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Longitudinal prevalence, oocyst shedding and molecular characterisation of *Cryptosporidium* species in sheep across four states in 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 3,412 faecal samples were collected from approximately 1,182 lambs across the 4 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 x10^6 and the median was 3.2 x 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.

Keywords: *Cryptosporidium*; lambs; qPCR; actin; 18S rRNA; *gp60*; *C. xiaoi*; *C. ubiquitum*; *C. parvum; C. scrofarum*

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 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 3,412 faecal samples were collected directly from the rectum of approximately 1,189 cross-bred lambs from 8 different farms across 4 states (Table 1). Lambs were sampled on 3 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 200mg of each faecal sample using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) or from 250mg 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, California), 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 sec, and a combined annealing and extension step of 60°C for 45 sec. 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., 1997).

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., 2013).

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 1 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 4 haploid sporozoites per oocyst. Therefore, every 4 copies of actin detected by qPCR were equivalent to 1 oocyst.

2.5 Investigation of inhibition and efficiency

Inhibition in faecal samples was measured using the IAC and amplification efficiency (*E*), (estimated by using the slope of the standard curve and the formula $E = -1+10^{(-1/\text{slope})}$) which was based on multiple qPCR reactions on serial dilutions of individual DNA samples (neat, 1:10, 1:100). The *Ct* values were then plotted versus the log base 10 of the dilution and a linear regression was performed using the Rotor-Gene 6.0. software.

2.6 Sequence analysis

Purified PCR products were sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California). Nucleotide sequences were analyzed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp).

147 *2.7 Statistical analysis*

148

149 Prevalences were expressed as the percentage of samples positive by PCR, with 95%
150 confidence intervals calculated assuming a binomial distribution, using the software Quantitative
151 Parasitology 3.0 (Rózsa et al., 2000). Chi-square and non-parametric analyses were performed using
152 SPSS 21.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA) to
153 determine if there was any association between the prevalence and concentration of
154 *Cryptosporidium* oocysts at different sampling times and across states.

155

156 **3 Results**

157

158 *3.1 Specificity, sensitivity and efficiency testing of the actin qPCR*

159

160 Evaluation of specificity of the actin qPCR assay revealed no cross-reactions with other
161 genera and detected all the *Cryptosporidium* isolates tested (data not shown). Sensitivity analysis
162 revealed that the assay could reliably detected 8 copies of the cloned *C. xiaoi* amplicon per µl of
163 faecal DNA extract which is equivalent to a sensitivity of 2 *Cryptosporidium* oocysts per µl of
164 faecal DNA extract. The mean RSQ was 0.99 and the % RDS = 1.5%. In our hands, the incidence
165 of PCR inhibition as determined by the IAC amplification was about 2%. If inhibition was evident,
166 then the sample was diluted and re-amplified. The mean efficiency for the *Cryptosporidium* qPCR
167 was 108.4%.

168

169 *3.2 Prevalence of Cryptosporidium from 8 farms across 4 states*

170

171 The overall prevalence of *Cryptosporidium* from 8 farms across 4 states over 3 sampling
172 periods (weaning, post-weaning and pre-slaughter) was 16.9% (576/3412) (Tables 2 and 3 and Fig.

1a). There was no relationship between prevalence and the 3 sampling times ($p>0.05$), as the peak prevalence occurred at different sampling times across the farms tested. There was however a significant difference between farms ($p>0.05$). The prevalence of *Cryptosporidium* at WA2 was significantly higher than all other farms ($p<0.05$). There were also significant differences between WA1 and SA1 and between NSW and SA1 ($p<0.05$). The prevalence of *Cryptosporidium* was highest in WA, which peaked at 43.9% and 37.1% at WA2 and WA1 respectively during weaning and at WA2 during pre-slaughter (36.4%). There were smaller peaks for *Cryptosporidium* at NSW (27.5% and 22.5% respectively during post-weaning and weaning respectively), at Vic 2 (21% at weaning), Vic1 (18.6% at post-weaning). In SA, the prevalence peaked at 19.2% at post-weaning at SA2. The overall prevalence in WA on the 3 farms was 25% (248/992). The prevalence in NSW was 20.7% (101/487), in Vic was 11.8% (117/989) and in SA, it was 11.3% (107/944), but these state-wide differences were not significant ($p>0.05$)(Fig 1b). Only 4, 2 and 1 lambs from WA, Vic and NSW respectively were positive across all 3 samplings.

