Molecular and serological dynamics of *Chlamydia pecorum* infection in a longitudinal study of prime lamb production

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26   **Abstract**

27   **Background**. *Chlamydia pecorum* is a globally significant livestock pathogen causing pathology

28   and production losses. The on-farm infection and serological dynamics and the relevance of

29   existing diagnostic tools for diagnosing *C. pecorum* in livestock remains poorly characterized. In

30   this study, we characterized the antigen and antibody dynamics of this pathogen in a longitudinal

31   study of prime lamb production, utilizing the infection focused *C. pecorum*-specific 16S rRNA

32   qPCR assay and serology based chlamydial Complement fixation Test (CFT).

33   **Methods.** The study consisted of 76 Border Leicester mixed sex lambs (39 females and 37 males)

34   that were sampled bimonthly from 2-10 months of age in a commercial farm operating in Central

35   NSW, Australia. Blood/plasma was analysed for CFT antibodies, and swabs from conjunctival,

36   rectal and vaginal sites were analysed for *C. pecorum* shedding using qPCR. We assessed the

37   temporal and overall dynamics of *C. pecorum* in lambs, including detailed description and

38   comparison of qPCR and CFT, the timing of first detection by either diagnostic method, the lag

39   between infection and antibody response; and the distribution of qPCR load and CFT antibody

40   titre over time.

41   **Results.** Over the study period, *C. pecorum* was highly prevalent (71.0% by qPCR, 92.1% by

42   CFT, 96.0% by both), with 21.1% (16/76) lambs shedding $\geqslant$ 1000 qPCR copies/µl (denoted as

43   high shedders). *C. pecorum* shedding (as evidence of infection) were first observed at 2 months

44   of age (14.4%) with a significant peak of infection occurring at 6 months of age (34.2%),

45   whereas seroconversions peaked at 8 months of age (81.5%). 52.6% of *C. pecorum* qPCR and

46   CFT positive lambs became qPCR negative by 10 months of age, indicating clearance of

47 chlamydial infection. Although CFT is utilised for on-farm detection of active infection, we

48 confirm that it lagged behind qPCR detection (average lag 1.7 ± 2.1 months) and that the

49 proportion of qPCR positives simultaneously identified by CFT was low with 2/11 (18.1%), 0/13,

50 17/25 (68.0%), 5/7 (71.4%) and 1/10 (10.0%) concurrent seroconversions occurring at 2, 4, 6, 8

51 and 10 months of age, respectively.

52 **Discussion.** This work reveals rapid rates of *C. pecorum* infection and widespread exposure

53 during lamb production. The comparison of molecular and serological diagnostic agreement

54 longitudinally, supports the use of qPCR as an important ancillary tool for the detection of active

55 infections in conjunction with chlamydial CFT for routine veterinary diagnostics. Development

56 of rapid Point-of-Care (POC) tools for diagnosing active infection would be valuable for

57 producers and veterinarians.

58

59 **Introduction**

60 Despite growing evidence that infections of the obligate intracellular bacterial pathogen,

61 *Chlamydia pecorum,* are ubiquitous among some of the most economically important livestock

62 species globally, the epidemiology and pathologies associated with this pathogen are poorly

63 understood (Walker et al. 2015). *C. pecorum* infections of sheep, cattle and goats are associated

64 with polyarthritis (Walker at al. 2016), keratoconjunctivitis (Polkinghorne et al. 2009), sporadic

65 bovine encephalomyelitis (SBE) and pneumonia (Jelocnik et al. 2014a). Sporadic cases of ovine

66 and caprine abortions due to *C. pecorum* have also been reported (Giannitti et al. 2016; Walker et

67 al. 2015). *C. pecorum* chlamydiosis in both sheep and cattle can limit growth and survival of

68 young rapidly growing stock and, such weight loss or failure to thrive as a result of *C. pecorum*

69 polyarthritis, is the primary economic concern for farmers (Poudel et al. 2012; Walker et al. 2015;

70   Walker et al. 2016). The latter disease is a compelling one for Australian producers with 2.1% of

71   lambs and 1.6% of calves condemned each year at Australian abattoirs as a result of polyarthritis,

72   estimated to cost the livestock industry around $30M annually (Walker et al. 2016). Similar

73   economic costs of arthritis have also been reported elsewhere (Dupuy et al. 2013). The specific

74   contribution of arthritis-associated losses by *C. pecorum* are yet to be established, however.

75   The complex relationship between *C. pecorum* infection and overt animal pathology makes the

76   diagnosis and control of infections challenging. Sub-clinical, asymptomatic infections are

77   common, characterised by the detection of *C. pecorum* in the faeces, gastrointestinal and/or

78   urogenital tract of so-called "shedder" animals (Reinhold et al. 2008; Reinhold et al. 2011).

