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PII: S0304-4017(10)00622-9
Reference: VETPAR 5545

To appear in: Veterinary Parasitology

Received date: 14-6-2010
Revised date: 28-10-2010
Accepted date: 29-10-2010

Please cite this article as: Ng, J., Yang, R., McCarthy, S., Gordon, C., Hijjawi, N., Ryan, U., Molecular characterization of Cryptosporidium and Giardia in pre-weaned calves in Western Australia and New South Wales., Veterinary Parasitology (2010), doi:10.1016/j.vetpar.2010.10.056

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Molecular characterization of Cryptosporidium and Giardia in pre-weaned calves in Western Australia and New South Wales.

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Abstract

A total of 364 fecal specimens from randomly selected pre-weaned calves, aged up to 4 months, from 5 different farms in the south of Western Australia and 1 farm from New South Wales were screened for the presence of Cryptosporidium and Giardia using PCR. There were substantial differences in prevalence between the farms and the overall prevalence was 22.3% (81/364) and 26.9% (98/364) respectively for Cryptosporidium and Giardia. For Cryptosporidium, 70 positives were identified at the 18S locus. At a unique diagnostic locus, an additional 12 C. parvum positives were identified. Sequence analysis at the 18S ribosomal RNA locus was successful for 59 of the 70 positive isolates; of these 14 were C. parvum, 28 were C. bovis, 15 were C. ryanae, 1 was pig genotype II and 1 was a mixed C. ryanae/ C. parvum infection. Subtyping analysis at the glycoprotein 60 (gp60) locus for 24 C. parvum isolates identified all as IIa; 17 were A17G2R1, 1 was A18G3R1 and 6 were A20G3R1. For Giardia, 75 positives were identified at the 18S locus and an additional 23 positives were identified at the gdh locus. The majority of the isolates sequenced were assemblage E, however assemblage A and B and mixed A and E and A, B and E infections as well as the quenda genotype were identified. The findings of the present study indicate that pre-weaned calves are not an important source of zoonotic Giardia species in Australia but may be an important source of zoonotic Cryptosporidium.

Keywords: Cryptosporidium, Giardia, pre-weaned calves, 18S rRNA, gdh, gp60.
1. Introduction

Cryptosporidium and Giardia spp. are the most common protozoan parasites that infect domestic animals and humans (Caccio and Ryan, 2008; Fayer, 2008). In cattle, cryptosporidiosis causes significant neonatal morbidity, causing weight loss and delayed growth, which leads to large economic losses (McDonald, 2000). Contamination of food or water by cattle manure has been identified as a cause of several foodborne and waterborne outbreaks of cryptosporidiosis (Glaberman et al., 2002; Blackburn et al., 2006). In case–control studies, contact with cattle was implicated as a risk factor for human cryptosporidiosis in the United States, United Kingdom, Ireland, and Australia (Robertson et al., 2002; Goh et al., 2004; Hunter et al., 2004; Roy et al., 2004).

Over the past 20 years, cattle have been thought to be one of the main reservoir hosts for the zoonotic C. parvum. However, studies worldwide suggest that cattle are infected with at least five Cryptosporidium parasites: C. parvum, C. bovis, C. andersoni, C. ryanae (previously called deer-like genotype) and C. suis (Xiao and Feng, 2008; Xiao, 2010).

There appears to be geographical differences in the age-related prevalence of different Cryptosporidium species in cattle. In parts of the US, Belgium, Ireland, Germany, Malaysia, the UK and Sweden, it has been reported that the zoonotic C. parvum is responsible for the majority of Cryptosporidium infections in pre-weaned calves and only a small percentage of Cryptosporidium infections in post-weaned calves and heifers (Brook et al., 2009; Geurden et al., 2007; Thompson et al., 2007; Xiao et al., 2007; Broglio et al., 2008; Halim et al., 2008; Fayer et al., 2010; Silverlås et al., 2010). Post-weaned calves were mostly infected with C. bovis, C. andersoni and C. ryanae (Fayer et al., 2010). Other studies in China, India, Georgia and western
North Dakota however, have reported that *C. bovis* was the most common species found in pre-weaned calves (Feng et al., 2007; Feltus et al., 2008).

