Murdoch
UNIVERSITY

MURDOCH RESEARCH REPOSITORY

This is the author's final version of the work, as accepted for publication following peer review but without the publisher's layout or pagination. The definitive version is available at
http://dx.doi.org/10.1016/j.tvjl.2014.05.037

Yang, R., Jacobson, C., Gardner, G., Carmichael, I., Campbell, A.J.D. and Ryan, U. (2014) Longitudinal prevalence and faecal shedding of Chlamydia pecorum in sheep. The Veterinary Journal, 201 (1). pp. 322-326.

http://researchrepository.murdoch.edu.au/22757/

Copyright: © 2014 Elsevier Ltd.

It is posted here for your personal use. No further distribution is permitted.

Accepted Manuscript

Title: Longitudinal prevalence and faecal shedding of *Chlamydia pecorum* in sheep

Author: Rongchang Yang, Caroline Jacobson, Graham Gardner, Ian Carmichael, Angus J.D. Campbell, Una Ryan

PII: S1090-0233(14)00234-2
DOI: http://dx.doi.org/doi:10.1016/j.tvjl.2014.05.037
Reference: YTVJL 4173

To appear in: *The Veterinary Journal*

Accepted date: 20-5-2014

Please cite this article as: Rongchang Yang, Caroline Jacobson, Graham Gardner, Ian Carmichael, Angus J.D. Campbell, Una Ryan, Longitudinal prevalence and faecal shedding of *Chlamydia pecorum* in sheep, *The Veterinary Journal* (2014), http://dx.doi.org/doi:10.1016/j.tvjl.2014.05.037.

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.

ACCEPTED MANUSCRIPT

Longitudinal prevalence and faecal shedding of *Chlamydia pecorum* in sheep

Rongchang Yang [a], Caroline Jacobson [a], Graham Gardner [a], Ian Carmichael [b], Angus J.D. Campbell [c], Una Ryan [a,*]

[a] *School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia, 6150, Australia*
[b] *South Australian Research and Development Institute, 33 Flemington Street, Glenside, South Australia 5065, Australia.*
[c] *Faculty of Veterinary Science, University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia*

* Corresponding author. Tel.: +61 8 9360 2482.
E-mail address: Una.Ryan@murdoch.edu.au (U. Ryan).

Accepted Manuscript

ACCEPTED MANUSCRIPT

Abstract

The prevalence and faecal shedding of *Chlamydia* spp. in sheep in Australia has not been well described. Two species-specific quantitative PCRs (qPCR) targeting the chlamydial outer membrane protein cell surface antigen gene (*ompA*) were validated and used to determine the prevalence and faecal shedding of *C. abortus* and *C. pecorum* from faecal samples of lambs at three sampling times (weaning, post-weaning and pre-slaughter) from eight farms in South Australia, New South Wales, Victoria and Western Australia. A total of 3412 faecal samples were collected and screened from approximately 1189 lambs across the four states. *C. abortus* was not detected in any of the samples screened. The overall prevalence of *C. pecorum* was 1027/3412 (30.1%) and median bacterial concentrations at weaning, post-weaning and pre-slaughter were 1.8×10^7, 1.2×10^7 and 9.6×10^5/g faeces, respectively. A subset of *C. pecorum* positive samples from each farm, ($n = 48$) were sequenced to confirm their identity. The present study demonstrates that *C. pecorum* is prevalent in Australian sheep, highlighting a need for further research on the impact of this bacterium on production.

Keywords: *Chlamydia pecorum*; Lambs; Quantitative PCR; *ompA*; Prevalence

Accepted Manuscript

ACCEPTED MANUSCRIPT

Introduction

Members of the genus *Chlamydia* cause disease in humans and animals, and most are zoonotic (Everett et al., 1999; Vlahovick and Lasta, 2006). Two species, *Chlamydia abortus (Chlamydia psittaci* serotype 1*)* and *Chlamydia pecorum* are known to infect sheep (Berri et al., 2009; Lenzko et al., 2011). Both species cause abortions in sheep and *C. pecorum* also causes enteritis in sheep (Berri et al., 2009). *Chlamydia abortus*, the causative agent of enzootic abortion of ewes (EAE), is also zoonotic (Rodolakis and Yousef Mohamad, 2010).