79   These same animals may also act as an important reservoir, facilitating infection of individuals

80   who exhibit symptomatic infections. Indeed, in Australia, the largest exporter of sheep globally,

81   *C. pecorum* was recently estimated to be present in 30% of the country's sheep flock, based on

82   faecal shedding alone (Yang et al. 2016). While these infections are common, it is apparent that

83   *C. pecorum* can also disseminate to other tissues where it can replicate in epithelial cells and

84   macrophages of the conjunctival, genital and intestinal sites, in synoviocytes of the joint tissue

85   and occasionally, the respiratory tract (Jelocnik et al. 2014a; Twomey et al. 2006). The factors

86   that influence dissemination and pathogenesis of these strains are currently unknown, although

87   molecular typing studies have suggested that genetic differences may exist between strains

88   associated with disease and those found asymptomatically colonising the gastrointestinal tract

89   (Jelocnik et al. 2013; Jelocnik et al. 2014b; Mohamad et al. 2014).

90   In Australia, diagnosis of sheep chlamydiosis is based on clinical history, symptoms and

91   presenting pathology, and is routinely confirmed by diagnostic laboratories using a Complement

92   Fixation Test (CFT). Chlamydial CFT detects antibodies (Ab) to either whole chlamydial

93   elementary bodies (EBs) or *Chlamydiaceae*-specific lipopolysaccharide and remains as the

94   recommended test for *Chlamydia* diagnosis by the World Organisation for Animal Health and

95   Sub-Committee for Animal Health Laboratory Standards (Sachse et al. 2009). The use of crude

96   or partially purified antigen in CFT depends on the binding of anti-*Chlamydiaceae* antibodies of

97   the host species to guinea pig complement, and has highly variable sensitivity depending on the

98   host species and antibody isotype (Kaltenboeck et al. 1997; Perez-Martinez et al. 1986).

99   Moreover, use of whole chlamydial elementary bodies (EBs) or *Chlamydiaceae*-specific

100   lipopolysaccharide as an antigen in this assay renders only genus-specificity and inevitable

101   serological cross-reactivity with Chlamydia-related organisms and gram negative bacteria

102   (Casson et al. 2007; Haralambieva et al. 2001). Detection of sero-conversion by CFT has

103   multiple purposes such as: (a) confirmation of chlamydiosis; (b) the presence or absence of

104   chlamydial infection; and (c) determination of immune status after vaccination (Sachse et al.

105   2009). The major issue is that CFT has a largely unknown relationship to either the acute,

106   convalescent or persistent phase of *C. pecorum* infection or even pathology itself (Griffiths et al.

107   1996; Kaltenboeck et al. 1997; Perez-Martinez et al. 1986). Alongside this unknown relationship,

108   *C. pecorum* shedding (measured by qPCR) at the gastrointestinal tract has not well correlation to

109   disease or pathology and has not been investigated further to date (Walker et al. 2016).

110   Beyond the use of CFT and qPCR for the diagnosis of *C. pecorum* cell culture or embryonated

111   hens' egg based isolation traditional methods exist that are often cumbersome and time

112   consuming for on-farm settings (Sachse et al. 2009). Here we conducted a longitudinal study of

113   *C. pecorum* infection in a prime lamb flock. Our aims were to: (a) describe the temporal and

114   overall dynamics of *C. pecorum* in lambs, including detailed description and comparison of

115   qPCR and CFT, the timing of first detection by either diagnostic method, and estimate the lag

116   between infection (qPCR) and antibody response (CFT); and (2) detail the distribution of

117   chlamydial qPCR load and CFT antibody titre over time. For both aims, we detail consistency

118   and variation in infection across three anatomical sampling sites: conjunctiva, rectum and vagina.

119

120   **Materials and Methods**

121   **Lamb husbandry.** This study followed 76 Border Leicester mixed sex lambs operating in a

122   commercial sheep farm in Central Western NSW. Lambs in this study were managed as per

123   normal farm husbandry practices (e.g. marking, feeding, weaning, vaccinations etc.). At time of

124   marking (two months of age), the lambs were uniquely ear tagged and sampled at bi-monthly

125   intervals (2, 4, 6, 8 and 10 months) until finishing which is at the 10 months of age.