3.3 Oocyst load

Oocyst numbers per gram of faeces (g^{-1}) were also determined using qPCR (Tables 2 and 3). The highest median concentration of *Cryptosporidium* oocysts were shed by lambs at WA2 (1.5×10^5 oocysts g^{-1}). Across the other farms, median *Cryptosporidium* oocysts concentration peaked during the pre-slaughter period at SA1, SA2, Vic 2 and WA3 (1.4×10^5, 8.3×10^4, 9.3×10^4 and 1.1×10^5 oocysts g^{-1} respectively). The median concentration of oocysts shed at Vic1 was low (1.6×10^3- 1.6×10^4), although individual sheep shed up to 3.7×10^9 oocysts g^{-1} during post-weaning. This corresponded with a peak prevalence of 18.6% at this time at Vic1. At NSW, the median concentration of oocysts shed was also low (6.3×10^3-1.7×10^4 oocysts g^{-1}) but individual sheep at NSW shed up to 2.1×10^8 and 1.4×10^7 oocysts during post-weaning and pre-slaughter respectively. Across both SA farms, the range of shedding at weaning was 375-7.9×10^6 and the median was 8.8

199 x 10^4. The range of oocyst shedding at weaning overall across all states was 63-7.9 x 10^6 and the
200 median was 3.2 x 10^4. At pre-slaughter, the range was 260-4.8 x 10^7 and the median was 6.3 x 10^4
201 (Table 3). Of the 7 samples that were positive at all 3 samplings, no trends were seen in oocysts
202 concentration; some were high at the weaning sampling (8.1 x 10^3-1.4 x 10^8) and continued to
203 decrease but others shed an increased concentration of oocysts at the pre-slaughter sampling.
204
205 *3.4 Cryptosporidium genotypes*
206
207 The 576 positives detected at the actin locus were screened using *C. parvum/C. hominis*
208 specific primers and the 18S primers and the positives sequenced. Of these, 500 were successfully
209 genotyped. The following species/genotypes were identified; *C. xiaoi* (69% - 345/500), *C.*
210 *ubiquitum* (17.6% - 88/500), *C. parvum* (9.8% - 49/500), *C. scrofarum* (previously pig genotype II)
211 (0.8% - 4/500), mixed *C. parvum* and *C. xiaoi* (2.4% - 12/500), *C. andersoni* (0.2% -1/500) and
212 sheep genotype 1 (0.2% -1/500) (Table 4).
213 Across the states, *C. xiaoi* was the most prevalent and peaked at 81.4% (57/70) of positive
214 samples for NSW (Fig. 3). *Cryptosporidium ubiquitum* was the second most prevalent species in SA
215 at 43.2% (43/88) positive samples and in Vic at 21.7% (20/92), whereas in NSW and WA, *C.*
216 *parvum* was the second most prevalent species at 10% (7/70) and 10.4% (26/250) compared to
217 8.6% (6/72) and 9.6% (24/250) respectively for *C. ubiquitum*. *Cryptosporidium scrofarum* was only
218 identified in Vic and mixed *C. parvum, C. xiaoi* infections as well as *C. andersoni* and sheep
219 genotype I were only identified in WA.
220
221 *3.5 Cryptosporidium subtypes*
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223 Subtyping of *C. parvum* identified IIa and IId subtype families. All *C. ubiquitum* isolates
224 typed (n=88) were XIIa (Table 4). The following *C. parvum* subtypes were identified; IIaA15G2R1

(n=5), IIdA19G1 (n=10), IIdA18G1 (n=23). The *C. parvum* subtype IIaA15G2R1 was identified at Vic1 (n=3) and NSW (n=2). Subtype IIdA19G1 was identified in SA1 (n=3), SA2 (n=3), Vic1 (n=1) and Vic2 (n=3). Subtype IIdA18G1 was identified in SA1 (n=1), NSW (n=3), WA1 (n=2), WA2 (n= 9) and WA2 (n=8).

4. Discussion

In the present study, a novel qPCR at the actin locus was developed and 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 four Australian states.

The qPCR assay was very specific for *Cryptosporidium,* as it detected all the *Cryptosporidium* species tested and did not cross-react with the non-*Cryptosporidium* isolates analysed. The sensitivity of the assay was determined by cloning the actin *C. xiaoi* PCR amplicon into a plasmid vector, and then spiking known amounts of plasmid into faecal samples, extracting the DNA and screening by qPCR. The assay could reliably detect 2 *Cryptosporidium* oocysts per µl of faecal DNA extract, which is similar to or better than sensitivities reported previously for *Cryptosporidium* qPCR detection assays (Hadfield et al., 2011; Koken et al., 2013).

