. abortus* can first be detected in the placenta (Buxton et al., 1990).
51 The organism establishes itself in the trophoblast cells of the fetal chorionic
52 epithelium, spreading to the surrounding intercotyledonary membranes, where it gives
53 rise to the typical thickened and necrotic placental lesions that are associated with the
54 disease (Buxton et al., 2002). Usually the first clinical manifestation of disease is
55 abortion in the last 2-3 weeks of gestation or when the ewe gives birth to stillborn or
56 weak lambs, although ewes may exhibit a vaginal discharge 1-2 days prior to abortion
57 occurring (Aitken and Longbottom, 2007). These discharges, and those occurring
58 following abortion or lambing, as well as the placentas, foetuses, and coats of lambs
59 contain large numbers of infectious organisms that are the major source of infection
60 for other susceptible animals (Longbottom and Coulter, 2003). During an extended
61 lambing period it is possible for other naïve ewes to pick up infection and abort in the
62 same pregnancy. Where ewes become infected after around 110 days of gestation (i.e.
63 within the last 5 weeks of pregnancy) they would not be expected to abort in that
64 pregnancy, although such animals may go on to abort in the subsequent pregnancy
65 (Aitken and Longbottom, 2007).

66 Infection can also be spread via vertical transmission from ewe to offspring,
67 however there is little evidence to suggest that this has a significant role to play in the
68 spread of disease (Rodolakis and Bernard, 1977). Although ewes develop immunity
69 and do not experience further EAE abortive episodes, this immunity is not thought to
70 be sterile. Two studies in the 1990s have suggested that ewes may become
71 persistently infected carriers, shedding chlamydiae during subsequent oestrus cycles,
72 thereby providing an opportunity for venereal transmission during breeding (Papp et
73 al., 1994; Papp and Shewen, 1996b). They may also shed infectious organisms at
74 subsequent lambing, thus contributing to the spread of infection to naïve animals
75 (Wilsmore et al., 1990).

76 Management strategies are principally concerned with the containment and
77 control of disease at abortion or lambing time to prevent exposure of infection to
78 naïve animals. Therefore, the suggestion by Papp et al. (1994, 1996a) that post-
79 abortion ewes continue to excrete organisms at oestrus raised new questions regarding
80 the possibility of venereal or mechanical transmission of EAE by rams. Yet despite
81 the potential importance of these findings, there have to date been no further
82 published studies confirming these findings. Furthermore, in the decade since these
83 initial studies were performed, there have been major advances in the development
84 and availability of molecular diagnostic techniques that allow the rapid and accurate
85 quantification of organisms, and, importantly, enable the discrimination of different
86 chlamydial species. Therefore, the purpose of this study was to reinvestigate the level
87 of chlamydial excretion that occurs at oestrus and subsequent lambings in post-
88 abortion ewes, using a highly sensitive and newly developed *C. abortus*-specific real
89 time PCR assay. The interpretation and potential impact of the results on the
90 epidemiology of EAE are discussed.

91

92 2. Materials and methods

93 2.1. Animals and Experimental Design

94 Twenty adult Scottish Blackface ewes, from a flock known to be free of EAE
95 and serologically negative for *C. abortus* antibodies by rOMP90-3 and rOMP90-4
96 indirect ELISA (Longbottom et al., 2002) were randomly assigned to 2 groups and
97 mated, following synchronisation with progesterone sponges (Veramix, Upjohn Ltd.,
98 Crawley, UK). At day 75 of gestation ten ewes received a subcutaneous injection,
99 over the left prefemoral lymph node, of 2×10^6 inclusion forming units (IFU) egg-
100 grown *C. abortus* strain S26/3 (McClenaghan et al., 1984) in a volume of 1ml. The
101 remaining group of 10 ewes were housed separately from the infected animals and
102 acted as negative control animals; receiving 1ml of control inoculum, which was
103 prepared from uninfected yolk sac material. All animals were monitored throughout
104 pregnancy and blood samples were taken at regular intervals for serological analysis.
105 At lambing or abortion, placentas and blood samples were collected for analysis.

106 Approximately 7 months post parturition, all previously infected ewes and
107 control ewes were synchronized, as described above, and successfully re-bred.
108 Vaginal swabs were taken during the oestrus cycle, between 2 weeks pre and post
109 ovulation. Blood was taken throughout pregnancy and 3 months post lambing for
110 serological analysis. At lambing, placentas were collected for analysis by real-time
111 PCR. Three months following parturition, ewes were euthanized by intravenous
112 injection of pentobarbitone sodium (Euthatal; Merial Animal Health Ltd., Harlow,
113 UK). Reproductive tract tissue and lymph nodes were removed at necropsy. The care
114 and use of experimental animals were approved by the Institute's Experiments and
115 Ethical Review Committee and complied with both Home Office Regulations and all
116 local animal health and welfare policies.