126   **Blood and swab samples collection.** Swab samples taken from conjunctiva, rectum and vagina,

127   and blood samples were collected from individual lambs across five bimonthly sampling time

128   points from 2-10 months of age. Collected swabs were used for in-house DNA amplification

129   assays, and the blood samples were submitted to the State Veterinary Diagnostic Lab, Elizabeth

130   Macarthur Agricultural Institute, Menangle, NSW, for serological testing by *Chlamydia* CFT. A

131   serum sample was considered positive by CFT with a titer of 16 or greater in this study. The

132   collection and testing of these swabs and blood samples was approved by the University of the

133   Sunshine Coast Animal Ethics Committee (AN/S/14/31).

134   **Swab processing and DNA extraction.** Clinical swabs were processed according to the in-

135   house swab processing methods (Jelocnik et al. 2013). Briefly, swabs were dispensed into 1.5ml

136   of sucrose-phosphate-glutamate buffer by vortexing and centrifugation. The resulting cell pellet

137   was resuspended in tris-EDTA buffer and heated at 95 C for 10 minutes to heat-inactivate the

138   elementary bodies (EBs) for further processing at room temperature. For all swabs, DNA was

139   extracted using a QIAamp DNA kit (Qiagen, Doncaster, Victoria, Australia), according to the

140   manufacturer's instructions. DNA purity and yield was determined using a NanoDrop

141   spectrophotometer ND-1000 (Thermo Fisher Scientific, Inc.).

142   ***C. pecorum* 16S species- specific qPCR screening.** Conjunctival, vaginal and rectal swabs

143   were screened for the presence of *C. pecorum* infections by a *C. pecorum*-specific quantitative

144   PCR targeting the 16S rRNA gene modified from previously published protocol (Marsh et al.

145   2011). The *C. pecorum* 16S 204 bp fragment (RT-Cpec -F: 5'-

146   AGTCGAACGGAATAATGGCT-3', RT-Cpec-R: 5'-CCAACAAGCTGATATCCCAC-3'; IDT)

147   was sub-cloned into pGem-T Easy (Promega) and amplified with M13 universal primers to

148   generate a M13-Cpec-16S fragment. Serial dilutions of the M13-Cpec-16S fragment were used

149   to produce a standard curve by mixing 5 μl of diluted fragment with RT-Cpec-F and RT-Cpec-R

150   primers (1μM final) and 1X QuantiTect SYBR® Green PCR mix (Qiagen) in a final volume

151   reaction of 20 μl. Cycling conditions were 95°C- 15 min, followed by 35 cycles of 94°C- 15 sec,

152   57°C- 15 sec, 72°C- 30sec, and a final amplification cycle of 72°C, 10 min. Diluted *C. pecorum*

153   strain MC/Marsbar served as a positive control while dH$_2$O was used as negative control. All

154   samples were tested in duplicates. The limit of detection load was 10 *C. pecorum* 16S rRNA

155   copies/μl.

156

157   **Statistical analysis**

158   We undertook a detailed individual assessment of the timing and diagnostic characteristics of *C.*

159   *pecorum* infection dynamics. This included the diagnostic infection and serological response

160   (qPCR and CFT) status at each sampling time point (Table 1), information on evidence of

161   individual clearance of infection (as determined by a lamb having both a qPCR and CFT

162   diagnosis across the sampling time points, but only being CFT positive by 10 months of age).

163   This individual level information facilitated flock-wide description of *C. pecorum* infection

164   dynamics and diagnostic evaluations, including overall and time dependent CFT and qPCR

165   positivity and the diagnostic states (qPCR+CFT+, qPCR+CFT-, qPCR-CFT+, qPCR-CFT-)

166   (Table 2). We evaluated the 'qPCR CFT agreement' as the percentage of qPCR positive and

167   negative lambs with matching CFT diagnosis

168    $$\frac{(a+d)}{(a+b+c+d)}x100$$

169   where a is diagnostic state qPCR+CFT+, b is qPCR+CFT-, c is qPCR-CFT+, and d is qPCR-

170   CFT-. We have specifically avoided the widely used 'sensitivity' and 'specificity' terminology

171   owing to the knowledge that qPCR and CFT are measuring inherently different things (bacterial

172   agent and antibodies, respectively). The comparison of CFT against qPCR is nevertheless

173   functional on applied grounds, owing to its relatively common use in veterinary settings to

174   diagnose active infection.

175   Focussing only on the month at which first qPCR diagnosis was made, individual lags of CFT

176   from qPCR diagnosis was made followed by average ± standard deviation to get the

177   population/flock lag. Lag in CFT from qPCR was calculated on the basis of animals that had

178   their qPCR detection first followed by/concurrent CFT detection (n=45) (Tables S1-S3). Lambs

179   that had CFT detection arise before qPCR (n=31) as a result of previous infections that were

180   missed due to bimonthly sampling frequency were not included in the lag analyses.

181   All data analyses was performed in Microsoft Excel 2013 and graphs outputs (Fig 1 and 2)

182   generated using GraphPad Prism version 7 (GraphPad Software, LaJolla, CA, USA).

