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

Rongchang Yang[1], Caroline Jacobson[1], Graham Gardner[1], Ian Carmichael[2], Angus J. D. Campbell[3] and Una Ryan[1*]

[1]*School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia, 6150.*

[2]*South Australian Research and Development Institute, 33 Flemington Street, Glenside, SA 5065, Australia.*

[3]*Faculty of Veterinary Science, University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia*

Corresponding author: Una Ryan

Phone: 08 9360 2482

Fax: 08 9310 4144

E-mail: Una.Ryan@murdoch.edu.au

Abstract

The prevalence of *Eimeria* in sheep in Australia has not been well described, therefore a quantitative PCR (qPCR) was developed, validated and used to study the prevalence and oocyst concentration in 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 *Eimeria* using this qPCR at the 18S ribosomal RNA (rRNA) locus. A subset of positives were typed by sequence analysis at the 18S locus. The overall prevalence was 18.1% (95% CI 16.8-19.3%) and of the 616 positives, 118 were successfully genotyped. The prevalence of *Eimeria* was highest in NSW and peaked at 70% during the post-weaning period. The range of oocyst shedding per gram of faeces (g^{-1}) at weaning, post-weaning and pre-slaughter overall across all states was 23-2.1 x 10^7, 23-1.3 x 10^7 and 23-2.1 x10^5, respectively. Median *Eimeria* shedding g^{-1} was higher during post-weaning (1.1x10^3) and pre-slaughter (1.1x10^3) than during weaning (206). The following species were identified: *E. crandallis, E. ahsata, E. ovinoidalis, E. weybridgensis and E. cylindrica.* Of these, *E. crandallis* and *E. ovinoidalis,* the most pathogenic species in sheep were responsible for 58.5% of infections typed. This highlights a need for further research to quantify the production impacts of *Eimeria* in sheep.

Keywords: *Eimeria*; lambs; qPCR; 18S rRNA; *E. crandallis; E. ahsata; E. ovinoidalis; E. weybridgensis; E. cylindrica.*

1. Introduction

Coccidiosis (Eimeriosis) of sheep is a widespread infection caused by the protozoan parasite, *Eimeria*, which develops in the small and the large intestine and affects young animals in particular (Chartier and Paraud, 2012). Although often asymptomatic in sheep, coccidiosis can be a serious economic enteric disease, resulting in diarrhea, inefficient weight gains, and occasionally death (Chartier and Paraud, 2012). Sheep of all ages are susceptible, although disease outbreaks are typically observed in young lambs 1–3 months old (2–3 weeks after weaning) following an incubation period of ~14–20 days (Platzer et al., 2005; Taylor et al., 2011), or when sheep are housed in barns or feedlots (O'Callaghan et al., 1987; Foreyt, 1990; Taylor and Catchpole, 1994; Wright and Coop, 2000). Chronic subclinical infections can also occur with animals shedding low numbers of *Eimeria* oocysts in their faeces and providing a continuous source of infection for other animals (Kaya et al., 2007). In addition, the presence of *Eimeria* parasites in the animal intestine has been correlated with increased susceptibility to secondary infection, especially bacterial diseases (Taylor et al., 1973).

The principal species of *Eimeria* in sheep worldwide are *E. ahsata, E. bakuensis*, *E. parva, E. pallida, E. crandallis, E. weybridgensis*, *E. ovina*, *E. ovinoidalis*, *E. granulosa, E. intricata*, *E. faurei* and *E. marsica* (Chartier and Paraud, 2012). Of these *Eimeria* species, *E. ovinoidalis* and *E. crandallis* are considered pathogenic and cause the most severe disease (Gregory and Catchpole, 1987, 1990; Taylor et al., 2003). Species of *Eimeria* can be distinguished by oocyst morphology, pre-patent period, site of infection or minimum sporulation time, but all of these methods are labour intensive, time consuming and can be very difficult and unreliable with a mixed sample as different species overlap in size and shape (Tenter et al., 2002; Haug et al., 2007), prompting the development of DNA-based molecular methods mainly for the detection and quantitation of *Eimeria* in poultry (Morgan et al., 2009; Vrba et al., 2010; Raj et al., 2013).

Effective diagnostic tools would be useful for the detection of pathogenic subclinical *Eimeria* infections in domestic livestock through faecal monitoring. Such technology could be

implemented in a basic control strategy where animals harbouring pathogenic subclinical infections could be isolated from uninfected animals preventing disease transmission (Kaya et al., 2007).

The prevalence and species of *Eimeria* in sheep in Australia has not been well described and therefore, the aim of the present study was to develop and validate a quantitative PCR (qPCR) assay for detecting *Eimeria* in sheep and to use this assay to determine the prevalence and oocyst shedding concentrations per gram of faeces (g^{-1}) in lambs in Western Australia, (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

Faecal samples were collected from 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). A total of 3,412 faecal samples were collected directly from the rectum of approximately 1,189 lambs over the 3 sampling periods. Lambs were born and reared in paddocks and were not housed indoors at any stage. 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 Power Soil DNA Kit (MolBio, Carlsbad, California) with the following modification; faecal samples were resuspended in lysis buffer, subjected to four freeze-thaw cycles and lysed at 95°C for 10 min prior to DNA extraction to ensure complete lysis of oocysts. A negative control (no faecal sample) was used in each extraction group.