117

118 *2.2. Sampling methods*

119 Placentas were collected, macroscopically assessed for EAE lesions, and
120 representative cotyledons removed for bacteriological and pathological analysis.
121 Cotyledons and surrounding intercotyledonary membrane were placed in 4ml sucrose-
122 phosphate-glutamate buffer (SPG) (Spencer and Johnson, 1983) and immediately
123 frozen at -20°C for subsequent recovery of organisms in cell culture and for real-time
124 PCR analysis. Similar samples were also stored at +4°C for the subsequent
125 preparation of smears. For the detection of organisms at oestrus, the external opening
126 of the vagina was firstly cleaned with chlorhexidine gluconate solution prior to
127 insertion of swabs. Two vaginal swabs per animal were then taken by rotating cotton-
128 tipped swabs (Barloworld Scientific, Stone, UK) around the wall of the vagina,
129 approximately 2 cm distal to the cervix, and placed in 2 ml SPG. All swabs were
130 stored at -20°C prior to DNA extraction. Sheep were bled at approximately monthly
131 intervals by jugular venipuncture. At necropsy, samples of vagina (2 cm distal to the
132 cervix) and cervix (2 cm distal to the uterus), as well as uterine, mesenteric and
133 prefemoral lymph nodes, were placed in cryovials, immediately snap frozen in liquid
134 nitrogen and stored at -70°C until required for DNA extraction. Rigorous procedures
135 were observed to ensure tissues were sampled from the same anatomical location in
136 each animal. Strict aseptic precautions, including the use of new sets of instruments
137 and blades, were applied between each sample to avoid cross-contamination.

138

139 *2.3. Bacteriological analysis*

140 Following parturition, smears of placental membranes or vaginal swabs were
141 prepared and stained by the modified Ziehl-Neelsen (mZN) method (Stamp et al.,
142 1950). Smears were then examined under high-power microscopy for the presence of
143 chlamydial elementary bodies (EBs) and any other contaminating bacteria. Isolation

144 of chlamydial organisms was attempted in cell culture. Placental cotyledons were
145 aseptically ground in SPG and dilutions (1/60) prepared in complete RPMI medium
146 containing 1 µg/ml cycloheximide (Sigma-Aldrich Company Ltd., Poole, UK), while
147 vaginal swabs were vortexed in SPG and diluted 1/5 and 1/10 in RPMI medium.
148 Diluted material was inoculated onto confluent McCoy cell monolayers grown in
149 complete RPMI medium on coverslips in trac bottles (Barloworld Scientific., Stone,
150 UK). The bottles were centrifuged at $3,000 \times g$ at room temperature for 2×15 min
151 and incubated at 37°C in 5% CO₂. After 72 h, coverslips were fixed in methanol,
152 stained using Giemsa 'Gurr' (Merck Ltd., Poole, United Kingdom), and examined for
153 the presence of chlamydial inclusions by light microscopy.

154

155 *2.4. Serological analysis*

156 Sera were analysed by rOMP90-3 & rOMP90-4 indirect enzyme-linked
157 immunosorbent assay (iELISA), as described previously, except TMB substrate was
158 used in place of OPD (Longbottom et al., 2001; Longbottom et al., 2002). Optical
159 densities (ODs) were measured at 450nm using a Labsystems iEMS MF microplate
160 reader (Thermo Life Science, Basingstoke, United Kingdom).

161

162 *2.5. DNA extraction*

163 Prior to DNA extraction, swab samples, stored in 2 ml SPG, were vortexed
164 vigorously for 20 seconds. 1ml was removed to a clean tube and centrifuged at 14,000
165 rpm for 10 minutes in a microcentrifuge. Genomic DNA was extracted from the
166 resulting pellet using a DNeasy[®] Blood & Tissue Kit (Qiagen Ltd., Crawley, UK),
167 according to the manufacturer's instructions. Tissue samples were finely chopped
168 using sterile blades prior to extracting DNA with the DNeasy[®] Blood & Tissue Kit.

169 All samples were eluted in 200 μ l of supplied buffer (10 mM Tris-HCl, 0.5 mM
170 EDTA, pH 9.0) for analysis by real-time PCR.

171

172 2.6. Real-time PCR

173 The *C. abortus* primers and fluorescent probe were selected following analysis
174 of the *ompA* gene, which encodes the major outer membrane protein (MOMP)
175 (GenBank accession number X51859), using ABI Primer Express software (Applied
176 Biosystems, Warrington, United Kingdom) and synthesized by MWG (MWG-
177 BIOTECH AG, Ebersberg, Germany). The specificity of the primers and probe was
178 confirmed following alignment of the *ompA* genes of all chlamydial species using
179 ClustalV multiple-sequence alignment software (Lasergene MegAlign, DNASTar Inc).
180 The sequences of the selected primers and the TaqMan[®] probe were as follows:
181 forward primer, 5'- GCGGCATTCAACCTCGTT-3'; reverse primer, 5'-
182 CCTTGAGTGATGCCTACATTGG-3'; and TaqMan[®] probe, 5'-
183 TGTTAAAGGATCCTCCATAGCAGCTGATCAG-3'. The TaqMan probe was
184 fluorescently labelled with a 6-carboxy-fluorescein (FAM) reporter molecule attached
185 at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) as the 3' end quencher
186 molecule. The size of the amplification product was 86 bp.