183

184   **Results**

185 **Temporal and overall dynamics of *C. pecorum* exposure in lambs.**

186 Diagnosis of *C. pecorum* infections revealed an overall positivity of 71.0% by qPCR, 92.1% by

187 CFT and 96.0% by either test over the study period (Table 1). The temporal patterns of qPCR

188 detection were broadly consistent among the samples from the conjunctiva, rectum and vagina,

189 with greatest number of detection at 6 months of age and similar among other months of

190 sampling (Table 1). While temporal patterns in qPCR were similar among sampling sites, greater

191 numbers of detection were made from the conjunctiva, relative to the rectum and vagina (Table

192 1). Detection of *C. pecorum* by CFT rose rapidly from 6 months of age, peaking at 8 months of

193 age and then declining by 10 months of age (Table 1). A notable number of CFT detection (n=10)

194 were made at 2 months of age, and possibly indicate the presence of maternal antibodies, or

195 infections that occurred and resolved < 2 months of age. Temporal agreement between qPCR and

196 CFT was greatest at 2 months of age, decreased to lowest levels at 8 months of age (owing to an

197 increased number of lambs that were positive by CFT, but negative by qPCR), and increased

198 again by 10 months of age (owing to an increased number of lambs negative by both diagnostic

199 methods) (Table 2). Approximately half of the lambs (52.6%; 40/76) sampled exhibited evidence

200 of infection clearance (only CFT detection by 10 months of age) within the sampling frame of

201 this study (Table 2)

202 In this study, 67.1% of lambs (51/76) tested positive by both CFT and qPCR (Table 2). However,

203 3/51 of these lambs had CFT antibodies arise before qPCR detection due to past infection that

204 went undiagnosed as a result of bimonthly sampling or detection of maternal antibodies at 2

205 months of age (Table S1). Interestingly, 3.9% (3/76) of lambs were qPCR positive and CFT

206 negative throughout the study period and 2/3 of these lambs had recurrent *C. pecorum* infection

207 as measured by repeated qPCR positivity (Table 2). We identified a further 25% (19/76) of

208   lambs that tested positive by CFT and negative by qPCR during the entire study period of 2 to 10

209   months of age, and 3.9% (3/76) of lambs tested qPCR and CFT negative during the entire study

210   period (Table 2).

211   We also investigated the timing of first detection by qPCR and CFT to gain an improved

212   understanding of when lambs first became infected and seroconverted (Table 3 and 4). Peaks in

213   timing of first detection by qPCR and CFT were 6 and 8 months of age, respectively (Table 3

214   and 4), which was broadly similar to what was observed when evaluating the flock as a whole

215   (Table 1). Most of the first seroconversions (CFT diagnoses) were detected concurrently with

216   first detection of infection, a smaller number lagged behind infection and a moderate number

217   occurred without detection of infection, which may indicate the 'window of infection' that was

218   missed due to the bi-monthly sampling intervals (Table 3 and 4). Overall, the sampling intervals

219   in this study led to an estimated average time lag between first qPCR detection and first CFT

220   detection of 1.7 ± 2.1 months.

221   **Distribution of *C. pecorum* bacterial load and antibody titre in lambs.**

222   *C. pecorum* bacterial load (qPCR copy number) and CFT antibody titer exhibited distinct

223   temporal patterns (Table 5, Fig 1a and b). From 2-6 months of age, lambs exhibited an overall

224   increase in chlamydial loads, peaking at 6 months of age (Table 5, Fig 1a). Across all time points,

225   a small number of lambs had loads exceeding 1000 16S rRNA gene copy numbers (Fig 1a, data

226   points in red). The temporal patterns in bacterial load varied among the sampling sites (Fig 2). In

227   the conjunctiva, loads trended downward from 2-10 months of age (Fig 2a). In contrast, bacterial

228   loads increased from 2-6 months of age at the rectal sampling site, diminishing markedly

229   thereafter (Fig 2b). For female lambs the greatest loads occurred from 6-10 months of age at the

230   vaginal sampling site (Fig 2c). Overall, 16/76 (21%) of lambs were shedding high loads of *C.*

231   *pecorum* DNA (≥ 1000 copies/µl) from their mucosal sites of conjunctiva (3/76), rectum (4/76)

232   and vagina (9/76), respectively (Fig 2). CFT titres were low at 2-4 months of age, and then

233   peaked from 6-8 months of age, followed by a slight decline in titre at 10 months of age (Table 5,

234   Fig 1b).

235

236   **Discussion**

237   Despite the significant economic loss in livestock due to chlamydiosis, *C. pecorum* infections

238   continue to be under recognized as a major endemic pathogen for producers globally (Walker et

239   al. 2015; Walker et al. 2016). Key factors contributing to this underestimation are the lack of

240   appreciation of infection dynamics, relative to antibody dynamics (Berri et al. 2009; Sachse et al.

