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

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ABSTRACT

The prevalence and faecal shedding of *Chlamydia* spp. in sheep in Australia has not been well described. Two species-specific quantitative PCRs (qPCRs) 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, ($n = 48$) was 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.

Introduction

Members of the genus *Chlamydia* cause disease in human beings 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 *chlamydial* 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.

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

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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 × Suffolk	June	Yes	15
Vic1	Rosedale, Vic	620	300	300 ewes	BL/Merino × Dorset and Southdown	Mid-July	No	10
Vic2	Ballarat, Vic	750	1960	7000	Merino × Suffolk	Early August	Yes	13
NSW	Armidale, NSW	495	2958	1000	BL/Merino	May-August	No	20
WA1	Pingelly, WA	450	1500	1350	Merino × Suffolk	Mid-July	No	12
WA2	West Arthur, WA	500	1250	1750	Merino × Suffolk	Early August	No	10
WA3	Frankland, WA	550	560	3300	Merino × 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).

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-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 pGEM-T (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 were used as positive controls for generating standard curves. A subset of two positive samples from each farm ($n = 48$) were 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 (RSD) 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 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 °C. 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 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 RSD for *C. abortus* and *C. pecorum* were 4.7% and 3.9%, respectively. The frequency of PCR inhibition, as determined using the IAC, 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.

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