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Longitudinal prevalence, oocyst shedding and molecular characterisation of *Cryptosporidium* species in sheep across four states in Australia

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

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

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ABSTRACT

The prevalence of *Cryptosporidium* in sheep in the eastern states of Australia has not been well described, therefore a study of the prevalence, oocyst concentration, species and subtypes of *Cryptosporidium* were assessed from lamb faecal samples at three sampling periods (weaning, post-weaning and pre-slaughter) from eight farms across South Australia, New South Wales, Victoria and Western Australia. A total of 3412 faecal samples were collected from approximately 1182 lambs across the four states and screened for the presence of *Cryptosporidium* using a quantitative PCR (qPCR) at the actin locus. Positives were typed at the 18S locus and at a second locus using *C. parvum* and *C. hominis* specific qPCR primers. The overall prevalence was 16.9% (95% CI: 15.6–18.1%) and of the 576 positives, 500 were successfully genotyped. In general, the prevalence of *Cryptosporidium* was higher in WA than the eastern states. *Cryptosporidium* prevalence peaked at 43.9% and 37.1% at Pingelly (WA2) and Arthur River (WA1), respectively during weaning and at Pingelly (WA2) during pre-slaughter (36.4%). The range of oocyst shedding at weaning overall across all states was 63–7.9×10^6 and the median was 3.2×10^4 oocysts g^{-1}. The following species were identified; *C. xiaoi* (69%—345/500), *C. ubiquitum* (17.6%—88/500), *C. parvum* (9.8%—49/500), *C. scrofarum* (0.8%—4/500), mixed *C. parvum* and *C. xiaoi* (2.4%—12/500), *C. andersoni* (0.2%—1/500) and sheep genotype 1 (0.2%—1/500). Subtyping of *C. parvum* and *C. ubiquitum* isolates identified IIa and IId subtype families within *C. parvum* (with IId as the dominant subtype) and XIIa within *C. ubiquitum*. This is the first published description of *C. parvum* subtypes detected in lambs in Australia.

1. Introduction

Cryptosporidium is an enteric protozoa parasite that causes diarrhoeal illness in humans and animals worldwide (Xiao, 2010). Currently there are approximately 25 valid species and more than 50 genotypes. As sheep may potentially contribute significantly to contamination of watersheds, it is important to understand the public health risk posed by *Cryptosporidium* infections in sheep. Molecular studies have identified at least eight *Cryptosporidium* species in sheep faeces including *C. parvum*, *C. hominis*, *C. andersoni, C. suis, C. xiaoi*, *C. fayeri*, *C. ubiquitum* and *C. scrofarum*, with *C. xiaoi, C. ubiquitum* and *C. parvum* most prevalent (Ryan et al., 2005; Santín et al., 2007; Soltane et al., 2007; Geurden et al., 2008; Mueller-Doblies

* Corresponding author. Tel.: +8 9360 2482; fax: +8 9310 4144/ +8 9360 2482.
E-mail address: Una.Ryan@murdoch.edu.au (U. Ryan).

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et al., 2008; Quílez et al., 2008a; Fayer and Santín, 2009; Giles et al., 2009; Paoletti et al., 2009; Yang et al., 2009; Díaz et al., 2010; Robertson et al., 2010; Wang et al., 2010; Fiuza et al., 2011; Shen et al., 2011; Sweeny et al., 2011; Cacciò et al., 2013; Connelly et al., 2013; Imre et al., 2013; Ye et al., 2013). Previous studies conducted in Australia have examined sheep and pre and post-weaned lambs (typically 4 months of age and older) in Western Australia (WA) only (Ryan et al., 2005; Yang et al., 2009; Sweeny et al., 2011). Therefore the aim of the present study was to determine the prevalence, oocyst shedding concentration and genotypes of *Cryptosporidium* lambs in WA, New South Wales (NSW), Victoria (Vic) and South Australia (SA) at three sampling periods (weaning, post-weaning and pre-slaughter) and compare this data between states.

2. Materials and methods

2.1. Animals and faecal sample collection

A total of 3412 faecal samples were collected directly from the rectum of approximately 1189 cross-bred lambs from eight different farms across four states (Table 1). Lambs were sampled on three occasions (i.e. the same animals were sampled on each occasion) at weaning (approx. 12 weeks of age), post-weaning (approx. 19 weeks) and pre-slaughter (approx. 29 weeks). All sample collection methods used were approved by the Murdoch University Animal Ethics Committee (approval number R2352/10).