*Giardia* is a ubiquitous enteric parasite that affects domestic animals and humans (Caccio and Ryan, 2008). *Giardia duodenalis* has been implicated as an etiological agent in dairy and beef calf diarrhea, worldwide (Olson et al., 2004; Castro-Hermida et al., 2006; Coklin et al., 2007; Guerden et al., 2008a). Surveys of dairy cattle worldwide have reported predominantly assemblage E, with lower levels of assemblage A and also assemblage B (Caccio and Ryan, 2008; Santin et al., 2009).

Few studies have been conducted to examine *Giardia* in pre and post-weaned animals (Becher et al., 2004; Trout et al 2004; 2005; Coklin et al., 2007; Santin et al., 2009). Two studies in the US and Australia reported that assemblage E but not assemblage A was detected in pre-weaned calves (Becher et al., 2004; Santin et al., 2009), whereas others have reported both assemblage A and E in pre-weaned cattle (Trout et al., 2004).

Little is known about the prevalence of *Cryptosporidium* and *Giardia* sp. in pre-weaned cattle in Western Australia (WA) and New South Wales (NSW). In the present study, fecal samples from pre-weaned cattle were screened for the presence of *Cryptosporidium* and *Giardia* with genotyping of any positives detected carried out in order to clarify the epidemiology of these infections in WA and NSW.

## 2. Materials and Methods

### 2.1 Faecal Sample Collection

A total of 364 fecal specimens from randomly selected, pre-weaned calves
aged up to 4 months, were collected between March 2006 and September 2008 from 5
different farms in the south of WA and 1 farm from NSW (Table 1). Fecal samples
were collected directly from the rectum of the animals using disposable gloves and
plastic containers.

2.2 DNA isolation

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

2.3 PCR amplification.

All samples were screened for the presence of Cryptosporidium and Giardia
by PCR. For screening of Cryptosporidium, amplification of the 18S rRNA gene locus
was carried out using a two-step nested PCR as previously described (Ryan et al.,
2003). All samples were also screened using a C. parvum and C. hominis specific
qPCR at a unique Cryptosporidium specific protein-coding locus (Cgd3_440 which
spans positions 52052–53389 of contig AAEE01000004 which maps to chromosome
3) as previously described (Yang et al., 2009; Morgan et al., 1996; 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). For
Giardia, screening was conducted using a two-step nested PCR protocol to amplify
the 18S rRNA gene locus and gdh gene locus as previously described (Read et al.,
2002, 2004). PCR contamination controls were used and the area for preparation of
PCR reactions was separated from the area for amplification. A spike analysis
(addition of 0.5 µL of C. hominis or G. duodenalis assemblage F positive control into each sample) was conducted on randomly selected negative samples from each group of DNA extractions to determine if negative results were due to PCR inhibition. The amplified DNA fragments from the secondary PCR product were separated by gel electrophoresis and purified using the freeze-squeeze method (Ng et al., 2006).

2.4 Sequence and phylogenetic analysis

Purified PCR products were sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer’s instructions with the exception that the annealing temperature was raised to 58ºC. 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://clustalw.genome.jp/). Cryptosporidium 18S rRNA and gp60 gene sequences and Giardia 18S rRNA and gdh gene sequences have been deposited in the GenBank database, accession no. HQ398316-HQ398327.

3. Results

3.1 Cryptosporidium Prevalence

The prevalence of Cryptosporidium in the different farms ranged from 2% to 37.5% with the highest prevalence in the WA farm 1 (Table 1). The overall prevalence was 22.5% (82/364). At the 18S rRNA locus, 70 positives were identified. At the unique diagnostic locus (Yang et al., 2009), an additional 12 C. parvum positives were identified. Sequence analysis at the 18S rRNA locus was successful for 59 of the 70 positive isolates; of these 14 were C. parvum, 28 were C. bovis, 15 were...
C. ryanae, 1 was pig genotype II and 1 was a mixed C. ryanae/ C. parvum infection (Table 2). Sub-typing analysis at the gp60 locus was successful for 24 of the 27 C. parvum isolates and all were identified as IIa; 17 were A17G2R1, 1 was A18G3R1 and 6 were A20G3R1 (Table 2).