Infections caused by *Chlamydia* spp. have long been underestimated due to difficulties in diagnosis of these obligate intracellular pathogens, which require growth in embryonated eggs or tissue culture (Nordentoft et al., 2011). Immunoassays have also been developed, but lack specificity (Jones et al., 1997; McCauley et al., 2010). Currently, *C. abortus* is believed to be absent from Australia based on culture and immunoassays (McCauley et al., 2010; Animal Health Australia, 2012). However, relatively few studies have been conducted on the prevalence of ovine *Chlamydia* infections in Australia (St George, 1971; McCauley et al., 2010; Jelocnik et al., 2013) and no molecular surveys for *C. abortus* have been undertaken.

The aim of the present study was to use species-specific quantitative PCR (qPCR) primarily for *C. pecorum* (but also for *C. abortus*) to determine the prevalence, faecal shedding concentrations and species of *Chlamydia* in lambs over a wide geographical area, representing the major sheep growing regions of Australia, specifically Western Australia (WA), New South Wales (NSW), Victoria (Vic) and South Australia (SA), at three sampling times (weaning, post-weaning and pre-slaughter), and to compare these data between states.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Materials and methods

Animals and collection of faecal samples

Faecal samples were collected from cross-bred lambs from eight different farms across four states of Australia (Table 1). Lambs were born and reared in paddocks and were not housed indoors at any stage. Lambs were sampled on three occasions (i.e. the same animals were sampled on each occasion) at weaning (~12 weeks of age), post-weaning (~19 weeks of age) and pre-slaughter (~29 weeks of age). A total of 3412 faecal samples from ~1189 lambs were collected directly from the rectum. All sample collection methods used were approved by the Murdoch University Animal Ethics Committee (approval number R2352/10).

DNA isolation

Genomic DNA was extracted from 200 mg of each faecal sample using a QIAamp DNA Mini Stool Kit (Qiagen) or from 250 mg of each faecal sample using a Power Soil DNA Kit (MolBio). A negative control (no faecal sample) was used in each extraction group.

PCR amplification, quantification and sequencing

A species-specific 76 base pair (bp) product was amplified from the *C. pecorum* outer membrane protein cell surface antigen gene (*ompA*) using the forward primer CpecOMP1 F 5'- CCATGTGATCCTTGCGCTACT- 3', the reverse primer CpecOMP1 5'-TGTCGAAAACATAATCTCCGTAAAAT-3' and the probe CpecOMP1-S 5'-CAL-Fluor Orange-560-TGCGACGCGATTAGCTTACGCGTAG-TAMARA-3', as described previously (Pantchev et al., 2010). A *C. abortus* species-specific qPCR, also based on the *ompA* gene, which produces an 86 bp product, was amplified using the forward primer CpaOMP1-F 5'-GCAACTGACACTAAGTCGGCTACA-3', the reverse primer CpaOMP1-R 5'- ACAAGCATGTTCAATCGATAAGAGA-3' and the probe CpaOMP1-Sb 5'-dFAM-

Accepted Manuscript

ACCEPTED MANUSCRIPT

TAAATACCACGAATGGCAAGTTGGTTTAGCG-BHQ-1-3', as described previously (Pantchev et al., 2009). In the original studies by Pantchev et al. (2009, 2010), these were single PCRs; however for the present study, both assays were multiplexed into a single reaction with detection in different channels.

An internal amplification control (IAC), consisting of a fragment of a coding region from Jembrana disease virus (JDV) cloned into pGEM-T (Promega), was used as described previously (Yang et al., 2013). The IAC primers were JDVF (5'-GGTAGTGCTGAAAGACATT-3') and JDVR (5'-ATGTAGCTTGACCGGAAGT-3'), and the probe was 5'-(Cy5)-TGCCCGCTGCCTCAGTAGTGC-BHQ2-3'. Each 15 µL PCR mixture contained 1x PCR buffer, 4 mM $MgCl_2$, 1 mM each deoxynucleotide triphosphate, 1.0 U KAPA DNA polymerase (MolBio), 0.2 µM each of forward and reverse primers, 0.2 µM each of forward and reverse IAC primers, 50 nM specific probe, 50 nM IAC probe, 10 copies of IAC template and 1 µL sample DNA. The PCR cycling conditions consisted of 95 °C for 3 min, followed by 45 cycles of 95 °C for 20 s and 60 °C for 45 s. PCR contamination controls were used, including negative controls and separation of preparation and amplification areas.