The overall prevalence of *Cryptosporidium* from 8 farms across 4 states over 3 sampling periods (weaning, post-weaning and pre-slaughter) was 16.9% (576/3412). There was no relationship between prevalence and lamb age, which ranged from ~12 weeks (weaning) to ~29 weeks (pre-slaughter). Previous studies in cattle have shown that the highest prevalence occurs in very young animals (~ 2 weeks of age) (Santín et al., 2008). In sheep, it has been shown that ewes (2-6 years in age) had a much higher prevalence of *Cryptosporidium* than lambs aged 7-21 days in age (Santín et al., 2007). However evidence suggests that *Cryptosporidium* prevalence is not highest in very young lambs. For example, previous studies in WA have reported prevalences by

PCR of 26% for slaughter age lambs in WA (Ryan et al., 2005) and 24.5% for pre-weaned lambs (aged 1-8 weeks) in WA (Yang et al., 2009). A recent study reported that the prevalence in 3-4 week-old and 15-16 week-old lambs was 18.4% and 26.7% respectively (Ye et al., 2013). Another study reported that the prevalence in 5-6 week old lambs increased from 15% to 25% in 6-10 week old lambs (Robertson et al., 2010). Further longitudinal research is required to better understand the relationship between the prevalence of *Cryptosporidium* and lamb age.

Oocyst concentration (numbers per gram of faeces) was also determined using qPCR. Accurate quantification of *Cryptosporidium* oocysts in animal faecal deposits on land is an essential starting point for estimating catchment *Cryptosporidium* loads (Davies et al., 2003). There are limited reports, however, on the concentration and environmental loading of *Cryptosporidium* oocysts as a result of faecal contamination by sheep. It is also important to note that oocyst recovery rates from faecal samples and across animal types can be highly variable. For example, recovery rates ranging 14-70% for adult cattle faeces, 0-83% for calf faeces, 4-48% for sheep faeces, 40-73% for kangaroo faeces, and 3-24% for pig faeces have been reported (Davies et al., 2003). Thus, oocyst shedding rates reported in various studies may underestimate the number of oocysts unless recovery efficiency is factored into the analysis. A previous study which examined a range of animal faeces in Sydney catchments, reported that the range of oocyst shedding concentration for adult sheep was 1-52,474 g^{-1} with a median of 148 g^{-1} whereas the range for juvenile sheep was 1-641 g^{-1} with a median of 275 g^{-1} (Davies et al., 2003). In the present study, oocyst numbers (concentration) were determined directly by qPCR from total DNA extractions from unpurified faecal samples, which obviates the need for recovery rate calculations. The average range of oocyst shedding concentration at weaning overall (across all states) was 63 - 7.9 $x10^6$ and the median was $3.2x10^4$ g^{-1}. At pre-slaughter, the average range was 260-4.8 x 10^7 and the median was $6.3x10^4$ g^{-1}. These shedding rates are higher than the previous study and highlights the advantages of using a method that does not require purification of oocysts and utilises a PCR-based detection method, which has been shown to be much more sensitive than microscopy (Ryan et al., 2005). The data

277 shows that although the prevalence in SA was lower than WA, oocyst shedding concentrations were
278 higher in SA.

279 A total of 6 genotypes were identified including *C. xiaoi*, *C. ubiquitum*, *C. parvum*, *C.*
280 *scrofarum, C. andersoni* and sheep genotype 1, with *C. xiaoi* and *C. ubiquitum* responsible for
281 86.6% of infections typed compared to 12.2% for *C. parvum* (includes the mixed *C. parvum, C.*
282 *xiaoi* isolates). *Cryptosporidium ubiquitum* is a common human pathogen (Xiao, 2010). In
283 Australia, *C. ubiquitum* has not been identified in the limited typing of Australian human
284 *Cryptosporidium* isolates that has been conducted to date (Ryan and Power, 2012), however *C.*
285 *ubiquitum* has been identified in source water in Australia (unpublished) and should be considered a
286 zoonotic species. *Cryptosporidium xiaoi* has only been reported once in two HIV-positive
287 individuals in Ethiopia (Adamu et al., 2013). *Cryptosporidium scrofarum* was detected in 4 lambs
288 from Vic and not in any other samples. It is primarily a porcine parasite (Kváč et al., 2013), but has
289 previously been identified in sheep and cattle in WA (Ryan et al., 2005; Ng et al., 2011) and has
290 been reported in an immunocompetent human (Kváč et al., 2009). Sheep genotype I was identified
291 in one sheep at WA1. This genotype has not been identified in humans and is genetically distinct at
292 both the 18S and actin loci but most closely related to *C. ubiquitum* (Sweeny et al., 2011).
293 *Cryptosporidium andersoni* was also identified in one isolate from WA. This is primarily a bovine
294 parasite but has previously been identified in sheep in WA (Ryan et al., 2005) and a human in NSW
295 (Waldron et al., 2011a). Therefore 30.8% (154/500) of the positive samples identified were
296 potentially zoonotic. This is the first report of ovine genotypes from NSW, Vic and SA. Previous
297 studies have also reported that *C. xiaoi* and *C. ubiquitum* are the dominant species infecting sheep
298 (Yang et al., 2009; Robertson et al., 2010; Wang et al., 2010; Fiuza et al., 2011), although other
299 studies have reported that *C. parvum* (Ryan et al., 2005; Mueller-Doblies et al., 2008; Cacciò et al.,
300 2013; Imre et al., 2013) and even *C. hominis* were more dominant than *C. ubiquitum* in sheep
301 (Connelly et al., 2013)