2.3 PCR amplification.

Primers and probes for *Eimeria* were designed using Primer 3 (http://frodo.wi.mit.edu/) and and Real-Time design software available from Biosearch technologies (https://www.biosearchtech.com/realtimedesign-software-access.aspx) based on sequences from the following *Eimeria* species: *Eimeria weybridgensis* (AY028972), *Eimeria ovinoidalis* (AF345997), *Eimeria crandallis* (AF336339) and *Eimeria ahsata* (AF338350). Primers and probes were synthesised by Biosearch technologies (Petaluma, CA, USA). All samples were screened at the 18S rRNA locus using a qPCR with the forward primer, Eim F1 5' CGAATGGCTCATTAAAACAGTTATAGTT 3', the reverse primer Eim R1 5' CGCATGTATTAGCCATAGAATTACCA 3' and the probe 5'-(Joe)-ATGGTCTCTTCCTACATGGA BHQ1 3' which produces an 85 bp product. An internal amplification control (IAC) consisting 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). The IAC primers were JDVF (5'- GGT AGT GCT GAA AGA CAT T) and JDVR (5'- ATG TAG CTT GAC CGG AAG T) and the probe was 5'- (Cy5) TGC CCG CTG CCT CAG TAG TGC (BHQ2). 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. PCR contamination controls were used including negative controls and separation of preparation and amplification areas.

2.4 Specificity and sensitivity testing of the 18S qPCR

The analytical specificity of the qPCR assay was assessed by testing DNA from *E. crandallis, E. weybridgensis, E. ovina, E. ovinoidalis, E. ahsata, E. tilliquae, E. trichosuri, Eimeria sp.* from kangaroos (K2175, K2336 and K2534) and non-*Eimeria* spp.: *Cryptosporidium parvum, C. hominis, C. bovis, C. ryanae*, *C. xiaoi, Isospora, Tenebrio, Giardia duodenalis, Cyclospora, Campylobacter spp., Salmonella spp., Yersinia enterocolitica, Staphylococcus epidermidis* (ATCC 12228), *Streptococcus bovis* (ATCC 33317), *Toxoplasma gondii, Trichostrongylus spp., Teladorsagia circumcincta, Haemonchus contortus, Streptococcus bovis* (ATCC 33317), *Serratia marcescens* ATCC 14756 (pigmented), *Enterococcus durans* (ATCC 11576), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633) as well as human, sheep and cattle DNA.

In order to determine the sensitivity of the assay, the PCR product amplified from an *E. crandallis* isolate was cloned into the pGEMT-vector (Promega) and transformed into *E. coli* (JM109) competent cells. Plasmid DNA was isolated by alkali\SDS lysis followed by column purification using QIAprep Spin Columns (Qiagen) in accordance with the manufacturer's protocol. Plasmid mini-preparations were sequenced using T7 sequencing primer (Stratagene, La Jolla, CA, USA) and clones with the correct sequence 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 and then spiked into 200 mg aliquots of faecal samples and the DNA extracted and eluted in 50 µl to generate DNA standards that had 100, 1,000, 10,000, 100,000, 1,000,000 copies of the genomic template per µl. These were then amplified as described above and mean detection limits, RSQ (R squared) values and % Relative Standard Deviation (RDS) were calculated. Target copy numbers were converted to estimates of oocyst numbers based on the fact that sporulated *Eimeria* oocysts contain eight haploid sporozoites/nuclei and the 18S ribosomal gene is present in 140 copies per haploid genome in *E. tenella* (Lim et al., 2012; http://www.genedb.org/Homepage/Etenella) (i.e. there are 1,120 18S target copies per oocyst). Therefore, for the plasmid detection, the mean number of targets detected by qPCR (for 3 replicate faecal samples) was then divided by 140 and then by 8 (or by 1,120) to equate to number of oocysts

detected per 15 µl reaction and then extrapolated up to oocysts per gram (OPG) of faeces. [Note: *Eimeria* oocysts collected directly from the rectum are unsporulated, i.e. diploid (containing two genomes). However due to transit times interstate, microscopic examination revealed that the vast majority of oocysts had sporulated. Hence our decision to base the calculations on sporulated oocysts].

2.5 Investigation of inhibition and efficiency

Inhibition in faecal samples was monitored using the IAC, as equal amounts of the IAC template (10 copies) was added to all faecal DNA samples to detect any PCR inhibitors present in the extracted DNA. If inhibition is present in a sample, the IAC will not produce a signal. If low levels of inhibition are present, then the Ct value for the IAC will higher than uninhibited samples. Amplification efficiency (*E*) (which is 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 on faecal samples serial dilutions of individual DNA samples (neat, 1:10, 1:100) were performed and multiple qPCR reactions were conducted on each dilution. 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 Comparison of the qPCR assay with microscopy (haemocytometer counted oocysts) in faecal samples

Ten random sheep faecal samples were analysed (in triplicate) via haemocytometer counting to determine OPG levels in these faecal samples by microscopy. These samples were then DNA extracted as described above and tested by qPCR and the data compared.