3.2 Giardia Prevalence

The prevalence of Giardia in the different farms ranged from 13.7% to 57.5% with the highest prevalence in farm 3 from WA (Table 1). The overall prevalence was 26.9% (98/364). At the 18S rRNA locus, 75 positives were identified. At the gdh locus, 39 positives were identified of which 23 were in addition to the positives detected at the 18S rRNA locus. Therefore a total of 98 isolates were positive for Giardia between the two loci. Sequence analysis at the 18S rRNA locus was successful for all 75 positives; 71 of which were assemblage E, 1 was assemblage B, 1 was a mixed A and E infection, 1 was a mixed A, B and E infection and 1 was the quenda genotype (Table 3). At the gdh locus, all 39 positive isolates were successfully sequenced; 35 were assemblage E, 2 were genotyped as A2, 2 were B4. There was good agreement between the two loci with the exception of one isolate that was genotype E at the 18S rRNA locus but typed as assemblage B4 at the gdh locus (Table 3).

4. Discussion

In the present study, the prevalence of Cryptosporidium and Giardia in pre-weaned cattle was 22.3% and 26.9% respectively. Farm 1 from WA had the highest prevalence of Cryptosporidium (37.5%) but Farm 6 from NSW had the highest proportion of C. parvum (39.5%; 15/38 positives). Farm 3 from WA had the highest
Giardia prevalence at 57.5% (23/40). The prevalence of Cryptosporidium and Giardia spp. appears to vary widely from state to state and within states in Australia. For example, a previous longitudinal study of Giardia and Cryptosporidium in dairy cattle from birth to weaning in Western Australia reported that Cryptosporidium and Giardia were detected in 48% (26/54) and 89% (48/54) of sampled calves (Becher et al., 2004). In that study, molecular characterization at the 18S rRNA locus identified six of the Cryptosporidium positive samples as C. parvum and 31 Giardia positive isolates as G. duodenalis assemblage E (Becher et al., 2004). Another study reported that 58% (21/36) of Western Australian post-weaned calves were positive for Giardia (O’Handley 2000). Genetic sequences from five isolates identified four as assemblage E and one as assemblage A (O’Handley et al., 2000). A study in NSW reported that 14 of 15 cattle-derived Cryptosporidium-positive isolates were C. parvum and 1 as a C. bovis/C. parvum mixture (Ng et al., 2008). In Victoria, a more recent study identified C. parvum in 124 of 268 (46.3%) individual calves on pasture-based dairy farms in three regions of Victoria, Australia (Nolan et al., 2009).

Studies worldwide have reported prevalences ranging from 3% to 64% for Giardia in cattle (Trout et al., 2007; Geurden et al., 2008b). For Cryptosporidium, studies have reported that the highest prevalence of infection occurred at weeks 4 and 5 of age with pre-weaned calves (<8 weeks of age) exhibiting the highest prevalence (60.8%), followed by post-weaned calves (3-12 months of age) (32.1%) and heifers (12-24 months of age) (11.4%) (Santin et al., 2009).

In the present study, C. parvum, C. bovis, C. ryanae and pig genotype II were identified in the cattle. All these species are morphologically identical but differ biologically and genetically. Cryptosporidium andersoni was not detected in the present study. Cryptosporidium species in cattle appear to be linked to different...
clinical manifestations. *Cryptosporidium andersoni* appears to be more common in older (post-weaned) and mature cattle, infects the abomasums of juvenile and mature cattle and although induces no apparent clinical signs, has been implicated as a cause of reduced milk production (Olson et al., 1997; Anderson 1998; Lindsay et al., 2000; Santín et al., 2004; Fayer et al., 2006; Feng et al., 2007). *Cryptosporidium parvum* typically causes disease in calves and has been identified as one of the primary etiologic agents of neonatal calf diarrhea (Naciri et al., 1999). Preliminary evidence however suggests that *C. bovis* and *C. ryanae* are not associated with any signs of disease (Fayer et al., 2005, 2008; Starkey et al., 2006). As the different species have different pathogenicity in cattle and different infectivity for humans, identifying the factors that contribute to the occurrence of these different species in cattle is therefore critical to the understanding of economic and public health importance and transmission of cryptosporidiosis in cattle.