At the *gp60* locus, two subtype families were identified (IIa and IId). At least 12 *C. parvum* subtype families (IIa-IIl) have been identified at this locus, but only IId and especially the most common subtype family, IIa, appear to be zoonotic (Xiao, 2010). Prior to the present study, ovine-derived *C. parvum* isolates from Australia had not been subtyped at the *gp60* locus. The *C. parvum* subtype IIaA15G2R1 was identified in lambs in Vic and NSW. This is a dominant subtype in ruminants and has been reported in humans and calves in Australia (O'Brien et al., 2008; Waldron et al., 2011b) and worldwide (Xiao, 2010; Abeywardena et al., 2012; Alyousefi et al., 2012, Silva et al., 2013). This is the first report of IIaA15G2R1 in lambs in Australia. This subtype was also previously seen in three lambs linked to a human infection in the United Kingdom (Chalmers et al., 2005).

The *C. parvum* IId subtype family is less common and has been reported mainly from sheep and goats but has also been reported in humans and cattle overseas (Xiao, 2010). The IId subtype family has not been reported in cattle in Australia (as previous studies have only identified IIa subtypes in cattle), but has been reported in humans (Waldron et al., 2009; Ng et al., 2010). In the present study, subtype IIdA19G1 was identified in lambs from SA and Vic and subtype IIdA18G1 was identified in SA, NSW and WA. Subtype IIdA18G1 was previously identified in lambs in Spain and subtype IIdA19G1 was identified in both lambs and goats in the same study (Quilez et al., 2008a). Both subtypes are rare and have not been reported in humans in Australia. Previous studies have identified IIdA15G1 (Ng et al., 2010) and IIdA24G1 (Waldron et al., 2009) in individual human patients. In Spain, where both IIa and IId have been identified, IIa subtypes appear to preferentially infect calves, whereas IId subtypes preferentially infect lambs and goat kids (Quilez et al., 2008a; 2008b). Of the 38 *C. parvum* subtypes identified in the present study, the IId subtype family accounted for 87% (33/38) of the subtypes identified. This data along with evidence from studies overseas suggest that subtype family IId is adapted to lambs (and goat kids), and may therefore be to be one of the most important reservoirs for this zoonotic group of *C. parvum* isolates (Quilez et al., 2008a, 2008b; Imre et al., 2013).

All *C. ubiquitum* isolates analysed at the *gp60* locus were typed as subtype XIIa. To date six subtype families (XIIa to XIIf) have been identified in *C. ubiquitum* (Li et al., 2013). XIIa has been found in ruminants world-wide, XIIb to XIId in rodents in the United States, XIIe and XIIf in rodents in the Slovak Republic. XIIa, XIIb, XIIc, and XIId have been found in humans, therefore XIIa is a potentially zoonotic subtype (Li et al., 2013).

In conclusion, the present study identified that *Cryptosporidium* is prevalent in lambs across Australia and that lambs are capable of harboring *Cryptosporidium* species that are known to be zoonotic as well as those that appear to be host-specific. In addition, lambs may contribute significant amounts of *Cryptosporidium* oocysts to catchments, which has important implications for catchment management. Further studies are required to determine the prevalence of *C. ubiquitum* in the human population in Australia, and the extent of economic loss associated with *Cryptosporidium* in sheep.

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489

Figure 1A. Prevalence (%) of *Cryptosporidium* in sheep faecal samples from 8 farms across 4 states (NSW, SA, Vic and WA) over 3 sampling times (weaning, post-weaning and pre-slaughter) as determined by qPCR. 1B. Overall *Cryptosporidium* prevalence per state.

Figure 2. The prevalence (%) of *Cryptosporidium* species in sheep faecal samples from SA, Vic, WA and NSW.