A standard curve for quantifying *Chlamydia* spp. DNA was generated by cloning the PCR products amplified from *C. pecorum* or *C. abortus* into pGEMT (Promega) and transforming *Escherichia coli* competent cells. Plasmid DNA for each pathogen was isolated by alkali sodium dodecyl sulphate lysis, followed by column purification using QIAprep Spin Columns (Qiagen). Plasmid mini-preparations were sequenced using the T7 sequencing primer (Stratagene) and clones with the correct sequence then used as positive controls for generating a standard curve. A subset of two positive samples from each farm (n = 48) were

Accepted Manuscript

ACCEPTED MANUSCRIPT

agarose gel purified using an in-house filter tip method and used for sequencing without any further purification, as described previously (Yang et al., 2013).

Specificity and sensitivity

The analytical specificity of the *C. abortus* and *C. pecorum* species-specific qPCR assays has been described previously (Pantchev et al., 2009, 2010), but was further assessed by testing DNA from a wide range of bacterial and parasitic species. To determine the sensitivity of the assay, 10-fold serial dilutions of plasmids were prepared containing the cloned PCR products amplified from *C. abortus* or *C. pecorum*, these were spiked into faecal samples and the DNA was extracted and amplified as described above. The mean detection limits, R squared (RSQ) values and % relative standard deviation (RDS) were calculated. Template copy numbers were converted to numbers of organism present on the basis that the targeted gene (*OmpA)* is a single copy gene (Lan and Igo, 1998) and bacterial genomes are haploid. Therefore, the detected plasmid numbers were equivalent to the numbers of *Chlamydia* spp.

Inhibition and efficiency

Inhibition in faecal samples was measured using the IAC, which was added to all faecal DNA samples to detect any PCR inhibitors. If inhibition is present in a sample, the IAC will not produce a signal. Amplification efficiency (*E*), a measure of inhibition, was estimated by using the slope of the standard curve and the formula $E = -1+10^{(-1/\text{slope})}$. A reaction with 100% efficiency will generate a slope of -3.32. A PCR efficiency less than or greater than 100% can indicate the presence of inhibitors in the reaction, but reaction efficiencies between 90 and 110% are typically acceptable (Nybo, 2011). To estimate amplification efficiency, serial dilutions of individual DNA samples (neat, 1:10, 1:100) were performed and multiple

Accepted Manuscript

ACCEPTED MANUSCRIPT

qPCR reactions were conducted at each dilution. The *Ct* values were then plotted vs. the $\log_{10}$ of the dilution and a linear regression was performed using Rotor-Gene 6.0 software.

Molecular typing and sequence analysis

A subset of *C. pecorum* positive samples from each farm (n = 48) were sequenced to confirm their identity. Purified PCR products were sequenced using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions with the exception that the annealing temperature was raised to 58 ºC. Nucleotide sequences were analysed using Chromas lite version 2.0[1] and aligned with reference sequences from GenBank using Clustal W[2].

Statistical analysis

Prevalences were expressed as the percentage of samples positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). χ^2 and non-parametric analyses were performed using SPSS 21.0 for Windows (SPSS/IBM) to determine if there was any association between the prevalence and concentration of *C. pecorum* at different sampling times and across states.

Results

Specificity, sensitivity and efficiency

Evaluation of specificity of the multiplex *C. abortus* and *C. pecorum* qPCR assay revealed no cross-reactions with other genera and only amplified the relevant bacterial species (data not shown). There was no cross-detection of *C. pecorum* with the *C. abortus* primers

[1] See: http://www.technelysium.com.au.
[2] See: http://www.clustalw.genome.jp.