2.2. DNA isolation

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

2.3. PCR amplification.

All samples were screened at the actin locus using a quantitative PCR (qPCR) using the forward primer, Allactin F1 5′ ATCGTGAAAGAATGACWCAAATTATGTT 3′, the reverse primer Allactin R1 5′ ACCTTCATAAATTGGAACGGTGTG 3′ and the probe 5′-(FAM)-CCAGCAATGTATGTTAATA BHQ1 3′ which produces a 161 bp product. An internal amplification control (IAC) consisted of a fragment of a coding region from Jembrana Disease Virus (JDV) cloned into a pGEM-T vector (Promega, USA) was used as previously described (Yang et al., 2013). Each 15 μl PCR mixture contained 1×PCR buffer, 5 mM $MgCl_2$, 1 mM dNTP's, 1.0 U Kapa DNA polymerase (MolBio, Carlsbad, CA), 0.2 μM each of forward and reverse primers, 0.2 μM each of forward and reverse IAC primers, 50 nM of the probe, 50 nM of IAC probe, 10 copies of IAC template and 1 μl of sample DNA. The PCR cycling conditions consisted of a pre-melt at 95 °C for 3 min and then 45 cycles of 95 °C for 30 s, and a combined annealing and extension step of 60 °C for 45 s. A standard curve for quantifying *Cryptosporidium* DNA was generated using a series of dilutions of standard oocyst DNA extracted from *C. parvum* (IOWA isolate).

Positives were also amplified at the 18S ribosomal RNA (rRNA) locus using a nested protocol previously described (Ryan et al., 2003). All positives were also screened using a *C. parvum* and *C. hominis* specific qPCR at a unique *Cryptosporidium* specific protein-coding locus previously described (Yang et al., 2009, 2013; Morgan et al., 1996).

Sub-genotyping of *C. parvum* isolates was performed using a two-step nested PCR to amplify a ~832 bp fragment of the *gp60* gene as described (Ng et al., 2008). Subtyping of *C. ubiquitum* was performed using a two-step nested PCR to amplify a ~948 bp fragment of the *gp60* gene as described (Li et al., 2014).

PCR contamination controls were used including negative controls and separation of preparation and amplification areas. The amplified DNA fragments from the secondary PCR product were separated by gel electrophoresis and purified using an in house filter tip method and used for sequencing without any further purification as previously described (Yang et al., 2013).

2.4. Specificity and sensitivity testing of the actin qPCR

The analytical specificity of the qPCR assay was assessed by testing DNA from *C. muris*, *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. andersoni*, *C. serpentis*, *C. canis*, *C. suis*, *C. bovis*, *C. fayeri*, *C. macropodum*, *C. ryanae*, *C. xiaoi*, *C. ubiquitum*, *C. tyzzeri*, mouse genotype II and *C. scrofarum* and non-*Cryptosporidium spp.*: *Isospora*, *Tenebrio*, *Giardia duodenalis*, *Cyclospora*, *Campylobacter spp.*, *Salmonella spp.*, *Toxoplasma gondii*, *Trichostrongylus spp.*, *Teladorsagia circumcincta*, *Haemonchus contortus*, *Streptococcus bovis* (ATCC 33317), *Enterococcus durans* (ATCC 11576), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633) and *Eimeria sp.*, as well as human, sheep and cattle DNA.

In order to determine the sensitivity of the assay, the PCR product amplified from *C. xiaoi* was cloned into the pGEMT-vector (Promega) and transformed into *E. coli* (JM109) competent cells. Plasmid DNA was isolated using a QIAprep Spin Columns (Qiagen) and sequenced using the T7 sequencing primer (Stratagene, La Jolla, CA, USA) and clones with the correct sequence were then used. The plasmid copy numbers were calculated based on the plasmid size (base pairs) and DNA concentration. 10-fold series dilutions of plasmid were conducted from 10,000 copies down to one copy of the genomic template for sensitivity testing and these were then spiked into faecal samples and the DNA extracted and amplified as described above and mean detection limits, RSQ (R squared) values and % Relative Standard Deviation (RDS) were calculated. Target copy numbers detected were converted to numbers of oocysts based on the fact that the actin gene in *Cryptosporidium* is a single copy gene (Kim et al., 1992) and there are four haploid sporozoites per oocyst. Therefore, every four copies of actin detected by qPCR were equivalent to one oocyst.