496 Table 1. Sheep farms sampled during the present study.
497

Farm	Farm location	Mean annual rainfall (mm)	Farm size	Number of sheep	Breed of sheep	Commencement of lambing	Winter stocking rate
SA1	Wirrega, SA	430	1040 ha	1800	Suffolk	mid April	10DSE/ha
SA2	Struan, SA	550	1500 ha	5500	BL/Merino x Suffolk	June	15DSE/ha
Vic1	Rosedale, Victoria	620	300 ha (winter)	300 ewes	BL/Merino x Dorset & Southdown	mid July	10DSE/ha
Vic2	Ballarat, Victoria	750	1960 ha	7000	Merino x Suffolk	early August	13DSE/ha
NSW	Armidale, NSW	495	2958 ha	1000	BL/Merino	May -August	20 DSE/ha
WA1	Pingelly, WA	450	1500 ha	1350	Merino x Suffolk	mid July	12DSE/ha
WA2	West Arthur, WA River	500	1250 ha	1750	Merino x Suffolk	Early August	10DSE/ha
WA3	Frankland, WA	550	560Ha	3300	Merino x Suffolk	Mid July	21 DSE/Ha

498 Note: DSE = dry sheep equivalent, is a standard unit frequently used to compare animal carrying capacity and potential
499 productivity of a given farm or area of grazing land. DNA from samples from Western Australia was extracted by Josh
500 Sweeny and previously analysed using different primers as described in Sweeny et al., (2011) and Sweeny (2012).
501

Table 2. Prevalence and number of *Cryptosporidium* oocysts per gram of sheep faeces (g^{-1}) (range and median) in samples collected from 8 farms in 4 states over 3 sampling periods. 95% confidence intervals are given in parenthesis

Farm	Sampling period	Total no of samples	No of positives	Prevalence %	Oocysts g^{-1} Range	Oocysts g^{-1} Median
SA1	Weaning	165	14	8.5 (4.2-12.7)	938-1.8 x 10^6	4.8 x10^4
	Post-weaning	148	9	6.1 (2.2-9.9)	3.9 x 10^3-9.8 x10^5	2.3 x10^4
	Pre-slaughter	159	15	9.4 (4.9-14.0)	7.3 x 10^3-1.7 x 10^5	1.4 x10^5
SA2	Weaning	169	30	17.8 (12.0-23.5)	375-7.9 x 10^6	3.1 x 10^3
	Post-weaning	156	30	19.2 (13.0-25.4)	313-3.0 x 10^5	4.7 x10^3
	Pre-slaughter	147	9	6.1 (2.2-10.0)	4.7 x 10^3-1.7 x10^6	8.0 x 10^4
Vic1	Weaning	180	6	3.3 (0.7-6.0)	125-8.7 x 10^5	1.6 x 10^4
	Post-weaning	172	32	18.6 (12.8-24.4)	390-3.7 x 10^9	5.1 x 10^3
	Pre-slaughter	160	14	8.8 (4.4-13.1)	1.6 x 10^3-7.8 x 10^4	1.6 x 10^3
Vic2	Weaning	176	37	21 (15.0-27.0)	313-4.8 x 10^5	7.8 x 10^3
	Post-weaning	173	16	9.2 (4.9-13.6)	1.0 x 10^3-7.1 x 10^6	1.8 x 10^3
	Pre-slaughter	128	12	9.4 (4.3-14.4)	937-6.0 x 10^6	9.0 x 10^4
NSW	Weaning	160	36	22.5 (16.0-29.0)	313-1.1 x 10^6	6.1 x 10^3
	Post-weaning	160	44	27.5 (20.6-43.3)	563-2.1 x 10^8	1.7 x 10^4
	Pre-slaughter	167	21	12.5 (7.5-17.6)	262-1.4 x 10^7	1.2 x 10^4
WA1-AR	Weaning	124	46	37.1 (28.6-45.6)	125-2.6 x 10^6	1.6 x 10^4
	Post-weaning	122	18	14.8 (8.5-21.0)	313-1.1 x 10^5	4.5 x 10^3
	Pre-slaughter	121	29	24.0 (16.4-31.6)	375-1.6 x 10^7	5.8 x 10^4
WA2-PL	Weaning	107	47	43.9 (34.5-53.3)	63-5.3 x 10^3	400
	Post-weaning	109	29	26.6 (18.3-34.9)	313-2.4 x 10^7	1.5 x 10^5
	Pre-slaughter	107	39	36.4 (27.3-45.6)	1.6 x10^3-2.9 x 10^7	2.0x10^4
WA3-FL	Weaning	101	19	18.8 (11.2-26.4)	313-4.7 x 10^5	2.3 x 10^4
	Post-weaning	101	7	6.9 (2.0-11.9)	313-3.7 x 10^6	5.9 x 10^3
	Pre-slaughter	100	14	14 (7.2-2-.8)	2.0x10^3-4.8 x 10^7	1.0 x 10^5
Total		**3412**	**576**	**16.9 (15.6-18.1)**	**63 – 3.7 x 10^9**	**2.6 x 10^4**

506 Table 3. *Cryptosporidium* oocyst concentration (g^{-1}) and prevalence across four states (pooled

507 values for farms). 95% confidence intervals are given in parenthesis.

