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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 C. parvum and C. 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/ocyst concentrations in positive faecal samples were $1.7 \times 10^3$ oocysts g$^{-1}$ (range 32 – 3.7 $\times 10^6$ oocysts g$^{-1}$) and $2.5 \times 10^3$ cysts g$^{-1}$ (range 143 – 7.5 $\times 10^5$ cysts g$^{-1}$) respectively. Cryptosporidium and Giardia were identified in 10.4% (10/96) and 5.2% (5/96) of abattoir effluent
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27 samples (respectively). Median *Cryptosporidium* and *Giardia* oo/cyst concentrations in positive
28 effluent samples was $1.3 \times 10^3$ cysts g$^{-1}$ (range 393-1.5x10$^4$) and $1.5 \times 10^4$ oocysts g$^{-1}$ (range 759-
29 4.8x10$^3$) respectively. These findings have important implications for the sheep meat industry
30 because *Cryptosporidium* and *Giardia* have both been associated with reduced carcase productivity
31 in sheep, and the contamination of lamb carcases and watersheds with zoonotic species have
32 important public health consequences.

34 Keywords: *Cryptosporidium*; *Giardia*; lambs; saleyards; abattoir effluent; prevalence

36 1. Introduction

37 *Cryptosporidium* and *Giardia* are enteric protozoan parasites associated with diarrhea and
38 illness in humans and livestock worldwide (Xiao, 2010; Ryan and Caccio, 2013). The primary
39 species infecting humans are *Cryptosporidium parvum*, *C. hominis* and *Giardia duodenalis*
40 although a range of species and genotypes have been reported (Feng and Xiao, 2011; Ryan et al.,
41 2014; Ryan and Caccio, 2013). To date eight major *G. duodenalis* genetic groups (assemblages)
42 have been identified, two of which (A and B) are found in both humans and animals (Feng and
43 Xiao, 2011; Ryan and Caccio, 2013). A range of zoonotic and non-zoonotic *Cryptosporidium* and
44 *Giardia* species have been identified in sheep with prevalences up to 26.7% and 55.6% reported for
45 *Cryptosporidium* and *Giardia* respectively (Feng and Xiao, 2011; Ye et al., 2013; Yang et al.,
46 2014a; 2014b).

47 Sheep can shed *Cryptosporidium* and *Giardia* in faeces at high concentrations (Yang et al.,
48 2014a; 2014b). Most studies have examined faecal shedding in sheep on farms with few studies
49 exploring the extent of faecal shedding of *Cryptosporidium* and *Giardia* in sheep at sale yards or
50 abattoirs. This is of relevance because sheep are often consigned for slaughter via saleyards where
51 multiple groups (lines) from different sources are purchased and mixed during the period prior to
52 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 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; Saleyard A (n=238) and Saleyard 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 (50ml) 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.
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2.2 DNA isolation

Genomic DNA was extracted from 200mg of each faecal and effluent sample 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 $3,500 \times g$ 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; 2014b). 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., 2013). *Giardia* positives were typed using assemblage specific primers at the triose phosphate isomerase (tpi) locus as previously described (Geurden et al., 2008).
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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 (P=0.040), July (P<0.001) and August (P=0.009). There was also a significant interaction between site and sampling occasion for Giardia cyst concentration in faeces (P=0.014) whereby cyst concentration was higher in samples from Saleyard B at the May sampling (P=0.017) with no significant differences observed for June or July.

3.2 Cryptosporidium and Giardia in abattoir effluent
Cryptosporidium and Giardia oocyst prevalence and concentration in abattoir effluent samples are shown in Table 2. Cryptosporidium was identified at all four sampling periods collected between April and July (Table 2). Point prevalence for Cryptosporidium in effluent samples ranged from 8.3% to 16.7% across the overall sampling period (Table 2). Overall, no difference in Cryptosporidium prevalence in effluent samples was observed between source (inlet or outlet; P>0.100) or period (P>0.100). Within sources (inlet or outlet), there was no effect of sampling period on Cryptosporidium point prevalence (P>0.100). Within periods (months), there was no effect of source (inlet/outlet) on Cryptosporidium prevalence (P>0.100).