2.7 Sequence analysis

For sequencing, a subset of 5 positives from each sampling at each farm (n = 120) were amplified at the 18S ribosomal RNA (rRNA) locus using a two-step nested protocol previously described which produced a 497-498 bp product (Pieniazek et al., 1996). 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). Purified PCR products were sequenced using an ABI Prism^{TM} Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. 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).

2.8 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). Chi-square and non-parametric analyses were performed using SPSS 21.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA) to determine if there was any association between the prevalence and concentration of *Eimeria* oocysts at different sampling times and across states.

3 Results

3.1 Specificity, sensitivity and efficiency testing of the 18S qPCR

Evaluation of specificity of the 18S qPCR assay revealed no cross-reactions with other genera and detected all the *Eimeria* isolates tested (data not shown). Sensitivity analysis revealed that the assay could reliably detect ~80 copies of the cloned *Eimeria* amplicon per µl of faecal DNA

extract which is equivalent to a sensitivity of <1 *Eimeria* oocyst per µl of faecal DNA extract. (i.e. for 100 spiked *Eimeria* plasmids, the actual mean numbers of target gene detected was 81.2 which equates to ~0.07 oocysts/µl (81.2/1,120). The mean RSQ was 0.98 and the % RDS = 1.1%. Therefore we can be confident that the qPCR assay is reliable and highly sensitive for *Eimeria*. In our hands, the incidence of PCR inhibition as determined by the IAC amplification was about 2%. If inhibition was evident, then the sample was diluted and re-amplified. The mean efficiency for the *Eimeria* qPCR was 95.3%.

3.2 Comparison of the qPCR assay with haemocytometer counted oocysts in faecal samples

To validate the relationship between hypothetical qPCR-derived OPG values and the actual OPG values that could be determined by microscopy, OPG values between haemocytometer counted oocysts and OPG determined by qPCR were compared on 10 random samples (Table 2). The correlation between qPCR and haemocytometer-counted OPG was significant (Pearson correlation = 0.979) but showed that OPGs calculated from qPCR was lower than OPG determined via microscopy (using a haemocytometer). The average OPG calculated by qPCR from 10 random samples was 1,396 (% RSD = 5.2) compared to 1,657 (% RSD = 6.9) for haemocytometer-counted oocysts. Therefore, microscopy detected on average ~16% more oocysts than qPCR, due to losses presumably from pipetting and DNA extraction (Table 2). This discrepancy was then factored into all the OPG values determined by qPCR (i.e. all were increased by 16%).

3.3 Prevalence of Eimeria from 8 farms across 4 states

The overall prevalence of *Eimeria* from 8 farms across 4 states over 3 sampling periods (weaning, post-weaning and pre-slaughter) was 18.1% (616/3,412) (Fig. 1 and supplementary Tables 4 and 5). 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 *Eimeria* at the NSW

farm was significantly higher than all other farms ($p<0.05$) and peaked at 70% and 47.5% during the post-weaning and weaning periods respectively. There were smaller peaks for *Eimeria* at WA2 (33.6%), Vic1 (33.1%) and SA1 (20.8%) (all at pre-slaughter) and at WA3 at weaning (20.8%). Only 3, 1, 2 and 1 lambs from NSW, Vic1, Vic2 and WA2 respectively were positive across all 3 samplings.

3.4 Oocyst load from qPCR with the adjustment based on the haemocytometer count

OPGs were also determined using qPCR (and adjusted up by 16%) (Fig. 2 and supplementary Tables 4 and 5). The highest median concentration of *Eimeria* oocysts g^{-1} was shed by lambs at weaning in SA2 (1.8×10^5 oocysts g^{-1}). Across the other farms, median *Eimeria* oocyst concentration peaked during the weaning period at Vic1 and WA3 (8.3×10^3 and 6.5×10^4 oocysts g^{-1} respectively). Median *Eimeria* oocyst concentrations peaked during the post-weaning period at SA1 and NSW (5.5×10^3 and 7.1×10^3 oocysts g^{-1} respectively). Median *Eimeria* oocysts concentration peaked during the pre-slaughter period at WA1 (2.4×10^3 oocysts g^{-1}). Median *Eimeria* oocyst concentrations in Vic2 were low at all samplings (290, 23 and 380 oocysts g^{-1} respectively). The range of oocyst shedding at weaning overall across all states was 23-2.1×10^7 and the median was 224. At post-weaning the range was 23-1.3×10^7 and the median was 1.1×10^3. At pre-slaughter, the range was 23-2.1×10^5 and the median was 1.1×10^3 (supplementary Table 4). Of the 7 samples that were positive at all 3 samplings, no trends were seen in oocysts concentration; some were low at the weaning sampling (89 to 774 oocysts g^{-1}) and continued to increase at the pre-slaughter sampling (6.5×10^3 to 3.5×10^4 oocysts g^{-1}) but others shed a decreased concentration of oocysts at the pre-slaughter sampling.

3.4 Eimeria species

Of the 616 positives, a subset of 5 positives from each sampling in each farm (n=120) were sequenced and of these, 118 sequences were successfully obtained (only 3 sequences were obtained

from WA3 at post-weaning). The following species were identified; *E. crandallis* (48.3%, 57/118), *E. ahsata* (28%, 33/118), *E. ovinoidalis* (10.1%, 12/118), *E. weybridgensis* (10.1%, 12/118) *and E. cylindrica* (4.2%, 5/118) (Table 3). No mixed infections were detected.