ACCEPTED MANUSCRIPT

and probe, and vice versa. Sensitivity analysis revealed that the mean limits of detection for *C. abortus* and *C. pecorum* were 5 and 5 organisms/μL, respectively, which equates to 1250 bacteria/g faeces. The mean RSQ values for *C. abortus* and *C. pecorum* qPCRs were 0.98 and 0.98, respectively. The RDS for *C. abortus* and *C. pecorum* were 4.7% and 3.9%, respectively. The frequency of PCR inhibition, as determined by the IAC amplification, was ~2%. If inhibition was evident, then the sample was diluted and re-amplified. The mean efficiencies for *C. abortus* and *C. pecorum* were 96.5% and 94.4%, respectively.

Prevalence of Chlamydia spp.

C. abortus was not detected in any samples. The overall prevalence of *C. pecorum* on eight farms across four states over three sampling times (weaning, post-weaning and pre-slaughter) was 1027/3412 (30.1%) (Fig. 1). Overall, there were significant differences in the prevalence of *C. pecorum* between states ($P < 0.01$); for example, the prevalence of *C. pecorum* was lower in WA than in the eastern states (Fig. 1). The *C. pecorum* prevalence was highest (94.2%) in SA2 during the post-weaning period, followed by 80.8% in NSW during the pre-slaughter period and 77% in SA1 during the post-weaning period. The highest prevalence for *C. pecorum* in WA was 48.6% at WA2 during the pre-slaughter period. There was no relationship between prevalence and the three sampling times ($P > 0.05$), since the peak prevalence occurred at different sampling times across the farms tested. A total of 422, 114, 309 and 152 lambs were positive for *C. pecorum* across all three samplings at SA, Vic, NSW and WA, respectively.

Concentration of Chlamydia spp. in faeces

Chlamydia spp. concentrations in faeces were also determined using the multiplex qPCR (Table 2; see Appendix A: Supplementary Table 1). The highest median number of *C.*

Accepted Manuscript

ACCEPTED MANUSCRIPT

188 *pecorum* organisms/g was detected in NSW at post-weaning, Vic1 at weaning and WA2 at
189 pre-slaughter (3.1×10^9, 2.3×10^9 and 1.4×10^9 organisms/g, respectively), with a maximum
190 pathogen load of 4.4×10^{12} organisms/g detected in one NSW sample at post-weaning. This
191 corresponded to *C. pecorum* prevalences of 72.5 % in NSW at post-weaning and 80.8% in
192 NSW at pre-slaughter. There were also peaks in the median numbers of organisms for the
193 weaning and post-weaning periods at SA2 (7.4×10^7 and 3.8×10^7 organisms/g, respectively)
194 and the post-weaning period at Vic 2 (6.9×10^7 organisms/g). The range of *C. pecorum*
195 shedding at weaning overall across all states was 2.5×10^2 to 3.8×10^{11} organisms/g and the
196 median was 1.8×10^7 organisms/g. At post-weaning, the range was 2.5×10^2 to 4.4×10^{12} and
197 the median was 1.2×10^7. At pre-slaughter, the range was 2.5×10^2 to 1.4×10^{11} and the
198 median was 9.6×10^5 (see Appendix A: Supplementary Table 1).
199
200 *Sequencing*
201 In subset of 48 positive samples (two from each sampling period from each farm), all
202 were confirmed by sequencing to be *C. pecorum* (data not shown).
203
204 **Discussion**
205 In this longitudinal study, the prevalence, concentration and species of *Chlamydia*
206 were determined in faecal samples collected from lambs at three sampling times (weaning,
207 post-weaning and pre-slaughter) from eight farms across four Australian states using species-
208 specific qPCR primers. Two species-specific qPCRs for *C. abortus* and *C. pecorum* (Pantchev
209 et al., 2009, 2010) were multiplexed into a single assay for rapid detection of both *Chlamydia*
210 spp. The multiplex PCR was specific for *C. abortus* and *C. pecorum*, since it only detected the
211 relevant species and did not cross-react with non-*Chlamydia* spp. isolates, in agreement with
212 the previous extensive specificity testing of these primers and probes (Pantchev et al., 2009,

Accepted Manuscript

ACCEPTED MANUSCRIPT

2010). In previous studies, the specificity was validated against 25 *Chlamydia* spp. isolates and 14 non-*Chlamydia* bacterial species (Pantchev et al., 2009, 2010). In the present study, the sensitivity of the species-specific qPCR assays was determined by cloning the PCR amplicons from *C. abortus* and *C. pecorum* sp. into a plasmid vector, then spiking known amounts of plasmid into faecal samples, extracting the DNA and screening by qPCR.