241   2009). To address these issues, the objective of our study was to describe infection (measured by

242   a species specific 16S qPCR assay) and serological (measured by CFT) dynamics using a

243   longitudinal on-farm sheep flock setting in Central NSW, Australia.

244   Whether detected by qPCR, CFT or both diagnostic methods, the overwhelming majority of

245   lambs were exposed to *C. pecorum* at some point over the study period, indicating rapid rates of

246   infection, and widespread exposure in the flock. Based on the sampling frequency employed, we

247   observed that lambs experienced peak chlamydial infections over a period of 2-6 months of age

248   followed by seroconversion at 6-8 months of age, and occasional detection of low-level recurrent

249   chlamydial infection at 10 months. The sudden rise in infection prevalence at six months

250   potentially reflects a response to the stress of marking, and associated herding of lambs into

251   yards, increasing contact between infected lambs, and precipitating spread of chlamydial

252   infections (Poudel et al. 2012; Stanley & Jones 2003). Marking or mulesing has been recorded as

253   the peak time for risk of infections by Erysipelas, the other major cause of bacterial arthritis in

254   lambs (Robson 2003). Peak seroconversion of lambs occurred at eight months of age, although a

255   subset of lambs had circulating antibodies as early as two months, likely owing to maternal

256   transfer of antibodies or early seroconversion in cases that were also qPCR positive. At early

257   time points (2 and 4 months), qPCR preceded CFT in detecting new infections, with a peak in

258   coincidental first detection by both methods occurring at six months. Based on these findings,

259   qPCR performs well at detecting infections early in life (2-6 months), new infections, and

260   potentially recurrent infections. Antibody responses appeared to confer a degree of protection, as

261   indicated by animals becoming qPCR negative in 52.6% (40/76) of cases by 10 months.

262   However, our results indicated that the protection was not long lasting, as both declining

263   seroprevalence, waning titres from 8-10 months of age and absence of CFT titres in some cases

264   was observed, and may have contributed to repeat infections detected during these months (n=8

265   and n=6 at 8 and 10 months, respectively).

266   CFT is periodically utilized for diagnosis of active *C. pecorum* infection in veterinary settings,

267   hence it was important to characterize its association to qPCR diagnosis. We found that using

268   CFT potentially obscures the ability to detect acute active chlamydial infections with 9/11

269   (81.8%), 13/13 (100.0%), 8/25 (32.0%), 2/7 (28.5%) and 9/10 (90.0%) qPCR detection

270   preceding seroconversion at 2, 4, 6, 8 and 10 months of age, respectively. This important finding

271   of poor matching between CFT and qPCR is similar to results reported in other *Chlamydia*

272   studies (Bas et al. 2001; McCauley et al. 2007; Persson & Boman 2000; Sachse et al. 2009).

273   Overall, our results show that CFT has limited value for diagnosis of acute active infections. In

274   this study, we also identified three lambs that were qPCR positive (2/3 lambs were repeatedly

275   qPCR positive) but had no detectable CFT antibodies longitudinally. In our previous study on

276   characterisation of humoral immune responses to naturally occurring *C. pecorum* infections we

277   have identified moderate antigen specific MOMP and PmpG IgG antibodies present in these

278   three lambs longitudinally (Bommana et al. 2017). These findings elude us to believe that these

279   animals are not passive shedders of *C. pecorum* and that in reality CFT as an assay has relatively

280   poor sensitivity. CFT has also been criticized for its requirement of technical expertise,

281   subjective interpretation, and its unsuitability for testing large numbers of specimens; when

282   testing *C. abortus* infections in sheep (McCauley et al. 2007; McCauley et al. 2010). Conversely,

283   our species-specific qPCR assay appears a much improved tool for early and active stages of

284   infection (Sachse et al. 2009). CFT as a diagnostic assay is suitable for seroprevalence surveys

285   and serological dynamic studies (such as this study) as it can detect antibodies to clinically

286   unapparent chlamydial infections. CFT is inappropriate for the retrospective diagnosis of

287   chlamydial mucosal infections, such as in oculo-genital sites that make antibodies specific to a

288   localised site of infection (Barnes 1989; Griffiths et al. 1996). The exception to this is diagnosis

289   of chlamydial polyarthritis or abortion in ruminants, wherein high exposure to *Chlamydia* elicits

290   a pronounced systemic increase in antibody levels (Perez-Martinez et al. 1986; Bommana et al.