There was no main effect observed for sample period (month) or source (inlet/outlet), nor any significant interaction between sample period and source on oocyst concentration for Cryptosporidium in effluent samples (P>0.100).

Giardia was identified at three of the four sampling periods (Table 2). Giardia point prevalence in effluent samples ranged from 0% to 16.7% across the overall sampling period (Table 2). Giardia was not identified in any of the effluent samples (inlet or outlet) collected in July or in the outlet sampling sites during the May and June sample collections. Overall, no difference in Giardia prevalence in effluent samples was observed between source (P>0.100) or period (P>0.100). Within sources (inlet or outlet), there was no effect of sampling occasion on Giardia point prevalence (P>0.100). Within periods (months), there was no effect of source on Giardia prevalence (P>0.100).

There was no main effect observed for sample period (month) or source (inlet/outlet), nor any significant interaction between period and source on cyst concentration for Giardia in effluent samples (P>0.100).

3.3 Cryptosporidium and Giardia genotypes in sheep faeces and abattoir effluent

Genotyping was only successful for Cryptosporidium and Giardia positive samples from the faecal samples (saleyards) because there was insufficient template and/or mixed chromatograms for
For Cryptosporidium, twenty-four out of thirty-one qPCR positive isolates from faeces were successfully genotyped. Three Cryptosporidium species were identified; C. parvum (16.7%, 4/24), C. ubiquitum (37.5%, 9/24) and C. xiaoii (45.8%, 11/24). Subtyping of C. parvum and C. ubiquitum isolates at the gp60 locus, revealed that all four C. parvum isolates belonged to the IId subtype (IIdA18G1) and the nine C. ubiquitum isolates belonged to XIIId subtype family.

For Giardia, twenty-seven out of the thirty positives were successfully genotyped. Of these, 8/27 (29.6%) were assemblage A and 22/27 (81.5%) were assemblage E.

4. Discussion

This study identified an overall prevalence of 6.5% for Cryptosporidium and 6.3% for Giardia in faeces collected from sheep at saleyards in Western Australia. Cryptosporidium and Giardia were also identified in 10.4% and 5.2% of abattoir effluent samples respectively. Zoonotic species were identified in samples from saleyards, but the genotypes from effluent samples were not able to be determined. Most published data to date has examined the prevalence of these organisms in sheep on farms, with few studies including sheep “post farm gate” such as at saleyards or abattoir lairage, and none have examined sheep abattoir effluent. This is important because sheep in the pre-slaughter period are subjected to stressors (including transport, mixing of groups, and deprivation of feed and water) that may impact the shedding patterns of pathogenic organisms. Shedding of protozoan pathogens has implications beyond the farm gate including important public health implications because shedding may result in contamination of meat carcasses with potentially zoonotic pathogens.

A previous study by Ryan et al. (2005) examined faeces from sheep (mixed ages) in lairage from September to January at a different abattoir on the south coast of Western Australia and identified prevalences of 26% (131/500) for Cryptosporidium and 44% (220/500) for Giardia using nested PCR. The higher prevalence observed by Ryan et al. (2005) could reflect a number of factors...
that could not be compared between the previous and present study including sheep age, point of
origin (location), seasonal/temporal variation, the management of sheep and stressors (duration of
transport, deprivation of feed and water etc) in the period prior to faecal sample collection that may
impact shedding of pathogens. Other studies have reported prevalences of 13.2% and 0.7% for
_Cryptosporidium_ in faeces from slaughtered sheep at slaughterhouses in Turkey and Nigeria
respectively (Faleke et al., 2006; Çiçek et al., 2008). Another study identified _C. andersoni_ in 7.3%
of cattle faecal samples at an abattoir in the UK (Moriarty et al., 2005). However, prevalence in
these studies was determined using microscopy, which is likely to underestimate prevalence
compared with more sensitive molecular methods (Ryan et al., 2005). More recently, Yang et al.
(2014a; 2014b) used the same qPCR approach described in the present study in a longitudinal study
to identify on-farm prevalence of _Cryptosporidium_ and _Giardia_ in lambs at three time-points
(weaning, post-weaning and pre-slaughter) on eight Australian farms. These studies identified point
prevalences in the pre-slaughter period in sheep on farm ranging 6.1 - 36.4% for _Cryptosporidium_
and 9.4% - 42.1% for _Giardia_ (Yang et al. 2014a; 2014b), so comparable with the overall
prevalences observed in the present study.