187 For use as a quantitative standard, *C. abortus* S26/3 genomic DNA was
188 extracted from purified elementary bodies using a DNeasy[®] Blood & Tissue Kit and
189 quantified using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, USA).
190 The number of genomes was determined using a calculated mass of 1.17×10^{-15} g per
191 *C. abortus* genome (molecular mass of genome 7.07×10^8 Da), and used to construct a
192 standard curve ranging from 10^1 to 10^6 genome copies per reaction. The specificity of
193 the PCR reaction was confirmed by testing genomic DNA prepared from the

194 following species: *C. abortus* (11 strains), *Chlamydophila caviae* (2), *Chlamydia suis*
195 (1), *Chlamydophila psittaci* (5), *Chlamydophila pecorum* (4) and *Chlamydophila felis*
196 (1), details of which are shown in Table 1. Genomic DNA prepared from each isolate
197 was quantified using the NanoDrop spectrophotometer and 10^3 genome copies were
198 added per PCR reaction and amplified, as described below. In addition, spiked and
199 negative control swabs were included to control for DNA extraction efficiency, as
200 well as potential contamination and inhibitory factors. Spiked swab samples consisted
201 of known numbers of chlamydial genomes, whereas negative swab samples were
202 taken from known uninfected animals.

203 The PCR reaction consisted of 12.5 μ l of 2X TaqMan[®] universal PCR master
204 mix (Applied Biosystems, Warrington, United Kingdom), 300 nM final concentration
205 of each primer, and 250 nM final concentration of fluorescent probe and 3 μ l gDNA
206 made up to a final volume of 25 μ l with sterile deionised water. Amplification and
207 detection were performed using an ABI Prism 7000 sequence detection system
208 (Applied Biosystems), following manufacturer's standard protocols. The thermal
209 cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles
210 of 95°C for 15 s and 60°C for 1 min.

211

212 2.7. Statistical analysis

213 The mean \pm SEM for the serological data were calculated for each time point
214 post infection (p.i.). For statistical analysis, serological data were analysed by
215 comparing antibody responses between different time points using a paired t-test,
216 calculating 95% confidence intervals and treating a P-value of less than 0.05 as
217 significant. Since within-animal variability was small relative to between-animal
218 variability, this approach gave a high power to determine whether changes in antibody
219 response over time were significant.

220

221 **3. Results**222 *3.1. Clinical outcome*

223 In the first year of the study all pregnant ewes experimentally infected with *C.*
224 *abortus* aborted in the final 3 weeks of pregnancy, on average at 136 days gestation
225 (Table 2). All recovered placentas from the infected group exhibited typical EAE-
226 associated macroscopic lesions: necrotic cotyledons, thickened and oedematous
227 surrounding intercotyledonary membranes and a pinkish, thick exudate on the surface
228 (Longbottom and Coulter, 2003). The presence of *C. abortus* was confirmed by both
229 mZN staining of placental smears and by isolation of the pathogen in cell culture. All
230 uninfected control ewes produced healthy lambs, with none of the placentas recovered
231 exhibiting any gross pathology. In addition, organisms could not be detected in
232 smears or by cell culture.

233 In contrast, in the subsequent pregnancy, in the second year of the study, the
234 previously infected ewes all lambed normally at an average of 144 days gestation
235 (Table 2). No EAE lesions were observed, and no organisms could be detected by
236 either mZN staining of placental smears or by culture. All control ewes again lambed
237 normally and no organisms were detectable.

238

239 *3.2. Serology*

240 Blood samples were collected throughout the course of the experiment over a
241 period of 17 months. Serum samples were tested by *C. abortus*-specific rOMP90B-3
242 and rOMP90B-4 ELISAs (Longbottom et al., 2002) and the average antibody
243 responses to both antigens are shown in Figure 1. Following infection, an immediate
244 and statistically significant antibody response to *C. abortus* was observed with both
245 tests ($P < 0.001$ for both tests), with the titres increasing to and peaking at the time of

246 abortion. The responses steadily declined over the remaining months but circulating
247 antibody levels remained elevated at the end of the study. Antibody responses to both
248 antigens showed very similar profiles over the course of the experiment, except that
249 the responses to rOMP90B-4 were slightly lower than for rOMP90B-3. On closer
250 examination of the data, there appeared to be a small increase in antibody response at
251 the time of oestrus, which upon statistical analysis was found to be a significant
252 increase compared to samples taken 4 weeks earlier ($P < 0.001$ for the rOMP90-3
253 iELISA results; $P = 0.003$ for rOMP90-4). Both ELISAs revealed a statistically
254 significant drop in antibody response at the time of lambing (mean 144 dg) compared
255 to the bleeds taken 3 weeks earlier at around 123 dg ($P < 0.001$ for both tests). No
256 antibody responses to *C. abortus* were observed in uninfected control animals by
257 either ELISA (Figure 1).

258

259 3.3. Real-time PCR results

260 The specificity of the real time PCR assay was assessed using genomic DNA
261 prepared from 24 isolates associated with chlamydial infections in animals (Table 1).
262 Amplification was observed only with the *C. abortus* isolates and not with any of the
263 other species tested. The limit of detection was 10 genome copies per reaction, which
264 gave reliable amplification in each of the triplicate wells.