291   2017). Potentially, there may exist early seroconversion or antibody markers for improved

292   serological diagnosis of acute-phase infection in livestock, overcoming the shortcomings of CFT.

293   Future research is needed to identify such markers by investigating proteomic profiles of

294   chlamydial infections to differentiate responses related to acute infections, cured past infection,

295   and persistent infection. Improved diagnostic tools for acute phase infection, or exposure, that

296   are simple to undertake and interpret, would be of significant value for routine use by

297   veterinarians and veterinary diagnostic laboratories (Jelocnik et al. 2017).

298   A notable proportion (21.1%; 16/76) of the flock were shedding high numbers ($>1\times10^4$ - 10^5) of

299   *C. pecorum*, which may have important implications for *C. pecorum* dynamics. "Supershedders"

300   are suspected to facilitate transmission of many pathogens (Lloyd-Smith et al. 2005; Matthews et

301   al. 2013). In this study lambs shedding high amounts of *C. pecorum* may facilitate transmission

302   through fecal-oral contact or greater environmental contamination (eg. water troughs, grazing

303   pasture) (Stanley & Jones 2003). Rapid POC diagnostic tools underway for *C. pecorum* and

304   *C.psittaci*, (Jelocnik et al. 2017) are needed to identify these individuals as part of a preventative

305   flock management program. Any animal that is identified early as a supershedder could be culled

306   to avoid further flock transmission and reduce infection rates. More broadly this observation fits

307   with a body of literature suggesting that a small proportion of individuals are commonly

308   responsible for the majority of transmission events (the 80:20 rule for supershedders and

309   superspreaders) across multiple systems (May & Anderson 1987; Woolhouse et al. 1997). One

310   similar example was recorded in the livestock industry when faecal sample analysis conducted at

311   a UK abattoir revealed that approximately 9% of the cattle examined over a 9-week period were

312   high *Escherichia coli* O157 shedders ($>10^4$ CFU/g) and they accounted for over 96% of the

313   bacteria shed by all animals tested (Omisakin et al. 2003).

314

315   **Conclusion**

316   In this study, we have described the on-farm molecular and serological dynamics of the

317   enigmatic pathogen, *C. pecorum* in lambs from 2 to 10 months of age. Lambs exhibiting *C.*

318   *pecorum* infections had high rates of infection at 6 months of age and seroconversion at 8 months

319   of age. Based on our individual diagnostic assessments of qPCR and CFT, *C. pecorum*-specific

320   qPCR was found to be a more useful tool in diagnosing acute active infections early on in life (2-

321   6 months), recurring infections, and also in detecting *C. pecorum* positivity in CFT negative

322   animals. Further integration of the findings in this study with detailed pathological investigations

323 in relation to chlamydial shedding, disease and impact are currently underway. Findings in this

324 study provide insights into the infection and antibody dynamics, and ways to improve diagnosis

325 of *C. pecorum* infections in sheep. While CFT is the primary assay in diagnosing chlamydial

326 infections of livestock in veterinary settings, *C. pecorum*-specific qPCR assay will serve as an

327 important ancillary tool when used in parallel with CFT in diagnosing active acute on farm

328 infections of this pathogen. Further development of rapid POC assays for routine veterinary

329 diagnostic testing of *C. pecorum* infections in Australian livestock would be valuable (Jelocnik et

330 al. 2017). This is particularly the case for clinical cases with variable presentations, such as

331 conjunctivitis, asymptomatic infections and/or conjunctivitis with polyarthritis. Considering the

332 ever increasing availability of species and strain specific qPCR assays for member species in the

333 genus *Chlamydia*, sensitive discriminatory assays for *C. pecorum* would be beneficial for both

334 producers and researchers (Jelocnik et al. 2017; Sachse et al. 2009). Such tools will be essential

335 if we are to truly understand the true prevalence and impact of these infections on livestock

336 production globally.

337

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341

342

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462   **Figure legends**

463   Figure 1. Age dependent distribution of *C. pecorum* bacterial load and antibody titers (A) qPCR

464   load, lambs shedding high loads of *C. pecorum* DNA ($\geq$ 1000 copies/µl) and CFT positive are

465   indicated as data points in red (B) CFT titer. From 2-6 months of age, lambs exhibited an overall

466   increase in mean chlamydial loads, peaking at 6 months of age (1128±6205). Mean CFT titres

467   were low at 2-4 months of age (9.5±4.5 and 8.3±2.8 at 2 and 4 months), and then peaked from 6-

468   8 months of age (29±27.8 and 33.5±21 at 6 and 8 months), followed by a slight decline in mean

469   titre at 10 months of age (18.8±24), respectively.