A total of three _Cryptosporidium_ species were identified in sheep faeces in the present
study: _C. parvum_, _C. ubiquitum_ and _C. xiaoii_, with the zoonotic species _C. parvum_ and _C. ubiquitum_
accounting for 54.2% of all positives samples typed. _Cryptosporidium parvum_ and _C. ubiquitum_ are
common human pathogens (Xiao, 2010). The IId gp60 subtype family identified in the _C. parvum_
isolates is known to be zoonotic and has been reported in humans in Australia (Waldron et al.,
2009; Ng et al., 2010). The IIdA18G1 subtype identified in the present study has previously been
identified in sheep in South Australia, New South Wales and Western Australia (Yang et al.,
2014a). Evidence from previous studies suggests that the IId subtype family is adapted to lambs
(and goat kids), and therefore lambs may be one of the most important reservoirs for this zoonotic
group of _C. parvum_ isolates (Imre et al., 2013; Yang et al., 2014a).
For *Giardia*, the non-zoonotic assemblage E was responsible for the majority (81.5%) of positive isolates typed, whereas the potentially zoonotic Assemblage A was identified in 29.6% of positive isolates typed. Previous studies have also reported that assemblages E and A are the dominant assemblages infecting sheep, with assemblage E usually more prevalent (Feng and Xiao, 2011; Yang et al., 2014b).

The range of *Cryptosporidium* oocyst shedding concentration in faeces collected at sale yards was similar to a previous study that examined oocyst shedding concentrations in lambs on Australian farms that reported that the range of oocyst shedding in lambs on-farm in the pre-slaughter period ranged from 260 to $4.8 \times 10^7$ oocysts g$^{-1}$ in positive samples (Yang et al., 2014a).

Similarly, the range of *Giardia* cyst shedding concentration in faeces collected at sale yards in the present study was comparable with a previous Australian study that reported *Giardia* cyst shedding concentration in lambs on-farm ranged from 63 to $4.7 \times 10^9$ cysts g$^{-1}$ in positive samples in the pre-slaughter period (Yang et al., 2014b).

The factors that determine oo/cyst shedding (both prevalence of animals shedding and concentration in faeces) in sheep are not well described, but age is likely to have a significant impact with younger animals more susceptible to infection. The role of common stressors in sheep meat enterprises (for example, transport, curfew-lairage periods during which feed and water is withheld, mixing of groups of animals, seasonal factors and management practices such as shearing) may affect meat quality, and potentially affect shedding of zoonotic pathogens in faeces, although the impact on these factors on pathogen shedding have not been well described. There is evidence that for bacteria at least, protracted fasting may cause the bacterial load to be more hazardous (Pointon et al., 2012). The time spent off feed prior to sampling was not known for the lines of sheep sampled in the present study. Prevalence and median shedding concentration of oo/cysts in faeces would be expected to increase where sheep are consigned to slaughter via saleyards (compared with consignment directly from farms) and therefore subjected to protracted fasting times (>24 hours) and additional stressors related to subsequent mixing of animals and
transport between saleyards and abattoirs. Further studies are required to determine the changes in prevalence and faecal concentration of pathogens resulting from pre-slaughter stressors that may occur between the farm gate and slaughter.

Apart from potential for contamination of carcasses and meat products, Cryptosporidium was identified in abattoir effluent (inlet or outlet) at all four sampling occasions and Giardia was identified at three out of four sampling occasions. This has implications for the management of effluent waste-water from abattoirs. Giardia was not identified in effluent samples collected in July or at the outlet location in May and June. This may reflect seasonal variation in shedding by sheep in lairage, different sources of sheep in lairage through that period, variation in cyst distribution through the effluent pond or the sample collection protocol (50ml samples collected at two locations) failing to identify Giardia cysts at low concentration in effluent.