States	Sampling periods	Oocysts g^{-1} Range	Oocysts g^{-1} Median	Prevalence %
SA	Weaning	375-7.9 x 10^6	8.8 x 10^4	13.2 (8.1-18.1)
	Post-weaning	313-9.8 x 10^5	2.2 x 10^4	12.8 (7.6-17.7)
	Pre-slaughter	4.7 x10^3-1.7 x 10^6	1.0 x 10^5	7.8 (3.6-12.0)
Vic	Weaning	125-8.7 x 10^5	1.2 x 10^4	12.1 (7.9-16.5)
	Post-weaning	390-3.7 x 10^{10}	4.1 x 10^3	13.9 (8.9-19.0)
	Pre-slaughter	937-6.0 x 10^6	4.8 x 10^4	9.0 (4.4-13.8)
NSW	Weaning	313- 1.1 x 10^6	6.1 x 10^3	22.5 (16.3-29.8)
	Post-weaning	563 – 2.1 x 10^8	1.7 x 10^4	27.5 (20.6-34.4)
	Pre-slaughter	262-1.4 x10^7	1.2 x 10^4	12.5 (7.5-17.6)
WA	Weaning	63 – 2.6 x 10^6	1.5 x 10^4	33.7 (24.8-41.8)
	Post-weaning	313-2.4 x 10^7	4.8 x 10^4	16.3 (9.6-22.6)
	Pre-slaughter	262-4.8x 10^7	6.0 x 10^4	25.0 (17-32.7)
All states	Weaning	63-7.9 x 10^6	3.2 x 10^4	19.9 (14.3-26.5)
	Post-weaning	313-3.7 x 10^{10}	2.6 x 10^4	16.2 (11.7-20.9)
	Pre-slaughter	260-4.8 x 10^7	6.3 x 10^4	14.0 (8.1-19.0)

508
509

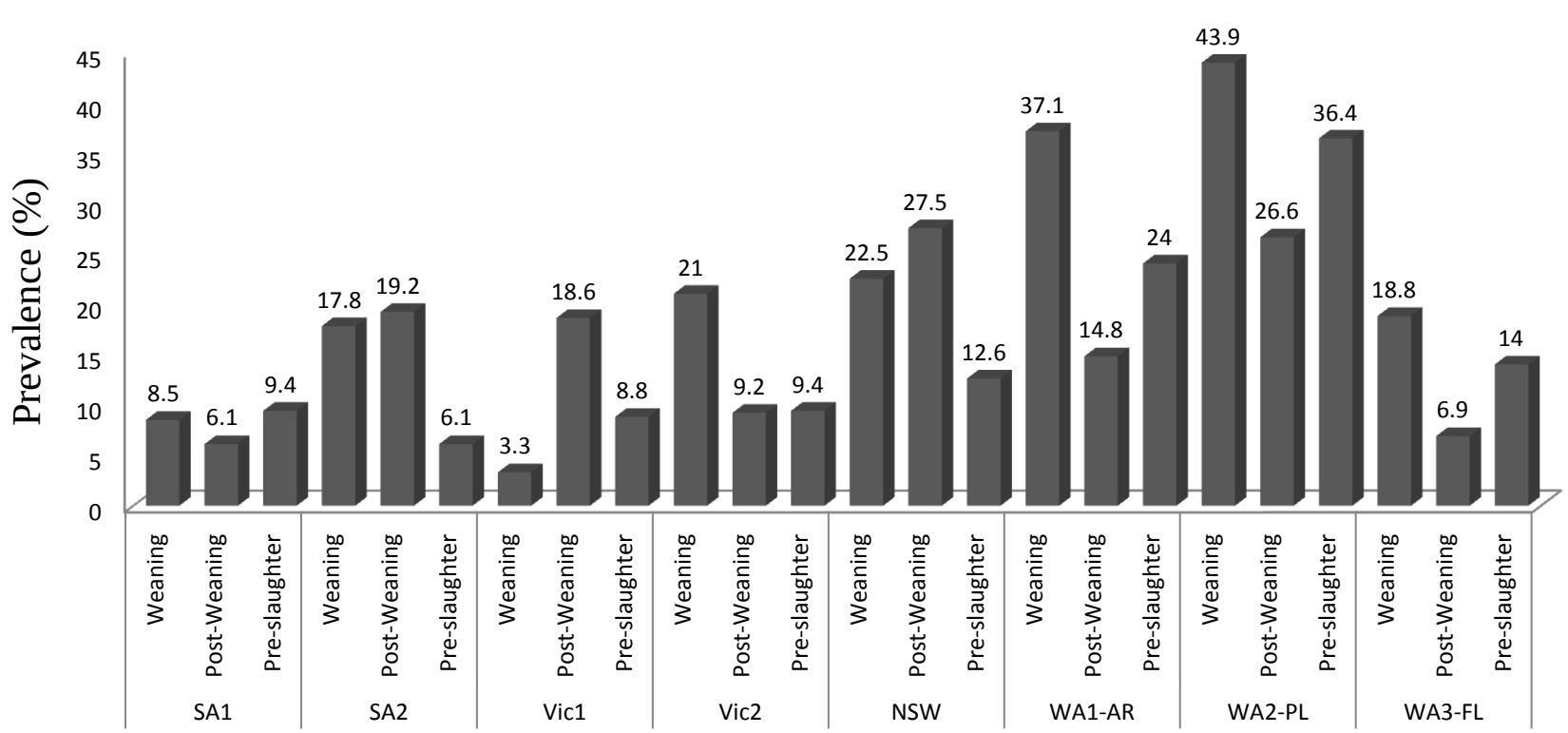
509 Table 4. Species and subtypes of *Cryptosporidium* detected on 8 farms across 4 states (NSW, SA,
510 Vic and WA) over 3 sampling times (weaning, post-weaning and pre-slaughter).
511