Of the 118 isolates sequenced, *E. crandallis* was the most prevalent across all states and peaked at 53.3% (8/15) for NSW (Fig. 3). *Eimeria ahsata* was the second most prevalent species across all states and peaked at 33.3% (10/30) in Vic, with peaks of 25.6-26.7 across the other states. In WA and NSW, *E. weybridgensis* was the third most prevalent species, which peaked at 16.3% (5/38) in WA and 13.3% (2/15) in NSW. However, the prevalences for SA and Vic were lower at 6.7% (2/30) and 3.3% (1/30) respectively. *Eimeria ovinoidalis* was the fourth most prevalent with a 10% prevalence (3/30) for both SA and Vic. In WA and NSW, *E. ovinoidalis* prevalence was at 11.6% (5/43) and 6.7% (1/15) respectively. *Eimeria cylindrica* was detected in SA and Vic only at a prevalence of 6.7% (2/30) and 10.0% (3/30) respectively. All *E. cylindrica* isolates sequenced (n=5) were 100% identical to each other and exhibited 2 single nucleotide polymorphisms (SNP's) from bovine-derived *E. cylindrica* (GenBank accession number AB769616). A representative 18S sequence from *E. cylindrica* obtained from sheep in the present study (isolate SA2A27) has been deposited in GenBank under accession number KF29583.

4 Discussion

In the present study, the prevalence, oocyst concentration and species of *Eimeria* were assessed from lamb faecal samples at three sampling periods (weaning, post-weaning and pre-slaughter) from eight farms across four Australian states using a novel qPCR at the 18S locus.

The qPCR assay was very specific for *Eimeria,* as it detected all the *Eimeria* species tested and did not cross-react with the non-*Eimeria* isolates analysed. The sensitivity of the assay was determined by cloning an *Eimeria* 18S 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 < 1 *Eimeria* oocyst per µl of faecal DNA extract.

Whilst numerous studies have conducted single point prevalence analysis by sampling a random selection of lambs or sheep within a flock at a specific time, few studies have conducted longitudinal analysis. This is important as the determination of prevalence at one sampling, may not provide a true indication of the overall prevalence in flocks over an extended period of time. Reported sporadic oocyst excretion by *Eimeria*, requires multiple faecal samples to be collected for an accurate diagnosis, as a negative specimen would indicate that an animal was not infected when there might actually be a pattern of intermittent oocyst excretion (Skirnisson and Hansson, 2006; Skirnisson, 2007).

In the present study, the overall prevalence of *Eimeria* from 8 farms across 4 states over 3 sampling periods (weaning, post-weaning and pre-slaughter) was 18.1% (616/3412). The prevalence varied widely between states and at different sampling points. The highest prevalence was identified in NSW, which peaked at 70% during the post-weaning period and the lowest prevalence was at WA1, which ranged from 5.8-10.7%. Differences in prevalence could be related to a wide range of factors, including environment, socking density and potential for contamination of feed/water. The sites chosen represented a range of different environments in which sheep are farmed throughout Australia. The WA farms were all located in southern Western Australia and experienced a Mediterranean environment (hot, dry summers and cool, wet winters) with a predominantly winter rainfall pattern and had similar average annual rainfall, ranging between 450 and 550 mm. The SA farms also experienced a Mediterranean climate with average rainfall ranging between 430 and 550 mm. The Vic farms experienced higher rainfall in winter with (620-750 mm/year) but milder summers whereas the NSW farm experienced relatively cold winters with warm wet summers (495 mm/year). However, given the lack of detailed weather information available for each site, it is difficult to link these prevalences with obvious environmental effects.

Previous prevalence studies on *Eimeria* in sheep, conducted in Australia, have been based on microscopy. One study conducted in South Australia detected *Eimeria* oocysts in 80% of sheep examined (n=136) (O'Callaghan et al., 1987). The most recent study, reported overall prevalences of 58.9% and 71.4% for *Eimeria* in two West Australian farms (Sweeny et al., 2011). In Papua New Guinea, a prevalence of 17% was reported for *Eimeria* in sheep by microscopy (Koinari et al., 2012).

Oocyst concentration (g^{-1}) was also determined using qPCR and validated using haemocytometer counted oocysts from the same samples. Results showed good agreement between both methods but that microscopy detected on average ~16% more oocysts than qPCR, due to losses presumably from pipetting and DNA extraction. Previous studies have reported a substantial decrease in sensitivity in PCR detection of *Eimeria* oocysts in DNA extracted from faecal samples compared with DNA amplification from purified oocyst DNA. For example, Morgan et al. (2009), developed a qPCR targeting rDNA ITS-2 and reported that the inclusion of faecal material decreased the sensitivity by 10 to 100-times depending on initial sample concentration. More recently, Kumar et al. (2014) targeted the 18S gene, spiked oocysts into faeces and found that the lower limit of detection was ~20,000 OPG. However in the latter study, a single round PCR amplifying ~1,790 bp region of the 18S gene based on primers by Schwarz et al. (2009) was used. In the present study, the amplicon size was only ~85 bp and would therefore be much more sensitive. In addition, we employed four freeze-thaw cycles, followed by DNA extraction using a Power Soil DNA Kit, which includes a mechanical bead disruption step using glass beads to increase the efficiency of DNA extraction. The paper by Morgan et al. (2009) was based on the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), which does not include a mechanical bead disruption step and their protocol did not include freeze-thaw cycles.