Of the 4 species identified, only *C. parvum* is a major pathogen in humans (Xiao, 2010). Of the 82 *Cryptosporidium* positive isolates typed at the 18S and diagnostic locus, 71 were typed and of these ~38% (27/71) were *C. parvum*. However, the majority of the *C. parvum* isolates detected were from the NSW farm (n =15). In the 5 WA farms, the combined prevalence of *C. parvum* was only ~15% (11/71). Subtype analysis at the gp60 locus identified 17 *C. parvum* isolates as IIaA17G2R1, 1 as IIaA18G3R1 and 6 as IIaA20G3R1. Subtype IIaA17G2R1 has previously been reported in calves and humans in NSW (Ng et al., 2008, Waldron et al., 2009), humans in WA (O’Brien et al., 2008; Ng et al., 2010) and cattle in Victoria (Nolan et al., 2009). Subtype IIaA18G3R1 has also been reported in both calves and humans in NSW (Ng et al., 2008, Waldron et al., 2009), calves and humans WA (O’Brien et al., 2008; Ng et al., 2010), humans in Victoria (Jex et al., 2007) and
humans in South Australia (Jex et al., 2008). Subtype IIA20G3R1 has been reported in calves and humans in NSW (Ng et al., 2008, Waldron et al., 2009), humans in WA (Ng et al., 2010) and humans in South Australia (Jex et al., 2008).

The zoonotic *G. duodenalis* assemblages A and B as well as the livestock-specific assemblage E and the quenda genotype were detected in the cattle. *Giardia* causes severe enteric disease in calves (Olsen et al., 1995) and a recent study reported that *Giardia* assemblage E may cause intestinal lesions leading to calf scours (Barigye et al., 2008). The zoonotic potential of *G. duodenalis* assemblages A and B detected in the present study is unclear, however, the assemblage A and B sequences identified were identical to sequences identified in humans (GenBank accession nos. GQ329675 and EU594663 respectively). A recent study in New Zealand identified assemblages A and B in 40 *Giardia* isolates from calves and 30 from humans, living in the same region and collected over a similar period, using the beta-giardin gene (Winkworth et al., 2008a). Genotype comparisons revealed a substantial overlap of identical genotypes from the two hosts for both assemblages. No assemblage E has been detected in New Zealand livestock to date (Winkworth et al., 2008a). The quenda genotype was identified in one cattle isolate from farm 1 in WA. The novel quenda genotype was identified in a previous study in 1/72 quendas (*Isoodon obesulus*) screened (Adams et al., 2004) and more recently in 11.8% (6/51) quendas screened (Thompson et al., 2010). This is the first report of the quenda genotype in cattle and it is at yet undetermined if the cattle was actually infected or simply passing cysts ingested from fecal contamination of grazing areas.

Prevention of contamination of drinking water catchments from oocysts shed in cattle manure is of great importance to water authorities. Studies in the US reported that the factors associated with environmental loading of *Cryptosporidium* oocysts
included cattle age class, 24 h precipitation, and cumulative seasonal precipitation, but not percent slope, lot acreage, cattle stocking number, or cattle density (Miller et al., 2008). Interestingly, the authors reported that vegetated buffer strips and straw mulch application significantly reduced the protozoal concentrations and loads in storm runoff, while cattle exclusion and removal of manure did not (Miller et al., 2008).

Similarly for *Giardia*, there were 26% fewer *Giardia* detected in runoff collected from recently planted vegetation strips compared with bare soil strips cleared of vegetation (P = 0.006) (Winkworth et al., 2008b).

In conclusion, the findings of the present study indicate that pre-weaned calves may not an important source of zoonotic *Giardia* genotypes in Western Australia and NSW but may be an important source of zoonotic *Cryptosporidium*. However, further research is required to determine if this holds true for pre-weaned calves across Australia and to understand why there are such dramatic geographic differences in the prevalence of zoonotic genotypes in pre-weaned calves.

**Acknowledgements**

We are grateful to Johanna Johnson for assistance with sample collection. This study was supported by Water Corporation, Perth, Western Australia (www.watercorporation.com.au).

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Table 1: Prevalence of *Cryptosporidium* and *Giardia* in pre-weaned cattle in five farms in Western Australia and one farm in NSW, by PCR analysis of the 18S rRNA and diagnostic locus for *Cryptosporidium* and 18S and *gdh* loci for *Giardia*.