265 In year one, all aborted placentas were found to contain high levels of *C.*
266 *abortus* genomic DNA (Table 3) ranging from $0.6 - 5.9 \times 10^7$ *C. abortus* genomes per
267 μg extracted total tissue DNA. In the subsequent pregnancy, *C. abortus* genomic
268 DNA was detected in the placentas of 3 animals, however the levels were very low in
269 comparison to the previous year and 2 of the 3 were at the limit of detection.

270 *C. abortus* genomic DNA was detected in vaginal swab material from all of
271 the ewes at some point during the oestrus cycle in the second pregnancy (Table 4).

272 The greatest number of positive samples originated from swabs taken around the time
273 of ovulation (± 2 days), when every animal had at least one positive sample. The
274 number of *C. abortus* genomes detected in individual samples also peaked during this
275 period. Although *C. abortus* was detected in ewe 398N on 4 consecutive days and in
276 animal 421N on 3 consecutive days, there was no distinct pattern to the frequency of
277 detection around the time of ovulation. The level of *C. abortus* genomes detected in
278 positive vaginal swabs was generally very low and many samples were found to be at
279 the limit of detection of the assay.

280 Samples of reproductive tract (vagina and cervix), and mesenteric, uterine and
281 prefemoral lymph nodes were obtained from 8 of the ewes at post-mortem. Of these
282 samples, *C. abortus* DNA could only be detected in tissues obtained from one ewe
283 (407N), specifically from the cervix and mesenteric lymph node (Table 3). Real-time
284 PCR analysis was repeated on samples a minimum of 3 times, and on 3 separate
285 sampled areas of tissue with similar results obtained on each occasion.

286

287 **4. Discussion**

288 In this study, we monitored ten *C. abortus*-infected sheep over two lambing
289 seasons to determine the number of organisms that are shed at oestrus, parturition and
290 in abortion material, using a sensitive quantitative real time PCR assay, to assess the
291 impact on the epidemiology of EAE. In the first year of the study, all experimentally
292 infected sheep aborted within the final 3 weeks of pregnancy, as expected. All
293 recovered placentas exhibited the typical gross pathology that is associated with the
294 disease (Longbottom and Coulter, 2003). Large numbers of organisms were detected
295 in these infected placentas following mZN staining of smears and recovery in tissue
296 culture, in agreement with previous studies (Buxton et al., 2002; Sammin et al., 2006).
297 Real-time PCR analysis of DNA extracted from placental samples identified an

298 average of 2.7×10^7 chlamydial genomes per microgram of total tissue DNA,
299 supporting the hypothesis that this material acts as a major source of infection for the
300 transmission of *C. abortus* within a flock.

301 Following abortion, ewes are considered to be immune to further lamb loss
302 (Aitken and Longbottom, 2007), as was observed in this study where all ewes carried
303 to full term (around 145 days gestation) delivering apparently healthy lambs in their
304 subsequent pregnancy. However, three studies in the 1990s suggested that ewes can
305 be chronically or persistently infected continuing to shed organisms at oestrus or at
306 subsequent lambings (Wilsmore et al., 1990; Papp et al., 1994; Papp and Shewen,
307 1996a). Papp and colleagues (Papp et al., 1994; Papp and Shewen, 1996a)
308 demonstrated the presence of chlamydial LPS in vaginal swabs taken at oestrus, using
309 the non-specific and non-quantitative Clearview™ Chlamydia LPS test. Detection of
310 LPS specifically correlated with ovulation, suggesting that venereal transmission or
311 the mechanical transfer of infectious organisms by the ram during mating could be a
312 significant route for the spread of infection within a flock. This idea was further
313 supported by a subsequent study (Papp et al., 1996b) in which they also demonstrated
314 the susceptibility of the vaginal mucosa to infection with *C. abortus*. In the current
315 study, we have also detected *C. abortus* DNA in vaginal swabs taken around oestrus,
316 which at least in part corroborates the findings in these previous studies. However, we
317 have shown using a more specific and quantitative real-time PCR assay that the
318 amount of detectable *C. abortus* DNA found in the vaginal swabs was low and
319 towards the limit of detection. This agrees with a previous study, in which the vaginal
320 swabs tested by the Clearview™ assay were barely positive (Papp et al., 1998).
321 Whether the low level of chlamydial DNA recovered from the vaginal swabs in the
322 current study correlates to a sufficient number of viable organisms to constitute an

323 infective dose of *C. abortus* is debateable. Unfortunately, attempted recovery of
324 organisms in cell culture proved unsuccessful as the vaginal swab material proved
325 toxic to the McCoys cells and so it was not possible to determine the number of viable
326 organisms. However, even if all the detected chlamydial genomes represented viable
327 organisms, these numbers are several fold lower than the experimental dose required
328 to either elicit abortion (Appleyard et al., 1985) or the birth of weak lambs (Papp and
329 Shewen, 1996b) following vaginal inoculation with *C. abortus*. Thus, although it is
330 attractive to postulate that a venereal or mechanical transfer of *C. abortus* may occur
331 during mating, it appears from this evidence to be unlikely or a low risk.