The overall median oocyst shedding (across all states) peaked during post-weaning and pre-slaughter (supplementary Table 5). This is in contrast to previous studies that have reported that peak oocyst shedding was reached around the period of weaning (Chartier and Paraud, 2012). For

example, in Bangladesh, it was reported that younger sheep passed higher numbers of *Eimeria* oocysts than older animals with a peak at around 4-6 weeks of age (7.9 x10^5 oocysts/g of faeces) (Karim et al., 1990), with a similar pattern seen in UK sheep (Gregory et al., 1983). Another study reported that *Eimeria* shedding in sheep in Iceland ranged from 300 to 4.9 x 10^4 g^{-1}, peaking just after weaning (Skirnisson and Hansson, 2006; Skirnisson, 2007). Other studies have reported a mean of ~2,838 g^{-1} for sheep in Sudan (Abakar et al., 2013), while a study in Kenya reported peaks of 1.1 x 10^4 in sheep with a mean of 1.1 x 10^3 in sheep < 1 year of age (Kanyari, 1993). One study suggested a genetic component in the determination of oocyst ouput (Yvore et al., 1992). The reasons for the higher oocyst output during post-weaning and pre-slaughter (compared to weaning) in the present study are unknown, but may be due to stress or to polyparasitism reducing host immunity as these sheep were known to be co-infected with *Giardia*, *Cryptosporidium*, bacteria and strongyle worms (Yang et al., 2014a; 2014b and unpublished).

There are some obvious limitations to the present study. DNA extractions are rarely 100% efficient and target copy numbers were converted to estimates of oocyst numbers based on the number of 18S gene copies present in *E. tenella* (Lim et al., 2012). The 18S copy number for *Eimeria* species in sheep is unknown and may not be the same. However, the ability to generate even crude OPG estimates using a rapid high through-put method has distinct advantages over microscopy, which is very time-consuming and lacks sensitivity (Haug et al., 2007). Future studies will include an in-depth comparison of qPCR data with faecal samples spiked with known numbers of oocysts.

Of the subset of 118 isolates sequenced in the present study, a total of 5 species were identified; *E. crandallis, E. ahsata, E. ovinoidalis, E. weybridgensis and E. cylindrica.* Of these, *E. crandallis* and *E. ovinoidalis,* the most pathogenic species in sheep were responsible for 58.5% of infections typed. This is the first report of ovine species of *Eimeria* from NSW, Vic and WA. A previous study in South Australia identified the following *Eimeria* species in sheep; *E. crandallis/E. weybridgensis* (76%), *E. ovina* (55%), *E. ovinoidalis* (54%), *E. granulosa* (49%), *E. parva/E.*

pallida (44%), *E. intricata* (37%), *E. ahsata* (31%), *E. faurei* (24%), and *E. punctata* (1%). (O'Callaghan et al., 1987). In the present study, only 5 species of *Eimeria* were detected, but this may be due to the fact that only a small subset of positives (118/616) were typed. Individual sheep faecal samples usually contain several species of *Eimeria* (O'Callaghan et al., 1987; Saratsis et al., 2011), however in the present study, no mixed infections were detected. This is likely due to the most abundant species being amplified by PCR. This could be overcome by the use of species-specific primers or by cloning each amplicon and sequencing multiple clones or via deep sequencing using next generation sequencing. However, this was beyond the scope of the present study. Around the world, it has been reported that in temperate areas like western Europe, the most prevalent *Eimeria* species are *Eimeria ovinoidalis* followed by *Eimeria weybridgensis/crandallis* in sheep (Reeg et al., 2005). In lambs in Germany, faecal samples with 3, 4 or 5 species of *Eimeria* are frequent with a predominance of *E. ovinoidalis* in high oocyst count samples (Dittmar et al., 2010; Reeg et al., 2005). In dry tropical areas such as Senegal, the main species of *Eimeria* in sheep are *E. ovinoidalis* (76%), *E. crandallis* (62%), *Eimeria ahsata* (28%) and *E. parva* (25%) (Vercruysse, 1982).

In the present study, *E. cylindrica* was detected in two out of 15 sheep in SA2 and 3 out of 15 sheep in Vic2. This species is a bovine *Eimeria* species (Duszynski et al., 2000) and has not been reported previously in sheep as *Eimeria* species are thought to be host-specific (Chartier and Paraud, 2012). Both SA2 and Vic2 properties also had cattle and thus it is possible that this was mechanical transmission due to ingestion of infected cattle faeces. Oocyst shedding numbers in these animals ranged from 19 to 3.2 x 10^5 g^{-1}. It may also be possible that ruminant *Eimeria* species are not as rigidly host-specific as previously thought as has been shown to be the case with *Cryptosporidium* (Xiao, 2010). However, it also be noted that only a single locus was used in the present study and no microscopic evidence of *E. cylindrica* in these samples was obtained. Further studies are required to determine if the identification of *E. cylindrica* in these sheep was due to an actual infection or mechanical transmission.