<table>
<thead>
<tr>
<th>Cattle breed</th>
<th>Dairy/Beef</th>
<th>Age</th>
<th><em>Cryptosporidium</em> prevalence</th>
<th>Giardia Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm 1</td>
<td>Holstein</td>
<td>Dairy</td>
<td>&lt; 1 week</td>
<td>37.5% (15/40)</td>
</tr>
<tr>
<td>n = 40</td>
<td></td>
<td></td>
<td></td>
<td>22.5-52.5 CI</td>
</tr>
<tr>
<td>Farm 2</td>
<td>Charolais/ Friesian Cross</td>
<td>Dairy</td>
<td>&lt;42 days</td>
<td>2% (1/51)</td>
</tr>
<tr>
<td>n = 51</td>
<td></td>
<td></td>
<td></td>
<td>0-5.8 CI</td>
</tr>
<tr>
<td>Farm 3</td>
<td>Friesian</td>
<td>Beef</td>
<td>&lt;4 months</td>
<td>7.5% (3/40)</td>
</tr>
<tr>
<td>n = 40</td>
<td>Holstein + Angus</td>
<td></td>
<td></td>
<td>0-15.7 CI</td>
</tr>
<tr>
<td>Farm 4</td>
<td>Angus</td>
<td>Beef</td>
<td>&lt;4 months</td>
<td>18.4 (9/49)</td>
</tr>
<tr>
<td>n = 49</td>
<td></td>
<td></td>
<td></td>
<td>7.5-29.2 CI</td>
</tr>
<tr>
<td>Farm 5</td>
<td>Angus</td>
<td>Beef</td>
<td>&lt;4 months</td>
<td>23.1% (15/65)</td>
</tr>
<tr>
<td>n = 65</td>
<td></td>
<td></td>
<td></td>
<td>12.8-33.3 CI</td>
</tr>
<tr>
<td>Farm 6</td>
<td>Angus</td>
<td>Beef</td>
<td>&lt;4 months</td>
<td>31.9 (38/119)</td>
</tr>
<tr>
<td>NSW</td>
<td></td>
<td></td>
<td></td>
<td>23.6-34.3 CI</td>
</tr>
<tr>
<td>n = 119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>22.3 (81/364)</strong></td>
</tr>
<tr>
<td><strong>n = 364</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>18.0-26.5 CI</strong></td>
</tr>
</tbody>
</table>
Table 2: *Cryptosporidium* genotypes in pre-weaned cattle in five farms in Western Australia and one farm in NSW.

<table>
<thead>
<tr>
<th>Farm</th>
<th>C. parvum 18S</th>
<th>C. bovis 18S</th>
<th>C. ryanae 18S</th>
<th>Pig II 18S</th>
<th>ND 18S</th>
<th>Additional C. parvum positives at diagnostic locus</th>
<th>gp60 subtype of C. parvum isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm 1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>5</td>
<td>IIa-A20G3R1</td>
</tr>
<tr>
<td>Farm 2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>IIa-A18G3R1</td>
</tr>
<tr>
<td>Farm 3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Farm 4</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Farm 5</td>
<td>0</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>IIa-A17G2R1</td>
</tr>
<tr>
<td>Farm 6 NSW</td>
<td>10*</td>
<td>12</td>
<td>10*</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>IIa-A17G2R1</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>28</td>
<td>16</td>
<td>1</td>
<td>11</td>
<td>12</td>
<td>24</td>
</tr>
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</table>

*1 mixed C. ryanae/C. parvum infection
ND = not determined
Table 3. *Giardia* assemblages/genotypes in pre-weaned cattle in five farms in Western Australia and one farm in NSW.

<table>
<thead>
<tr>
<th>Farms</th>
<th>18S</th>
<th></th>
<th></th>
<th>Quenda genotype</th>
<th></th>
<th></th>
<th></th>
<th>gdh</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>E</td>
<td>B</td>
<td></td>
<td>A</td>
<td>E</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Farm 1</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>1 B4</td>
<td></td>
</tr>
<tr>
<td>Farm 2</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Farm 3</td>
<td>0</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Farm 4</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>1 B4</td>
<td></td>
</tr>
<tr>
<td>Farm 5</td>
<td>2</td>
<td>10*</td>
<td>2*</td>
<td>0</td>
<td>2 A2</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Farm 6</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
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</tr>
<tr>
<td>NSW</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>71</td>
<td>1</td>
<td>1</td>
<td>2 A2</td>
<td>35</td>
<td>2 B4</td>
<td></td>
</tr>
</tbody>
</table>

*2 mixed infections; one with assemblage A and E, one with assemblages A, B and E.*