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471   Figure 2. Age dependent distribution of *C. pecorum* bacterial load at (A) Conjunctiva (B)

472   Rectum and (C) Vagina. Lambs shedding high loads of *C. pecorum* DNA ($\geq$ 1000 copies/µl) and

473   CFT positive are indicated as data points in red. The mean conjunctival bacterial loads trended

474   downward from 2-10 months of age (26.5±161.1 to 3.8±14.4, respectively), in contrast, mean

475   bacterial loads increased from 2-6 months of age (16.3±115.3 to 849.6±6143) at the rectal

476   sampling site, diminishing markedly thereafter (0.8±2.3 at 10 months). For female lambs the

477   greatest bacterial loads occurred from 6-10 months of age (544.2±1538 and 78.3±352.3 at 6 and

478   10 months) at the vaginal sampling site.

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Characteristics of *C. pecorum* positivity of lambs (n=76), as tested by qPCR at each sampling site (conjunctiva, rectum and vagina) and CFT.

1   Table 1. Characteristics of *C. pecorum* positivity of lambs (n=76), as tested by qPCR at each

2   sampling site (conjunctiva, rectum and vagina) and CFT.

Age (months)	qPCR+					CFT+	
	Conj. No.	Rect. No.	Vag. No.	Combined No.	Combined %	No.	%
2	3	5	3	11	14.4	10	13.1
4	12	4	2	18	21	1	1.3
6	10	9	11	21	34.2	42	55.2
8	5	3	5	13	15.7	62	81.5
10	9	1	2	12	15.7	25	32.8
Overall	**39**	**22**	**23**	**54**	**71.0**	**70**	**92.1**

Characteristics of qPCR CFT agreement, and the % of lambs that cleared their infection by 10 months of age (as determined by a lamb having both a qPCR and CFT diagnosis across the sampling time points, but only being CFT positive by 10 months of age).

1   Table 2. Characteristics of qPCR CFT agreement, and the % of lambs that cleared their infection by 10 months of age (as determined

2   by a lamb having both a qPCR and CFT diagnosis across the sampling time points, but only being CFT positive by 10 months of age).

Age (months)	qPCR+CFT+ No.	qPCR+CFT- No.	qPCR-CFT+ No.	qPCR-CFT- No.	qPCR CFT agreement %	Infection clearance %
2	2	6	8	60	81.5	_
4	0	16	1	59	77.6	_
6	22	4	23	27	64.4	_
8	9	3	53	11	26.3	_
10	5	7	20	44	64.4	_
Overall	**51**	**3**	**19**	**3**	**71.0**	**52.6 (n=40)**

First detection of *C. pecorum* in lambs at each sampling site (conjunctiva, rectum and vagina) by qPCR (infection).

1   Table 3. First detection of *C. pecorum* in lambs at each sampling site (conjunctiva, rectum and

2   vagina) by qPCR (infection).

Age (months)	qPCR+ (first detections)				
	Conj. No.	Rect. No.	Vag. No.	Combined No.	Combined %
2	3	5	3	11	14.4
4	9	2	2	13	17.1
6	10	5	10	25	32.8
8	3	2	2	7	9.2
10	7	1	2	10	13.1

First detection of *C. pecorum* in lambs by CFT (antibodies) in relation to age.

Lambs with their first CFT detection were further categorised into (a) new CFT detection due to concurrent seroconversion *i.e.,* matching qPCR detection, (b) new CFT detection due to delay in seroconversion as a result of previous qPCR positive infection, and (c) new CFT detection due to suspected previous infection that was missed due to bi-monthly sampling.

1   Table 4. First detection of *C. pecorum* in lambs by CFT (antibodies) in relation to age. Lambs

2   with their first CFT detection were further categorised into (a) new CFT detection due to

3   concurrent seroconversion *i.e.,* matching qPCR detection, (b) new CFT detection due to delay in

4   seroconversion as a result of previous qPCR positive infection, and (c) new CFT detection due to

5   suspected previous infection that was missed due to bi-monthly sampling.

Age (months)	CFT+		CFT+ due to seroconversion	CFT+ due to lag in seroconversion	CFT+ due to suspected previous exposure
	No.	%			
2	10	13.1	2	0	8
4	0	0	0	0	0
6	30	39.4	17	5	8
8	20	26.3	5	9	6
10	1	1.3	1	0	0

Characteristics of *C. pecorum* bacterial load (qPCR load) and CFT antibody titers from 2 to 10 months of age. The qPCR load and CFT titers are expressed as mean and standard deviation.