The mean limit of detection for *C. abortus* and *C. pecorum* was 5 and 5 organisms/µL faecal DNA extract, respectively. These detection limits are similar to or better than other studies on qPCR detection of *Chlamydia* spp. (Jee et al., 2004; Pelletier et al., 2006; Yang et al., 2006; Berri et al., 2009; Pantchev et al., 2009, 2010). PCRs can be inhibited in faecal specimens by factors such as bile acids, bilirubin, haem and complex carbohydrates (Wilson, 1997). In the present study, PCR inhibition (as determined by the IAC amplification) occurred in ~2% of samples.

Whilst numerous studies have conducted single point prevalence analysis by sampling a random selection of sheep within a flock at a specific time, few longitudinal studies have been conducted. The prevalence at one time of sampling may not provide a true indication of the overall prevalence in flocks over an extended period of time. In the present study, the overall prevalence of *C. pecorum* was 30.1%. However, the prevalence varied widely among states and at different sampling times. For example, the prevalence of *C. pecorum* was highest (94.2%) in SA2 during the post-weaning period, but was only 12.2% during the pre-slaughter period ~10 weeks later. Differences in prevalence could be related to a wide range of factors, including environment, stocking density and potential for faecal contamination of feed or water.

Accepted Manuscript

ACCEPTED MANUSCRIPT

238 *C. pecorum* was the only *Chlamydia* spp. identified in Australian sheep in the present
239 study. This is supported by a serological survey of 891 sheep from 109 properties across
240 southern Australia (McCauley et al., 2010). An obvious limitation of the present study is that
241 vaginal swabs were not screened and, therefore, conclusive evidence for the absence of *C.*
242 *abortus* in Australian sheep is lacking. However, *C. abortus* has been detected in faeces, as
243 well as the genital tract, of sheep (Tsakos et al., 2001; Lenzko et al., 2011).
244
245 PCR is more sensitive than traditional microscopy, culture and immunoassays for
246 detecting *Chlamydia* spp. (Amin, 2003; Nordentoft et al., 2011; Hazlett et al., 2013). In the
247 present study, the prevalence of *C. pecorum* across all states was highest in NSW (42.5-
248 80.8%) and lowest in Vic using qPCR (4.9-18.3%). A previous study using culture identified
249 *C. pecorum* in 4.8% of sheep in Egypt (Osman et al., 2011). A study in Germany using a
250 *Chlamydiaceae*-specific qPCR or conventional PCR identified *C. pecorum* in 13/32 (40.6%)
251 flocks, with in-flock prevalences of 9.1-27.3% (Lenzko et al., 2011). *Chlamydia* (thought to
252 be *C. pecorum*) has been detected in sheep faeces in Australia (St George, 1971) and, in the
253 most recent study conducted in Australia, *C. pecorum* was detected by PCR in 17-50% of
254 rectal swabs in sheep in New South Wales (Jelocnik et al., 2013).
255
256 The source of *C. pecorum* infections in Australian sheep is unclear. The organism is
257 also prevalent amongst koala populations (Jackson et al., 1999) and there is potential for *C.*
258 *pecorum* 'spillover' or 'spillback' between infected livestock and/or wildlife infections.
259 However, a recent *C. pecorum*-specific multilocus sequence analysis (MLSA) of koala-
260 derived and Australian sheep-derived isolates showed that the koala isolates formed a distinct
261 clade, with limited clustering with *C. pecorum* isolates from Australian sheep (Jelocnik et al.,
262 2013).

Accepted Manuscript

ACCEPTED MANUSCRIPT

Across all states, *C. pecorum* had median bacterial concentrations at weaning, post-weaning and pre-slaughter of 1.8×10^7, 1.2×10^7 and 9.6×10^5 organisms/g, respectively. However, individual sheep shed up to 4.4×10^{12} organisms/g (one lamb sampled post-weaning in NSW). The reasons for the higher bacterial shedding during weaning and post-weaning compared to pre-slaughter in the present study are unknown, but may be due to stress or to polyparasitism reducing host immunity, sine these sheep were co-infected with *Giardia*, *Cryptosporidium* and *Eimeria* spp., as well as strongyles (Yang et al., 2014a and b; R. Yang unpublished).