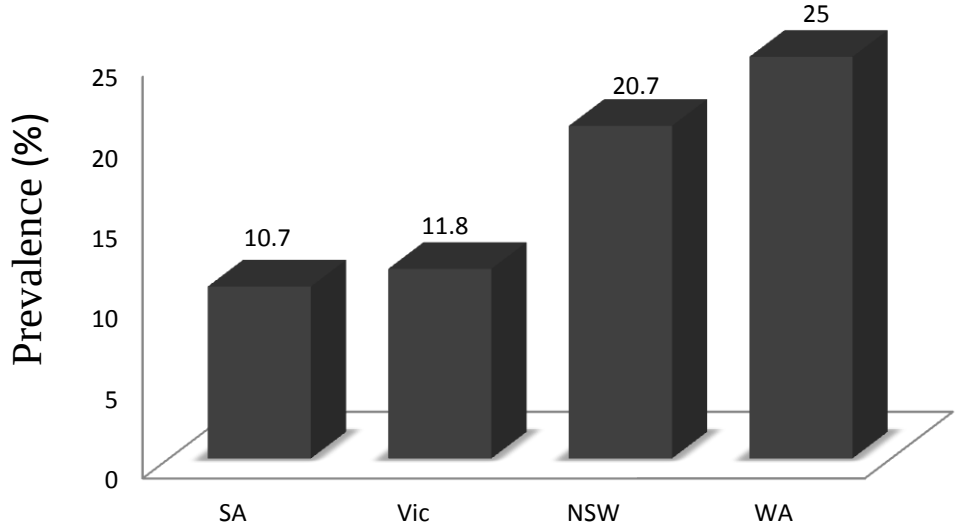
Farm	Sampling period	*C. xiaoi*	*C. ubiquitum*	*C. parvum*	*C. scrofarum*	Others and mixed infections
SA1	Weaning (n=165)	8	3	3		
	Post-Weaning (n=148)	7	1	1		
	Pre-slaughter (n=159)	9	6			
SA2	Weaning (n=169)	8	19			
	Post-Weaning (n=156)	7	6	1		
	Pre-slaughter (n=147)	4	3	2		
Vic1	Weaning (n=180)	4	1	2		
	Post-Weaning (n=172)	9	3	3	1	
	Pre-slaughter (n=160)	7	5	1	1	
Vic2	Weaning (n=176)	27		1	1	
	Post-Weaning (n=173)	9	4		1	
	Pre-slaughter (n=128)	3	7	2		
NSW	Weaning (n=160)	17		3		
	Post-Weaning (n=160)	34	3	1		
	Pre-slaughter (n=167)	6	3	3		
WA1	Weaning (n=124)	35	4			5 (*C. xiaoi* + *C. parvum*), 1 (*C. andersoni*) 1 (sheep genotype 1)
	Post-Weaning (n=122)	15	3	2		
	Pre-slaughter (n=121)	24	2	2		1 (*C. xiaoi* + *C. parvum*)
WA2	Weaning (n=107)	37	2	6		2 (*C. xiaoi* + *C. parvum*)
	Post-Weaning (n=109)	21	2	4		2 (*C. xiaoi* + *C. parvum*)
	Pre-slaughter (n=107)	31	2	4		2 (*C. xiaoi* + *C. parvum*)
WA3	Weaning (n=101)	10	3	6		
	Post-Weaning (n=101)	3	3	1		
	Pre-slaughter (n=100)	10	3	1		