332 Following primary infection, serum IgG antibody levels remained elevated for
333 the duration of the study, as has been observed previously (Papp et al., 1994; Papp
334 and Shewen, 1996b). This occurred despite preventing possible re-exposure to any
335 infective material. Whether this antibody response contributes to protection or is just
336 an indicator of the immune response is not fully understood: although antibody is
337 thought to play a minor role in primary infection, with T lymphocytes and cytokines
338 interferon-gamma and tumour necrosis factor alpha being more important, antibody
339 appears to be more important during secondary infection (Moore et al., 2002;
340 Morrison and Morrison, 2005). Papp et al. (1994) observed a slight, but non-
341 significant increase in anti-chlamydial activity during the periovulation period in 3 of
342 8 sheep, whereas in this study we observed a similar, but significant, rise in 9 of the
343 10 ewes. Thus, although there may some multiplication of organisms at this time, it is
344 possible that the circulating anti-chlamydial antibody titre is contributing to immunity
345 by limiting chlamydial multiplication and keeping the number of persisting organisms
346 low, and so preventing further abortive episodes. This would then also explain the
347 lack of any increased antibody response or the detection of organisms at parturition,
348 where there were no macroscopic lesions visible on any recovered placenta, no

349 organisms detectable in cell culture or in smears, and where real-time PCR failed to
350 detect chlamydial DNA in all but three of the placental samples. Furthermore, in the
351 three positive placental samples, only very low numbers of chlamydial genomes could
352 be detected, and these were reduced by over five orders of magnitude in comparison
353 to the post-abortion placental samples. As no viable organisms could be recovered in
354 cell culture and as PCR detects both viable and non-viable organisms it is unlikely
355 that these low numbers would significantly impact on the transmission of infection.

356 Previously, it has also been shown that following abortion, a chronic
357 chlamydial infection can become established in the reproductive tract of sheep (Papp
358 and Shewen, 1996a; Papp et al., 1998), suggesting that persistence in these tissues
359 could provide a reservoir of organisms that infect placental tissues in subsequent
360 pregnancies. In this study we sampled vaginal and cervical tissue, as well as selected
361 lymph nodes for evidence of chlamydial DNA by quantitative real-time PCR. No *C.*
362 *abortus* DNA was detected in any tissue sample analysed other than for one ewe,
363 where both the cervical tissue and mesenteric lymph nodes were repeatedly found to
364 be positive. Although only observed in one animal, the results are intriguing and
365 provide some evidence of a chronic infection of the reproductive tract. But it should
366 be noted that although this was also one of the ewes in which chlamydial DNA was
367 detected in the placenta, the numbers were very low and at the limit of assay
368 detection. Furthermore, whilst all attempts were made to sample tissues at the same
369 anatomical sites in all sheep, the size and complexity of these tissues and the small
370 size of sample required for PCR analysis means that we cannot rule out the possibility
371 that we inadvertently failed to sample infected areas in the other sheep.

372 Thus, while the results of the current study do corroborate the findings of
373 previous reports, we believe that the number of chlamydial genomes detected during
374 the periovulation period does not support the idea of the excretion of *C. abortus* at

375 oestrus as a major contributory factor in the transmission of EAE via the ram during
376 mating. Furthermore, although we found evidence of infection in the cervix and
377 lymph nodes of one ewe at post mortem, the absence or low levels of chlamydial
378 DNA detected in year 2 placental samples, particularly compared to year 1 post-
379 abortion samples, also suggests that ewes do not shed significant numbers of
380 infectious organisms at subsequent lambings. This, of course, does not discount the
381 possibility of a chronic low level of infection persisting in post-abortion ewes, but just
382 that low levels of organisms may be shed that do not significantly impact on the
383 transmission of EAE. Thus, the products of abortion represent the major source of
384 infection for transmission to naïve ewes and should be of the greatest concern in terms
385 of flock management to limit spread between ewes.

386

387 **Conflict of interest statement**

388 None of the authors (M. Livingstone, N. Wheelhouse, S. W. Maley or D.
389 Longbottom) has a financial or personal relationship with other people or
390 organisations that could inappropriately influence or bias the paper entitled
391 “Molecular detection of *Chlamydia abortus* in post-abortion sheep at oestrus and
392 subsequent lambing”.

393

394 **Acknowledgements**

395 The authors would like to thank David Buxton, Gary Entrican, Mara Rocchi,
396 Kevin Aitchison, Kim Wilson, Sean Wattedgera and the Bioservices Division for
397 their help with ensuring the care and welfare of the animals throughout this study. We
398 would also like to thank Iain McKendrick and Jill Sales for statistical advice. This
399 work was supported by funding from the Scottish Government Rural and
400 Environment Research and Analysis Directorate.

401

402 **References**

403

404 Aitken, I.D., Longbottom, D., 2007. Chlamydial abortion. In: Aitken, I.D. (Ed.),
405 Diseases of Sheep. Blackwell Publishing, Oxford, pp. 105-111.