In conclusion, the present study identified that *Eimeria* is prevalent in lambs across Australia and that individual lambs can shed large amounts of *Eimeria* oocysts. It also revealed that of the 118 isolates typed, pathogenic *Eimeria* species were found in 58.5% of samples. Further studies are required to determine the extent of economic loss associated with *Eimeria* in sheep.

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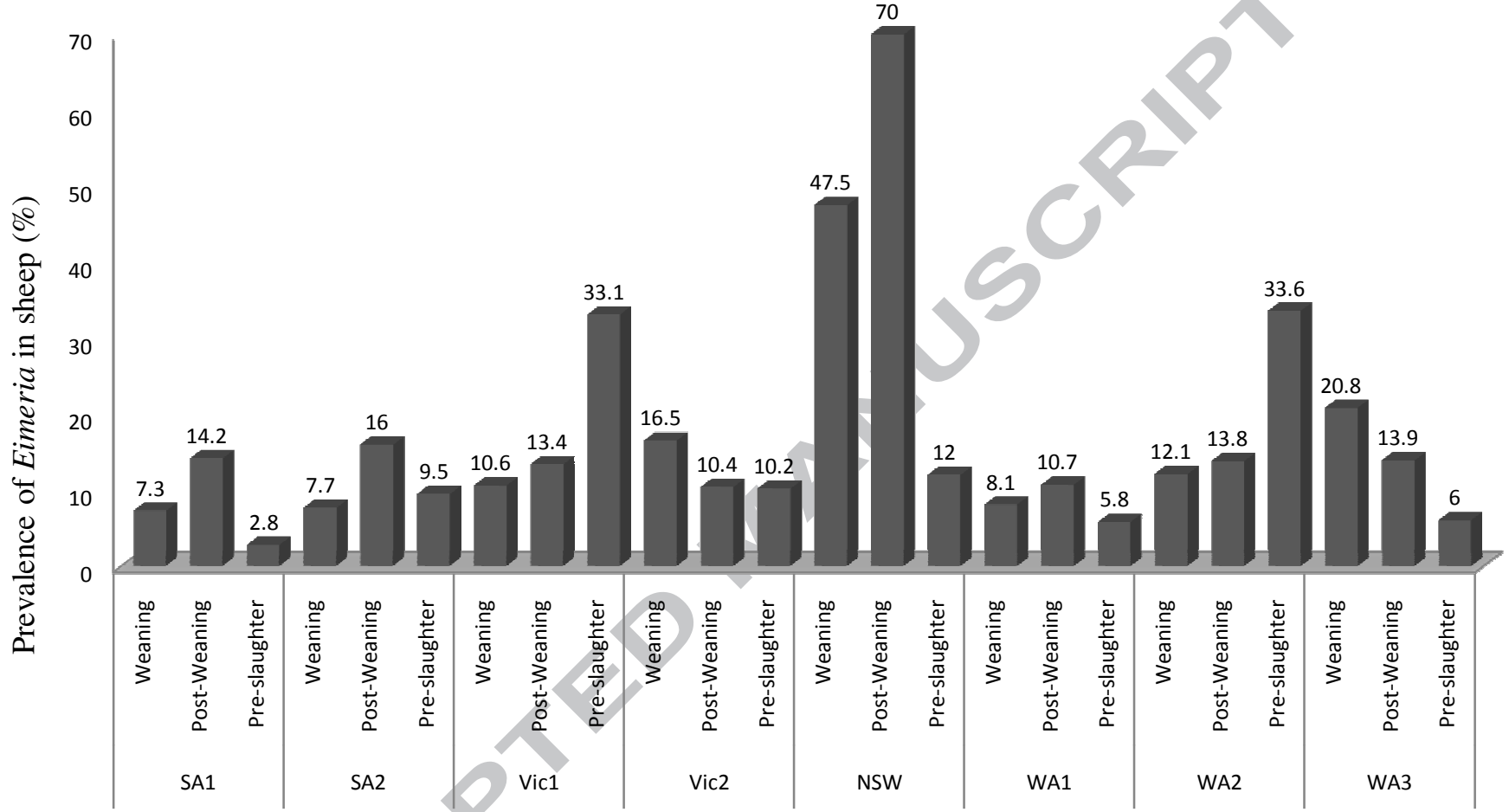
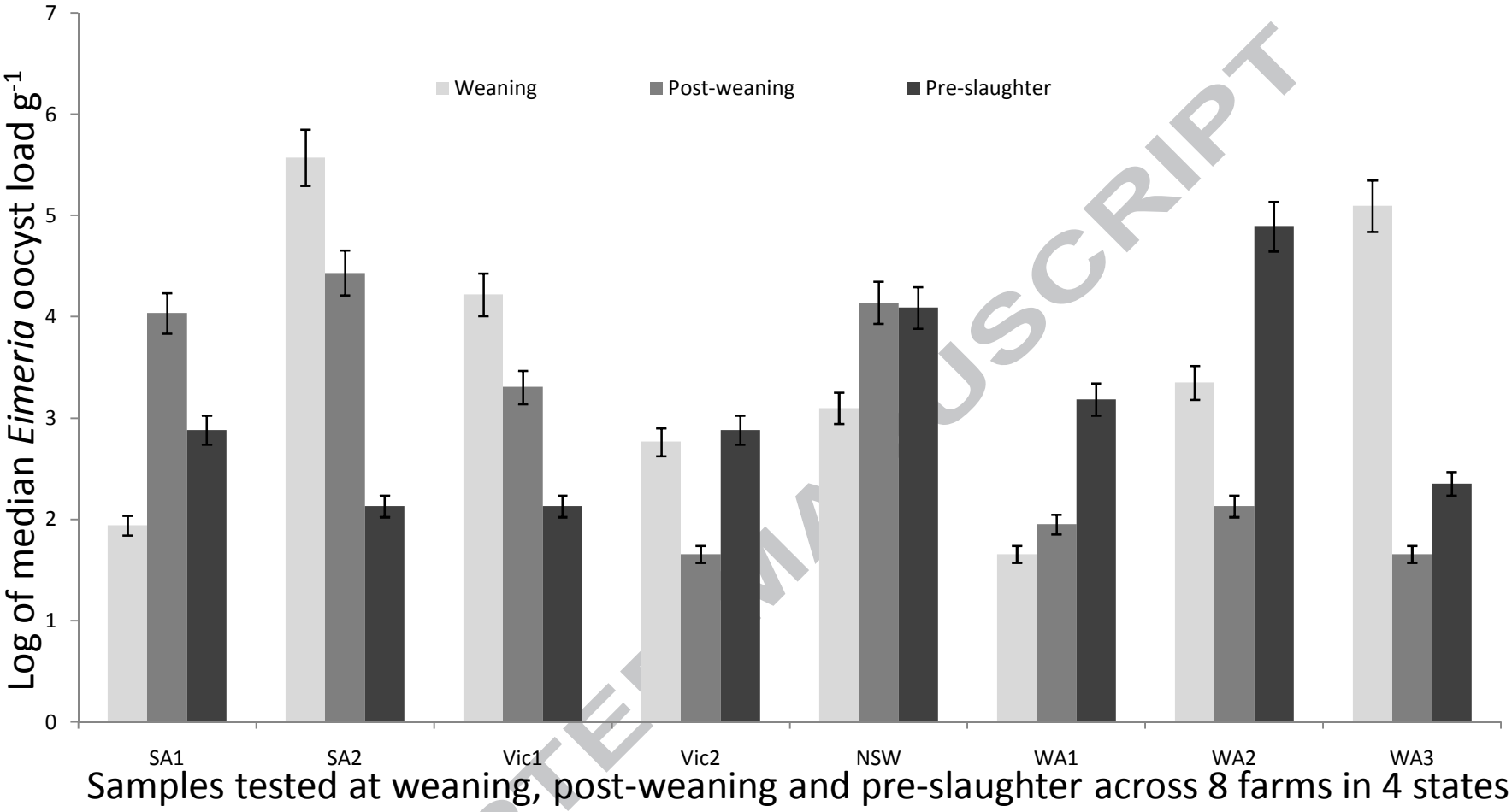
Figure 1. Prevalence (%) of *Eimeria* in sheep faecal samples from 8 farms across 4 states (NSW, SA, Vic and WA) over 3 sampling times (weaning, post-weaning and pre-slaughter) as determined by qPCR.