Total	**345**	**88**	**49**	4

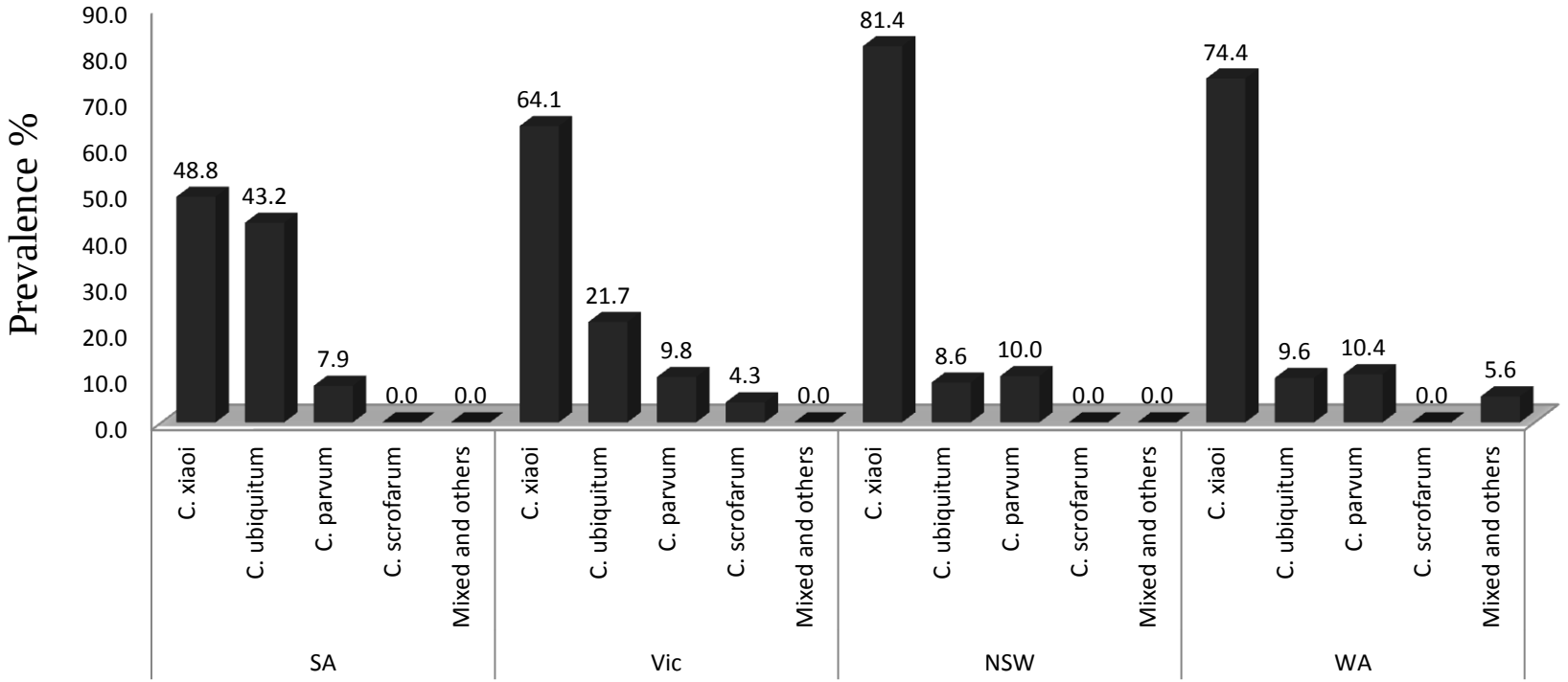
*=all *C. ubiquitum* isolates subtyped at the *gp60* locus were XIIa.

Figure


A
Prevalence (%)
45
40
35
30
25
20
15
10
5
0
8.5
6.1
9.4
17.8
19.2
6.1
3.3
18.6
8.8
21
9.2
9.4
22.5
27.5
12.6
37.1
14.8
24
43.9
26.6
36.4
18.8
6.9
14
Weaning
Post-Weaning
Pre-slaughter
SA1
SA2
Vic1
Vic2
NSW
WA1-AR
WA2-PL
WA3-FL


B
Prevalence (%)
25
20
15
10
5
0
10.7
11.8
20.7
25
SA
Vic
NSW
WA

Figure


Prevalence %
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
48.8
43.2
7.9
0.0
0.0
64.1
21.7
9.8
4.3
0.0
81.4
8.6
10.0
0.0
0.0
74.4
9.6
10.4
0.0
5.6
C. xiaoi
C. ubiquitum
C. parvum
C. scrofarum
Mixed and others
SA
Vic
NSW
WA