Conclusions

This study identified a high prevalence of faecal shedding of *C. pecorum* by sheep in four states in Australia. *C. abortus* was not detected in sheep faecal samples, but further screening of vaginal swabs is required to confirm the absence of *C. abortus* in sheep in Australia. Further studies are required to determine production losses associated with *C. pecorum* infection in sheep.

Conflict of interest statement

The study was financed by Meat and Livestock Australia (MLA), Australian Wool Innovation Limited (AWI) and the Australian Government, which had no influence on study design, data evaluation or manuscript preparation. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

Accepted Manuscript

ACCEPTED MANUSCRIPT

This study was funded by MLA, AWI and the Australian Government. We thank the participating farmers for providing access to sheep for collection of samples. We thank Justin Hoad for providing faecal samples from NSW. Samples from the WA farms were collected and DNA extracted by Joshua Sweeny. Thanks also go to Josephine Ng-Hublin for DNA extraction of faecal samples collected from the eastern states.

References

Amin, A.S., 2003. Comparison of polymerase chain reaction and cell culture for the detection of *Chlamydophila* species in the semen of bulls, buffalo-bulls, and rams. The Veterinary Journal 166, 86-92.

Animal Health Australia, 2012. Animal health status. In: Animal Health in Australia Report: Terrestrial Animal Health. http://www.animalhealthaustralia.com.au (accessed 19 September 2012).

Berri, M., Rekiki, A., Boumedine, K.S., Rodolakis, A., 2009. Simultaneous differential detection of *Chlamydophila abortus*, *Chlamydophila pecorum* and *Coxiella burnetii* from aborted ruminant's clinical samples using multiplex PCR. BMC Microbiology 9, 130.

Everett, K.D., Bush, R.M., Andersen, A.A., 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. International Journal of Systematic Bacteriology 49, 415-440.

Hazlett, M.J., McDowall, R., DeLay, J., Stalker, M., McEwen, B., van Dreumel, T., Spinato, M., Binnington, B., Slavic, D., Carman, S. et al., 2013. A prospective study of sheep and goat abortion using real-time polymerase chain reaction and cut point estimation shows *Coxiella burnetii* and *Chlamydophila abortus* infection concurrently with other major pathogens. Journal of Veterinary Diagnostic Investigation 25, 359-68.

Jackson, M., White, N., Giffard, P., Timms, P., 1999. Epizootiology of *Chlamydia* infections in two free-range koala populations. Progress in Veterinary Microbiology and Immunology 65, 255-264.

Jee, J., Degraves, F.J., Kim, T., Kaltenboeck, B., 2004. High prevalence of natural *Chlamydophila* species infection in calves. Journal of Clinical Microbiology 42, 5664-5672.

Jelocnik, M., Frentiu, F.D., Timms, P., Polkinghorne, A., 2013. Multilocus sequence analysis provides insights into molecular epidemiology of *Chlamydia pecorum* infections in Australian sheep, cattle, and koalas. Journal of Clinical Microbiology 51, 2625-32.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Jones, G.E., Low, J.C., Machell, J., Armstrong, K., 1997. Comparison of five tests for the detection of antibodies against chlamydial (enzootic) abortion of ewes. Veterinary Record 141, 164-168.

Lan, C.Y., Igo, M.M., 1998. Differential expression of the OmpF and OmpC porin proteins in *Escherichia coli* K-12 depends upon the level of active OmpR. Journal of Bacteriology 180, 171-174.

Lenzko, H., Moog, U., Henning, K., Lederbach, R., Diller, R., Menge, C., Sachse, K., Sprague, L.D., 2011. High frequency of chlamydial co-infections in clinically healthy sheep flocks. BMC Veterinary Research 7, 29.

McCauley, L.M., Lancaster, M.J., Butler, K.L., Ainsworth, C.G., 2010. Serological analysis of *Chlamydophila abortus* in Australian sheep and implications for the rejection of breeder sheep for export. Australian Veterinary Journal 88, 32-38.