Figure 2. Log of median *Eimeria* oocyst numbers per gram of faeces (g^{-1}) 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.

Figure 3. The prevalence (%) of *Eimeria* species from a subset of 118 sheep faecal samples from SA, Vic, WA and NSW.





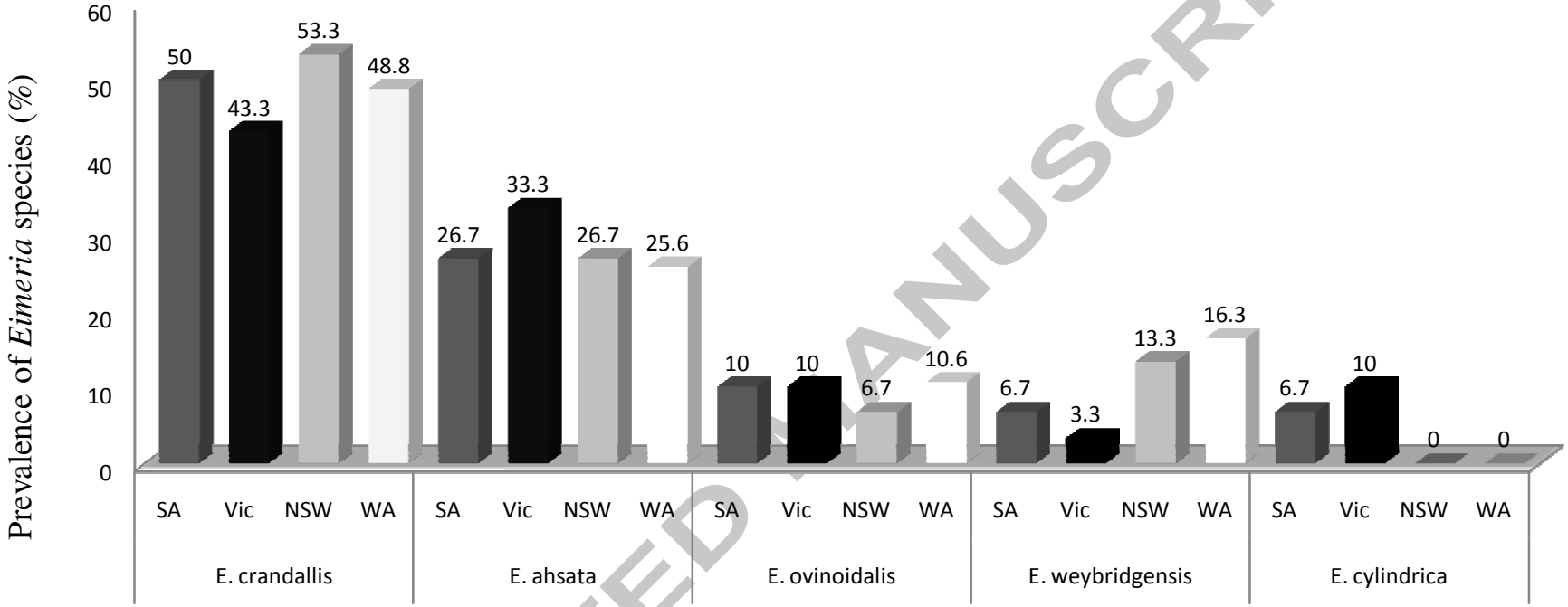


Table 1. Sheep farms sampled during the present study.

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

Note: DSE = dry sheep equivalent, is a standard unit frequently used to compare animal carrying capacity and potential productivity of a given farm or area of grazing land. DNA from samples from Western Australia were extracted by J. Sweeny as described in Sweeny et al., (2011) and Sweeny (2012).

Table 2. Comparison of *Eimeria* OPG counted using a haemocytometer with OPG calculated using the qPCR assay.

	Haemcytometer counted OPG						**OPG calculated using qPCR**					
Sample	I	II	III	Mean	STDEV	%RSD	I	II	III	Mean	STDEV	%RSD
Sample 1	2255	2180	2360	2265	73.8	3.3	1816	1753	1865	1811	56.1	3.1
Sample 2	1570	1450	1270	1430	123.3	8.6	1307	1378	1420	1368	57.1	4.2
Sample 3	1155	1260	1350	1255	79.7	6.3	876	885	820	860	35.2	4.1
Sample 4	1680	1530	1285	1498	162.8	10.9	1502	1430	1389	1440	57.2	4.0
Sample 5	1860	1745	1550	1718	128.0	7.4	1530	1620	1489	1546	67.0	4.3
Sample 6	2690	2565	2485	2580	84.4	3.3	2320	2338	2189	2282	81.3	3.6
Sample 7	3025	2685	2750	2820	147.4	5.2	2743	2520	2718	2660	122.2	4.6
Sample 8	1010	1230	1150	1130	90.9	8.0	785	823	770	793	27.3	3.4
Sample 9	1350	1430	1175	1318	106.5	8.1	983	856	789	876	98.5	11.2
Sample 10	500	600	560	553	41.1	7.4	350	289	325	321	30.7	9.5
Average	1710	1668	1594	1657	103.8	6.9	1421	1389	1377	1396	63.3	5.2

Table 3. Species of *Eimeria* from a subset of 118 isolates typed from 8 farms across 4 states (NSW, SA, Vic and WA) over 3 sampling times (weaning, post-weaning and pre-slaughter).

Farm	Sampling period	No. of genotyped Isolates	*E. crandallis*	*E. ahsata*	*E. ovinoidalis*	*E. weybridgensis*	*E. cylindrica*
SA1	Weaning	5	3	1	1		
	Post-weaning	5	2	2		1	
	Pre-slaughter	5	3	1	1		
SA2	Weaning	5	2	2		1	
	Post-weaning	5	3	1			1
	Pre-slaughter	5	2	1	1		1
Vic1	Weaning	5	3	2			
	Post-weaning	5	2	1	1	1	
	Pre-slaughter	5	3	2			
Vic2	Weaning	5	2	2			1
	Post-weaning	5	2	1	1		1
	Pre-slaughter	5	1	2	1		1
NSW	Weaning	5	3	1		1	
	Post-weaning	5	3	1	1		
	Pre-slaughter	5	2	2		1	
WA1	Weaning	5	3	1		1	
	Post-weaning	5	2	2		1	
	Pre-slaughter	5	4	1			
WA2	Weaning	5	2	1	1	1	
	Post-weaning	5	3	1	1		
	Pre-slaughter	5	1	1	1	2	
WA3	Weaning	5	2	2		1	
	Post-weaning	3	2	1	1		
	Pre-slaughter	5	2	1	1	1	
Total		**118**	**57**	**33**	**12**	**12**	**5**

Highlights

- First comprehensive study of *Eimeria* in sheep across Australia
- Novel qPCR developed
- Prevalence, oocyst shedding and species analysed
- Longitudinal study of 3,412 samples over 3 sampling times
- Pathogenic *Eimeria* species in 58.5% of samples typed

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