In conclusion, this study identified Cryptosporidium and Giardia, including potentially zoonotic species, in faeces from sheep at saleyards and in abattoir effluent. These organisms have potential to contaminate sheep carcasses with important implications for public health. Further studies are required to determine the prevalence of Cryptosporidium and Giardia in water used within abattoirs and to determine the extent (if any) of contamination of carcasses and meat products.

Conflict of interest
None declared.

Acknowledgements
This study was funded by Meat and Livestock Australia (MLA), Australian Wool Innovation Limited (AWI) and the Australian Government. We thank Malcom Boyce and Khama Kelman for collection of the faecal samples. We thank the participating farmers and saleyards for their support and providing access to sheep for sample collection. We thank the participating abattoir for providing effluent samples for this study.


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Table 1

Cryptosporidium and Giardia prevalence (%) and 95% confidence interval and oo/cysts concentration (range and median for positive samples only) of oo/cysts in effluent samples from a Western Australian abattoir over four sampling occasions.

<table>
<thead>
<tr>
<th>Period</th>
<th>Inlet/outlet</th>
<th>Samples (n)</th>
<th>Cryptosporidium</th>
<th>Giardia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prevalence (%) (95% CI)</td>
<td>Concentration in positive samples (ooocyst g⁻¹)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>April</td>
<td>Inlet</td>
<td>60</td>
<td>8.3 (1.3-15.3)</td>
<td>17.3 x 10⁰</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>60</td>
<td>16.7 (0-37.8)</td>
<td>393.2 x 10⁰</td>
</tr>
<tr>
<td>May</td>
<td>Inlet</td>
<td>58</td>
<td>5.0 (0-10.5)</td>
<td>25.3 x 10⁰</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>58</td>
<td>5.0 (0-10.5)</td>
<td>25.3 x 10⁰</td>
</tr>
<tr>
<td>June</td>
<td>Inlet</td>
<td>58</td>
<td>5.0 (0-10.5)</td>
<td>25.3 x 10⁰</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>58</td>
<td>5.0 (0-10.5)</td>
<td>25.3 x 10⁰</td>
</tr>
<tr>
<td>July</td>
<td>Inlet</td>
<td>60</td>
<td>5.0 (0-10.5)</td>
<td>96.3 x 10⁰</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>60</td>
<td>5.0 (0-10.5)</td>
<td>96.3 x 10⁰</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>238</td>
<td>5.9 (2.9-8.9)</td>
<td>175.3 x 10⁰</td>
</tr>
</tbody>
</table>

Table 2

Prevalence of Cryptosporidium and Giardia and concentration (range and median for positive samples only) of oo/cysts in effluent samples from a Western Australian abattoir over four sampling periods.

<table>
<thead>
<tr>
<th>Period</th>
<th>Inlet/outlet</th>
<th>Samples (n)</th>
<th>Cryptosporidium</th>
<th>Giardia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prevalence (%) (95% CI)</td>
<td>Concentration in positive samples (ooocyst g⁻¹)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>April</td>
<td>Inlet</td>
<td>12</td>
<td>16.7 (0-37.8)</td>
<td>393.2 x 10⁰</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>12</td>
<td>8.3 (0-24)</td>
<td>587.587</td>
</tr>
<tr>
<td>May</td>
<td>Inlet</td>
<td>12</td>
<td>8.3 (0-24)</td>
<td>1.2 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>12</td>
<td>8.3 (0-24)</td>
<td>1.2 x 10⁴</td>
</tr>
<tr>
<td>June</td>
<td>Inlet</td>
<td>12</td>
<td>8.3 (0-24)</td>
<td>750-750</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>12</td>
<td>8.3 (0-24)</td>
<td>894-894</td>
</tr>
<tr>
<td>July</td>
<td>Inlet</td>
<td>12</td>
<td>16.7 (0-37.8)</td>
<td>1.9 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>Outlet</td>
<td>12</td>
<td>8.3 (0-24)</td>
<td>1.3 x 10³</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>96</td>
<td>10.4 (4.3-16.5)</td>
<td>393.15 x 10⁰</td>
</tr>
</tbody>
</table>

CI: Confidence interval
*Within column (parasite), sample (site) prevalence values with different superscript are significantly different (P<0.05)*
Graphical abstract

Cryptosporidium and Giardia

qPCR screening (sheep at saleyards and sheep abattoir effluent)