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Prevalence and pathogen load of *Cryptosporidium* and *Giardia* in sheep faeces collected from saleyards and in abattoir effluent in Western Australia

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ABSTRACT

The prevalence of *Cryptosporidium* and *Giardia* in faeces collected from sheep at sale yards in Western Australia and for abattoir effluent was determined using a quantitative multiplex PCR (qPCR). A total of 474 faecal samples were collected from sheep at two saleyards on four occasions (April–July 2014) and 96 effluent samples were collected from an abattoir over a four month period (April–July). The overall prevalence of *Cryptosporidium* in sheep faeces was 6.5% (31/474), with the zoonotic species *Cryptosporidium parvum* and *Cryptosporidium ubiquitum* accounting for 54.2% of the typed positive samples. Subtyping of the *C. parvum* and *C. ubiquitum* positives at the gp60 locus identified four *C. parvum* positives as IIdA18G1 and nine *C. ubiquitum* isolates as the XIId subtype. The overall prevalence of *Giardia* in sheep faeces was 6.3% (30/474), with the non-zoonotic assemblage E responsible for the majority (81.5%) of positive isolates typed. Median *Cryptosporidium* and *Giardia* oo/cyst concentrations in positive faecal samples were 1.7×10^3 oocysts g^{-1} (range 32–3.7×10^6 oocysts g^{-1}) and 2.5×10^3 cysts g^{-1} (range 143–7.5×10^5 cysts g^{-1}) respectively. *Cryptosporidium* and *Giardia* were identified in 10.4% (10/96) and 5.2% (5/96) of abattoir effluent samples (respectively). Median *Cryptosporidium* and *Giardia* oo/cyst concentrations in positive effluent samples was 1.3×10^3 cysts g^{-1} (range 393–1.5×10^4) and 1.5×10^4 oocysts g^{-1} (range 759–4.8×10^3) respectively. These findings have important implications for the sheep meat industry because *Cryptosporidium* and *Giardia* have both been associated with reduced carcase productivity in sheep, and the contamination of lamb carcases and watersheds with zoonotic species have important public health consequences.

1. Introduction

Cryptosporidium and *Giardia* are enteric protozoan parasites associated with diarrhea and illness in humans and livestock worldwide (Xiao, 2010; Ryan and Cacciò, 2013). The primary species infecting humans are *Cryptosporidium parvum*, *Cryptosporidium hominis* and *Giardia duodenalis* although a range of species and genotypes have been reported (Feng and Xiao, 2011; Ryan et al., 2014; Ryan and Cacciò, 2013). To date eight major *G. duodenalis* genetic groups (assemblages) have been identified, two of which (A and B) are found in both humans and animals (Feng and Xiao, 2011; Ryan and Cacciò, 2013). A range of zoonotic and non-zoonotic *Cryptosporidium* and *Giardia* species have been identified in sheep with prevalences up to 26.7% and 55.6% reported for *Cryptosporidium* and *Giardia*, respectively (Feng and Xiao, 2011; Ye et al., 2013; Yang et al., 2014a,b).

Sheep can shed *Cryptosporidium* and *Giardia* in faeces at high concentrations (Yang et al., 2014a,b). Most studies have examined faecal shedding in sheep on farms with few studies exploring the extent of faecal shedding of *Cryptosporidium* and *Giardia* in sheep at sale yards or abattoirs. This is of relevance because sheep are often consigned for slaughter via saleyards where multiple groups (lines) from different sources are purchased and mixed during the period prior to slaughter. Furthermore, sheep are subjected to management practices in the pre-slaughter period that may impact pathogen shedding, including deprivation of feed and water and stressors related to transport and mixing of groups. Understanding the shedding patterns of *Cryptosporidium* and *Giardia* in sheep pre-slaughter is important because the fleece/hides, meat products and abattoir waste can become contaminated with potentially zoonotic pathogens, and this has public health consequences. Contamination of abattoir effluent with zoonotic pathogens is particularly

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relevant in circumstances where effluent is treated and re-used in abattoirs (Barros et al., 2007). Therefore the aim of this study was to determine the prevalence, oo/cyst shedding concentration and genotypes of *Cryptosporidium* and *Giardia* in faeces of sheep at saleyards and in abattoir effluent in Western Australia.