406 Appleyard, W.T., Aitken, I.D., Anderson, I.E., 1985. Attempted venereal transmission
407 of *Chlamydia psittaci* in sheep. Vet. Rec. 116, 535-538.

408 Buxton, D., Anderson, I.E., Longbottom, D., Livingstone, M., Wattedegera, S.,
409 Entrican, G., 2002. Ovine chlamydial abortion: characterization of the
410 inflammatory immune response in placental tissues. J. Comp. Pathol. 127,
411 133-141.

412 Buxton, D., Barlow, R.M., Finlayson, J., Anderson, I.E., Mackellar, A., 1990.
413 Observations on the pathogenesis of *Chlamydia psittaci* infection of pregnant
414 sheep. J. Comp. Pathol. 102, 221-237.

415 Longbottom, D., Coulter, L.J., 2003. Animal chlamydioses and zoonotic implications.
416 J. Comp. Pathol. 128, 217-244.

417 Longbottom, D., Fairley, S., Chapman, S., Psarrou, E., Vretou, E., Livingstone, M.,
418 2002. Serological diagnosis of ovine enzootic abortion by enzyme-linked
419 immunosorbent assay using a recombinant protein fragment of the
420 polymorphic outer membrane protein POMP90 of *Chlamydophila abortus*. J.
421 Clin. Microbiol. 40,

422 Longbottom, D., Psarrou, E., Livingstone, M., Vretou, E., 2001. Diagnosis of ovine
423 enzootic abortion using an indirect ELISA (rOMP91B iELISA) based on a
424 recombinant protein fragment of the polymorphic outer membrane protein
425 POMP91B of *Chlamydophila abortus*. FEMS. Microbiol. Lett. 195, 157-161.

- 426 McClenaghan, M., Herring, A.J., Aitken, I.D., 1984. Comparison of *Chlamydia*
427 *psittaci* isolates by DNA restriction endonuclease analysis. *Infect. Immun.* 45,
428 384-389.
- 429 Moore, T., Ananaba, G.A., Bolier, J., Bowers, S., Belay, T., Eko, F.O., Igietseme,
430 J.U., 2002. Fc receptor regulation of protective immunity against *Chlamydia*
431 *trachomatis*. *Immunology.* 105, 213-221.
- 432 Morrison, S.G., Morrison, R.P., 2005. A predominant role for antibody in acquired
433 immunity to chlamydial genital tract reinfection. *J. Immunol.* 175, 7536-7542.
- 434 Papp, J.R., Shewen, P.E., 1996a. Localization of chronic *Chlamydia psittaci* infection
435 in the reproductive tract of sheep. *J. Infect. Dis.* 174, 1296-1302.
- 436 Papp, J.R., Shewen, P.E., 1996b. Pregnancy failure following vaginal infection of
437 sheep with *Chlamydia psittaci* prior to breeding. *Infect. Immun.* 64, 1116-
438 1125.
- 439 Papp, J.R., Shewen, P.E., Gartley, C.J., 1994. Abortion and subsequent excretion of
440 chlamydiae from the reproductive tract of sheep during estrus. *Infect. Immun.*
441 62, 3786-3792.
- 442 Papp, J.R., Shewen, P.E., Thorn, C.E., Andersen, A.A., 1998. Immunocytologic
443 detection of *Chlamydia psittaci* from cervical and vaginal samples of
444 chronically infected ewes. *Can. J. Vet. Res.* 62, 72-74.
- 445 Rodolakis, A., Bernard, K., 1977. Isolement de chlamydia des organes genitaux de
446 beliers atteints d'epididymite. *Bulletin de l'Academie Veterinaire de France* 50,
447 65-70.
- 448 Sammin, D.J., Markey, B.K., Quinn, P.J., McElroy, M.C., Bassett, H.F., 2006.
449 Comparison of fetal and maternal inflammatory responses in the ovine
450 placenta after experimental infection with *Chlamydophila abortus*. *J. Comp.*
451 *Pathol.* 135, 83-92.

- 452 Spencer, W.N., Johnson, F.W.A., 1983. Simple transport medium for the isolation of
453 *Chlamydia psittaci* from clinical material. Vet. Rec. 113, 535-536.
- 454 Stamp, J.T., McEwen, A.D., Watt, J.A.A., Nisbet, D.I., 1950. Enzootic abortion in
455 ewes. I. Transmission of the disease. Vet. Rec. 62, 251-254.
- 456 Wilsmore, A.J., Izzard, K.A., Wilsmore, B.C., Dagnall, G.J., 1990. Breeding
457 performance of sheep infected with *Chlamydia psittaci* (ovis) during their
458 preceding pregnancy. Vet. Rec. 126, 40-41.
- 459
- 460

461 **Figure Legend**

462

463 Figure 1. Antibody responses in sheep over two lambing seasons following *C. abortus*464 infection. Sheep were initially infected with *C. abortus* strain S26/3 at 75 days

465 gestation (0 weeks post-inoculation) and bled over an 80 month period. Samples were