Nordentoft, S., Kabell, S., Pedersen, K., 2011. Real-time detection and identification of *Chlamydophila* species in veterinary specimens by using SYBR green-based PCR assays. Applied and Environmental Microbiology 77, 6323-6330.

Nybo, K., 2011. qPCR efficiency calculations. Biotechniques. 51, 401-402.

Osman, K.M., Ali, H.A., El Jakee, J.A., Galal, H.M., 2011. *Chlamydophila psittaci* and *Chlamydophila pecorum* infections in goats and sheep in Egypt. Revue Scientifique et Technique (Office International des Epizooties) 30, 939-948.

Pantchev, A., Sting, R., Tyczka, J., Bauerfeind, R., Sachse, K., 2009. New real-time PCR tests for species-specific detection of *Chlamydophila psittaci* and *Chlamydophila abortus* from tissue samples. The Veterinary Journal 181, 145-150.

Pantchev, A., Sting, R., Bauerfeind, R., Tyczka, J., Sachse, K., 2010. Detection of all *Chlamydophila* and *Chlamydia* spp. of veterinary interest using species-specific real-time PCR assays. Comparative Immunology, Microbiology and Infectious Diseases 33, 473-484.

Pelletier, C., Chartier, S., Berthillier, J., Dégletagne, E., Rigaud, C., Berthet, H., Valognes, A., Reynaud, A., Véry, P., 2006. Validation of an internal method for the diagnosis of infections with *Chlamydophila abortus* and *Coxiella burnetii* by real-time multiplex PCR. Journal of Developmental Biology 126, 219-26.

Rodolakis, A., Yousef Mohamad, K., 2010. Zoonotic potential of *Chlamydophila*. Veterinary Microbiology 140, 382-391.

Rózsa, L., Reiczigel, J. Majoros, G., 2000. Quantifying parasites in samples of hosts. Journal of Parasitology 86, 228-232.

St George, T.D., 1971. The isolation of chlamydiae from faeces of sheep in Australia. Australian Veterinary Journal 47, 74.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Sweeny, J.P., Ryan, U.M., Robertson, I.D., Yang, R., Bell, K., Jacobson, C., 2011. Longitudinal investigation of protozoan parasites in meat lamb farms in southern Western Australia. Preventive Veterinary Medicine 101, 192-203.

Talafha, A.Q., Ababneh, M.M., Ababneh, M.M., Al-Majali, A.M., 2012. Prevalence and risk factors associated with *Chlamydophila abortus* infection in dairy herds in Jordan. Tropical Animal Health and Production 44, 1841-1846.

Tsakos, P., Siarkou, V., Guscetti, F., Chowdhury, H., Papaioannou, N., Vretou, E., Papadopoulos, O., 2001. Experimental infection of pregnant ewes with enteric and abortion-source *Chlamydia abortus*. Veterinary Microbiology 82, 285-291.

Vlahovick, D.A., Lasta, P., 2006. Zoonotic aspects of animal chlamydioses - a review. Veterinary Archives 76, 259-263.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63, 3741-3751.

Yang, J.M., Liu, H.X., Hao, Y.X., He, C., Zhao, D.M., 2006. Development of a rapid real-time PCR assay for detection and quantification of four familiar species of *Chlamydiaceae*. Journal of Clinical Virology 36, 79-81.

Yang, R., Murphy, C., Song, Y., Ng-Hublin, J., Estcourt, A., Hijjawi, N., Chalmers, R., Hadfield, S., Bath, A., Gordon C., Ryan, U.M., 2013. Specific and quantitative detection and identification of *Cryptosporidium hominis* and *C. parvum* in clinical and environmental samples. Experimental Parasitology. 135, 142-147.

Yang, R., Jacobson, C., Gardner, G., Carmichael, I., Campbell, A.J.D., Ryan, U., 2014a. Longitudinal prevalence, cyst shedding and molecular characterisation of *Giardia* in sheep across four states in Australia. Experimental Parasitology 137, 46-52.