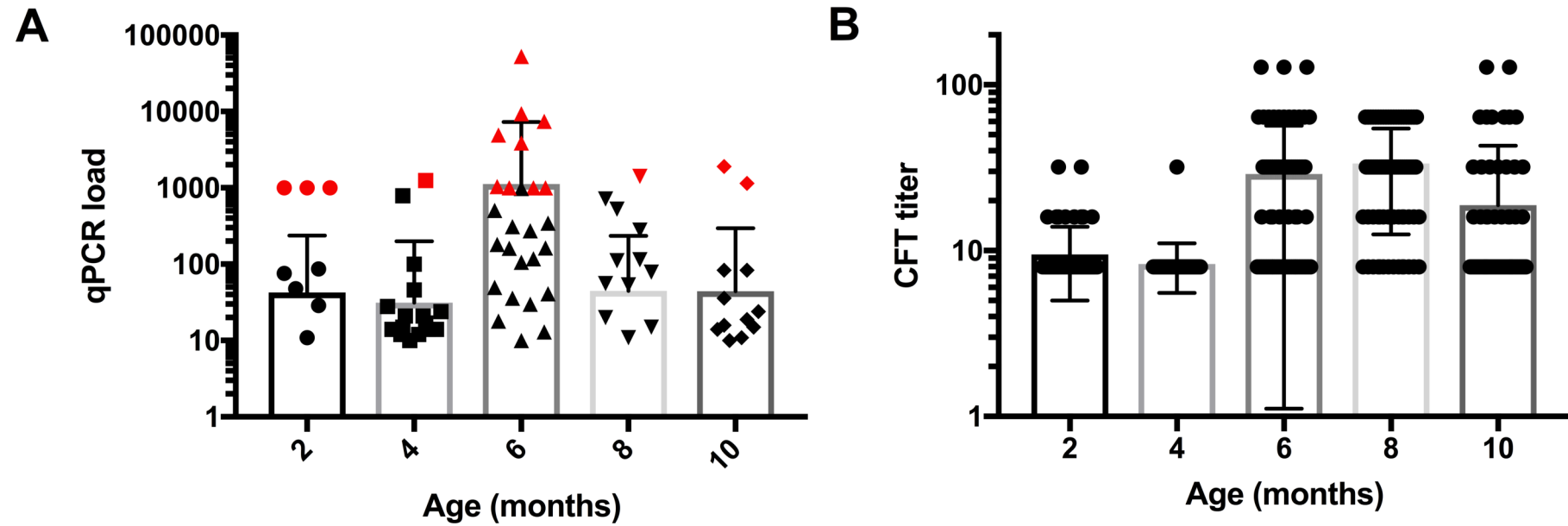
1 Table 5. Characteristics of *C. pecorum* bacterial load (qPCR load) and CFT antibody titers from

2 2 to 10 months of age. The qPCR load and CFT titers are expressed as mean and standard

3 deviation.

Age (months)	qPCR combined		Conjunctiva		Rectum		Vagina		CFT	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2	42.8	195.9	26.5	161.1	16.3	115.3	9.5	46.4	9.5	4.5
4	31.6	169.4	13.5	91.1	19.03	144.7	0.9	4.1	8.3	2.8
6	1128	6205	13.3	50.7	849.6	6143	544.2	1538	29	27.8
8	44.8	192.1	9.6	61.3	3.4	18.4	64.3	253.8	33.5	21
10	44.3	253.6	3.8	14.4	0.3	2.8	78.3	352.3	18.8	24

Age dependent distribution of *C. pecorum* bacterial load and antibody titers (A) qPCR load, lambs shedding high loads of *C. pecorum* DNA (≥ 1000 copies/µl) and CFT positive are indicated as data points in red (B) CFT titer.

From 2-6 months of age, lambs exhibited an overall increase in mean chlamydial loads, peaking at 6 months of age (1128±6205). Mean CFT titres were low at 2-4 months of age (9.5±4.5 and 8.3±2.8 at 2 and 4 months), and then peaked from 6-8 months of age (29±27.8 and 33.5±21 at 6 and 8 months), followed by a slight decline in mean titre at 10 months of age (18.8±24), respectively.

Age dependent distribution of *C. pecorum* bacterial load at (A) Conjunctiva (B) Rectum and (C) Vagina.

Lambs shedding high loads of C. pecorum DNA ($\geq$ 1000 copies/µl) and CFT positive are indicated as data points in red. The mean conjunctival bacterial loads trended downward from 2-10 months of age (26.5±161.1 to 3.8±14.4, respectively), in contrast, mean bacterial loads increased from 2-6 months of age (16.3±115.3 to 849.6±6143) at the rectal sampling site, diminishing markedly thereafter (0.8±2.3 at 10 months). For female lambs the greatest bacterial loads occurred from 6-10 months of age (544.2±1538 and 78.3±352.3 at 6 and 10 months) at the vaginal sampling site.