466 analysed by rOMP90-3 and rOMP90-4 indirect ELISAs (Longbottom et al., 2002),

467 and the mean optical density (OD) \pm SEM are shown. Abortion occurred in year 1 at

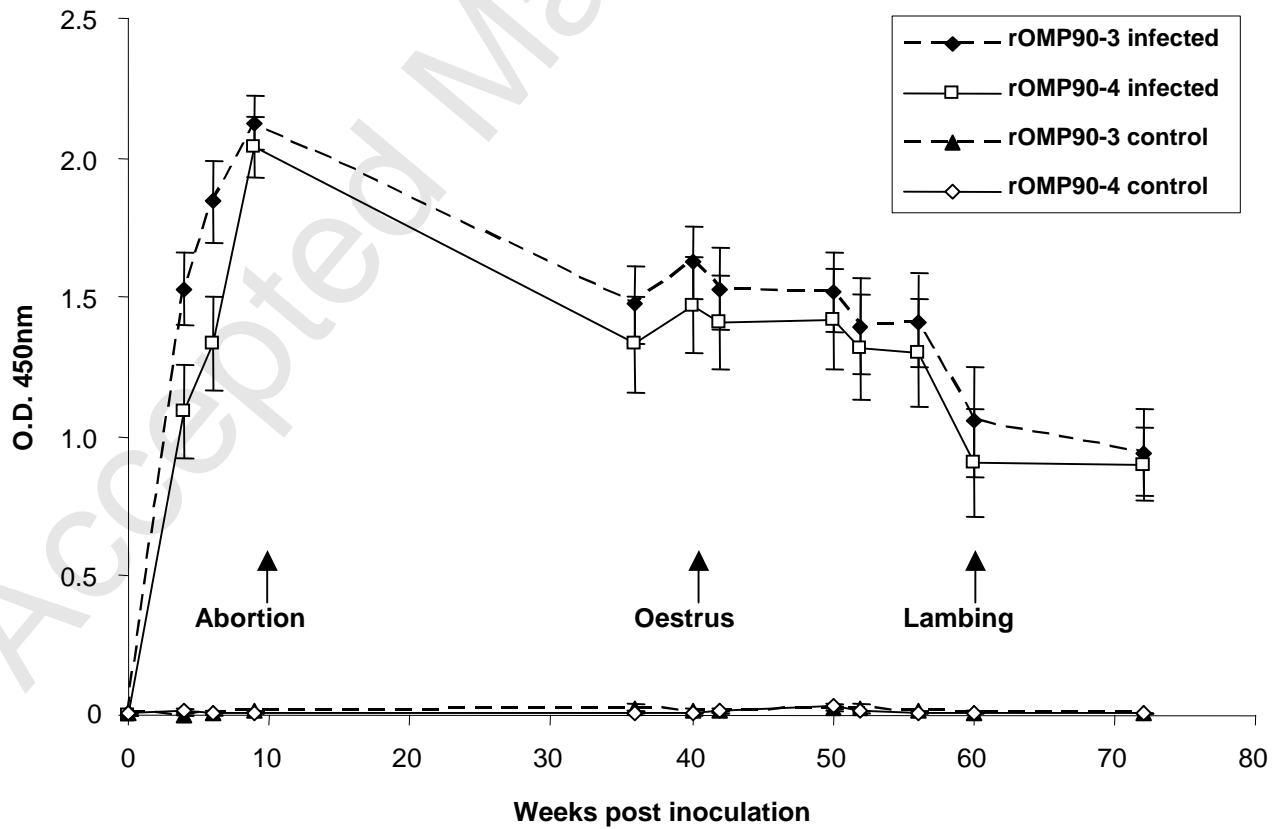
468 around 10 weeks post-inoculation, whereas oestrus and lambing in year 2 occurred at

469 around 40 and 60 weeks, respectively, post-inoculation. Details of the sampling

470 procedures and analyses are in the Materials and Methods.

471

472



1 Table 1. Chlamydial isolates used for real-time PCR validation

Species	Isolate	Host	Clinical condition
<i>C. abortus</i>	S26/3 ^a	Sheep	Abortion
	A58 ^b	Goat	Abortion
	VS 88-576 ^c	Sheep	Abortion
	T17 ^d	Sheep	Abortion
	T19 ^d	Sheep	Abortion
	T20 ^d	Sheep	Abortion
	T28 ^d	Sheep	Abortion
	83/12 ^a	Sheep	Abortion
	84/504 ^a	Sheep	Abortion
	90/214 ^a	Sheep	Abortion
	28/68 ^a	Sheep	Pneumonia
<i>C. pecorum</i>	1710S ^c	Pig	Abortion
	H4 ^b	Rabbit	Conjunctivitis
	W73 ^c	Sheep	Normal (faeces)
	JP-I-751 ^e	Sheep	Normal (faeces)
<i>C. suis</i>	S45 ^e	Pig	Normal (faeces)
<i>C. psittaci</i>	Cal 10 ^f	Human	Meningopneumonitis
	ISN 1670 ^f	Budgerigar	Psittacosis
	ISN1528 ^f	Parrot	Psittacosis
	ISN1263 ^f	Parakeet	Psittacosis
	4482 ^b	Duck	Ornithosis
<i>C. felis</i>	FePn ^e	Cat	Pneumonia
<i>C. caviae</i>	Z432 ^b	Guinea pig	Conjunctivitis
	64-H-281-R-8 ^e	Guinea pig	Conjunctivitis