2. Materials and methods

2.1. Faecal and effluent sample collection

A total of 474 faecal samples were collected from healthy sheep (fit for transport and sale) at two saleyards in Western Australia; Saleyards A ($n = 238$) and B ($n = 236$). Samples were collected on four occasions between April and August 2014 (Table 1). On each sampling occasion, sheep from six separate lines (approximately 10 sheep sampled per consigned line) at each sale yard were randomly selected for faecal sample collection. At Saleyard A the consigned lines selected for sampling were a mixture of age classifications (lambs through to adult sheep). At Saleyard B, all lines sampled were lambs/yearling (aged approximately 9–15 months). Age and gender for each sample were not recorded. Faecal samples were collected directly from the rectum of sheep. The sample collection method was approved by the Murdoch University Animal Ethics Committee (approval number R2352/10).

Effluent sampling was conducted at an abattoir in southern Western Australia. Effluent samples (50 ml) were collected in triplicate (3 samples per day) from two sampling points (inlet and outlet) once daily over a four-day period (12 samples per sampling point per month) for four months (April–July 2014), giving a total of 96 effluent samples.

2.2. DNA isolation

Genomic DNA was extracted from 200 mg of each faecal and effluent sample using a using a Power Soil DNA Kit (MolBio, Carlsbad, California). Effluent samples were processed before DNA extraction by mixing, then 50 ml for each sample was centrifuged at 3500 × g for 10 min, the supernatant discarded and the pellets were saved for DNA extraction. A negative control (no faecal sample) was used in each extraction group.

2.3. PCR amplification and genotyping

All samples were screened at the actin locus for *Cryptosporidium* and the glutamate dehydrogenase (*gdh*) locus for *Giardia* using quantitative PCR (qPCR) assays previously described (Yang et al., 2014a,b). 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.

Positives were also amplified at the 18S ribosomal RNA (rRNA) locus using a nested protocol previously described (Ryan et al., 2003). All positives were screened using a *C. parvum* and *C. hominis* specific qPCR at a unique *Cryptosporidium* specific gene (Clec) that codes for a novel mucin-like glycoprotein that contains a C-type lectin domain (CTLD) previously described (Yang et al., 2009; Bhalchandra et al., 2013; Yang et al., 2009). 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). *Giardia* positives were typed using assemblage specific primers at the triose phosphate isomerase (*tpi*) locus as previously described (Geurden et al., 2008).

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. 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.genome.jp/tools/clustalw/).

2.5. Statistical analysis

Descriptive statistics (range and median) were presented with sheep/faecal sample or effluent sample as the experimental unit. Prevalences were expressed as proportion (%) of samples positive by qPCR and 95% confidence intervals calculated assuming a binomial distribution using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). Oo/cyst concentrations were transformed (Log_{10} (oocyst concentration +10) for analyses. Analyses of prevalence and oo/cyst concentration were performed using the software SPSS Statistics version 21 (IBM). Prevalences were compared using Pearson Chi-square test or Fisher's Exact Test for two-sided significance. Oo/cyst shedding concentrations were compared using univariate general linear models and least square differences post hoc tests. For analyses of oo/cyst concentration in faecal samples collected at saleyards, the site (saleyard) and month (sampling occasion) were included as fixed factor variables. For analyses of oo/cyst concentration in effluent samples, sample source (inlet or outlet pipe) and month (sampling period) were included as fixed factor variables.

3. Results

3.1. Prevalence and oo/cyst concentration of Cryptosporidium and Giardia in sheep faeces at saleyards

Cryptosporidium and *Giardia* oocyst prevalence and faecal concentration (shedding) in faecal samples collected at saleyards are shown in Table 1. *Cryptosporidium* point prevalence ranged from 5.0 to 10.3% across the sampling occasions (Table 1). No difference in *Cryptosporidium* prevalence was observed between sites (saleyards) overall, nor for any specific occasion where samples were collected from both sites ($P > 0.100$; Table 1). Within sites, there was no effect of sampling occasion on *Cryptosporidium* prevalence for either saleyard ($P > 0.100$).

There was no significant main effect of site (saleyard) or sample occasion, nor any significant interactions between site or sample occasion on oocyst shedding concentration for *Cryptosporidium* ($P > 0.100$).

There was no difference in *Giardia* prevalence between sites (saleyards) overall, with May the only occasion where there was a difference in point prevalence between saleyards, whereby Saleyard B had 14% higher prevalence than Saleyard A ($P = 0.031$; Table 1). Within sites (saleyards), there was an effect of sampling occasion on *Giardia* prevalence only for Saleyard B ($P < 0.001$), whereby the point prevalences observed at the May and June collections were higher than for July (Table 1).

Main effects for *Giardia* cyst concentration were observed for both site ($P = 0.049$) and month ($P = 0.002$), with higher cyst concentration observed in May compared with April ($P = 0.015$), June