Yang, R., Jacobson, C., Gardner, G., Carmichael, I., Campbell, A.J.D., Ng-Hublin, J., Ryan, U., 2014b. Longitudinal prevalence, oocyst shedding and molecular characterisation of *Cryptosporidium* species in sheep across four states in Australia. Veterinary Parasitology 200, 50-58.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Table 1
Sheep farms sampled during the present study.

Farm	Farm location	Mean annual rainfall (mm)	Farm size (Ha)	Number of sheep	Breed	Commencement of lambing	Goats and/or cattle on property?	Winter stocking rate (DSE/Ha)
SA1	Wirrega, SA	430	1040	1800	Suffolk	Mid-April	No	10
SA2	Struan, SA	550	1500	5500	BL/Merino x Suffolk	June	Yes	15
Vic1	Rosedale, Vic	620	300	300 ewes	BL/Merino x Dorset and Southdown	Mid-July	No	10
Vic2	Ballarat, Vic	750	1960	7000	Merino x Suffolk	Early August	Yes	13
NSW	Armidale, NSW	495	2958	1000	BL/Merino	May-August	No	20
WA1	Pingelly, WA	450	1500	1350	Merino x Suffolk	Mid-July	No	12
WA2	West Arthur, WA	500	1250	1750	Merino x Suffolk	Early August	No	10
WA3	Frankland, WA	550	560	3300	Merino x Suffolk	Mid-July	No	21

DSE, dry sheep equivalent; BL, Border Leicester; SA, South Australia; Vic, Victoria; NSW, New South Wales, WA, Western Australia.
DNA from samples from Western Australia was extracted as described in Sweeny et al., (2011).

Accepted Manuscript

ACCEPTED MANUSCRIPT

424 **Table 2**

425 Concentration of *Chlamydia pecorum* in faecal samples collected from sheep on eight farms in four states of
426 Australia over three sampling times as determined by qPCR.
427

Location	Age of sheep	Number/g sheep faeces	
		Median	Range
SA1	Weaning	1.9×10^4	500 to 9.0×10^9
	Post-weaning	1.3×10^5	250 to 6.6×10^7
	Pre-slaughter	1.7×10^5	250 to 2.4×10^6
SA2	Weaning	7.4×10^7	1.7×10^4 to 1.6×10^{11}
	Post-weaning	3.8×10^7	1.3×10^3 to 3.0×10^{10}
	Pre-slaughter	8.4×10^4	4.5×10^3 to 1.0×10^6
Vic1	Weaning	2.3×10^9	3.0×10^3 to 2.3×10^{11}
	Post-weaning	1.7×10^5	250 to 1.6×10^8
	Pre-slaughter	1.9×10^4	250 to 2.8×10^5
Vic2	Weaning	0	0
	Post-weaning	6.9×10^7	7.9×10^5 to 8.2×10^{10}
	Pre-slaughter	1.3×10^3	500 to 6.3×10^3
NSW	Weaning	7.7×10^6	250 to 3.8×10^{11}
	Post-weaning	3.1×10^9	4.5×10^4 to 4.4×10^{12}
	Pre-slaughter	8.9×10^5	250 to 4.0×10^9
WA1-	Weaning	3.5×10^6	250 to 1.8×10^9
AR	Post-weaning	9.3×10^5	1.3×10^3 to 1.7×10^9
	Pre-slaughter	9.4×10^4	3.0×10^3 to 1.1×10^6
WA2-PL	Weaning	1.5×10^6	1.5×10^3 to 2.9×10^7
	Post-weaning	1.0×10^6	250 to 1.7×10^8
	Pre-slaughter	1.4×10^9	1.1×10^6 to 1.4×10^{11}
WA3-FL	Weaning	0	0
	Post-weaning	5.2×10^8	1.5×10^3 to 1.6×10^{11}
	Pre-slaughter	1.3×10^3	250 to 8.9×10^7
Total		8.1×10^5	0 to 4.4×10^{12}

428

Accepted Manuscript

ACCEPTED MANUSCRIPT

Figure legend

Fig. 1. Prevalence (%) of *Chlamydia pecorum* in ovine faecal samples from eight farms across four states (NSW, SA, Vic and WA) over three sampling times (weaning, post-weaning and pre-slaughter) as determined by qPCR.

Accepted Manuscript