2

3 Origin of isolates: ^a Moredun Research Institute; ^b H. Krauss, Department of Hygiene4 and Infectious Diseases, Justus-Liebig-University, Giessen, Germany; ^c B. Markey,

5 Department of Veterinary Microbiology and Parasitology, University College Dublin,
6 Dublin, Republic of Ireland; ^dH. Phillips, Department of Veterinary Clinical Science
7 and Animal Husbandry, University of Liverpool, Liverpool, England; ^eJ. Storz,
8 Department of Veterinary Microbiology and Parasitology, School of Veterinary
9 Medicine, Louisiana State University, USA; and ^fA. Andersen, Avian Diseases
10 Research Unit, National Animal Disease Center, US Department of Agriculture,
11 Ames, Iowa, USA.

Accepted Manuscript

1 Table 2. Pregnancy and clinical outcome of sheep infected with *C. abortus*

2

Ewe No	Year 1					Year 2			
	Length of gestation (days)	No. of lambs born	No. of fetuses aborted	Proportion of placenta affected (%)			mZN/culture	Length of gestation (days)	No. of lambs born
				Plac 1	Plac 2	Plac 3			
358N	140	0	1	75			++++	144	1
360N	138	1	1	80	NF ^b		+++	146	1
395N	134	1	2	NF	100	100	++++	144	2
398N	139	0	2	100	10		++++	145	2
406N	137	0	3	100	100	90	++++	144	1
407N	139	1	1	100	90		++++	143	2
408N	138	1	1	NF	NF		+++	141	1
421N	125	0	2	NF	100		++++	145	2
441N	136	0	2	100	100		++++	145	1
454N	135	0	2	100	NF		++++	145	2

3

4 In year 2 there were no abortions, there was also no macroscopic evidence of gross placental pathology and no organisms could be detected by
5 mZN staining of placental or vaginal smears or could be recovered in cell culture. ^a Plac/VS, for multiple births mZN score represents average
6 score for all placentas recovered or, where placentas were not available, for all vaginal swabs tested (occasional numbers of EBs, ‘+’; low, ‘++’;
7 moderate, ‘+++’; high, ‘++++’); ^b NF, not found.

1 Table 3. Number of *C. abortus* genomes detected per μg total tissue DNA

Ewe No.	Placental samples		Necropsy samples				
	Year 1	Year 2	Vagina	Cervix	ULN	MLN	PFLN
358N	1.7 (0.4) $\times 10^7$	0	0	0	0	0	0
360N	2.4 (0.4) $\times 10^7$	0	0	0	0	0	0
395N	5.9 (1.9) $\times 10^7$	<10	N/A	N/A	N/A	N/A	N/A
	4.6 (1.0) $\times 10^7$						
398N	1.0 (0.3) $\times 10^7$	0	0	0	0	0	0
	2.8 (1.1) $\times 10^7$						
406N	4.7 (1.2) $\times 10^7$	0	0	0	0	0	0
	0.7 (0.2) $\times 10^7$						
407N	1.4 (0.7) $\times 10^7$	<10	0	5275 (1819)	0	58 (11)	0
408N	1.1 (0.1) $\times 10^7$	0	N/A	N/A	N/A	N/A	N/A
421N	0.6 (0.1) $\times 10^7$	91 (18)	0	0	0	0	0
441N	4.5 (1.2) $\times 10^7$	0	0	0	0	0	0
454N	3.4 (1.1) $\times 10^7$	0	0	0	0	0	0

2

3 ULN, uterine lymph node; MLN, mesenteric lymph node; PFLN, prefemoral lymph

4 node; N/A, not available. Results for one or two placentas recovered per ewe and

5 necropsy samples are expressed as mean (\pm SEM).

6

1 Table 4. The number of *C. abortus* genomes detected in vaginal swabs

Ewe No.	Number of <i>C. abortus</i> genomes detected per test sample						
	14d pre ^a	2d pre	Ovulation	1d post ^b	2d post	6d post	13d post
358N	0	21 (13)	0	0	0	0	<10
360N	0	<10	0	10	0	<10	<10
395N	0	37 ^c	0	0	0	<10	0
398N	16 (10)	<10	15 (9)	12 (6)	0	0	<10
406N	<10	0	<10	0	<10	0	<10
407N	0	<10	0	<10	0	0	0
408N	0	14 (2)	0	22 (10)	0	0	<10
421N	0	0	12 (3)	<10	<10	0	0
441N	0	<10	15 (9)	0	<10	0	<10
454N	<10	0	<10	26 (12)	0	<10	0

2

3 ^a pre and ^b post, the number of days pre and post ovulation. Results are expressed as4 mean (\pm SEM). ^c only 1 sample was available for analysis.