Molecular characterization of Cryptosporidium spp. in pre-weaned calves in Shaanxi Province, north-western China

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Cryptosporidium, a worldwide protozoan parasite, is one of the most common causes of diarrhoea in humans and animals. The aim of the present study was to determine Cryptosporidium species/ genotypes in pre-weaned calves in Shaanxi Province using PCR and sequencing based on the small subunit rRNA gene. A total of 258 faecal samples were collected from pre-weaned calves in 19 different farms from six areas in Shaanxi Province, north-western China. Cryptosporidium infection was detected in 14 of 19 farms (73.7%), with a total prevalence of 20.2% (52/258). Both dairy and Oinchuan (beef) cattle were found with Cryptosporidium infection. Three Cryptosporidium species, namely Cryptosporidium bovis (n=26), Cryptosporidium andersoni (n=14) and Cryptosporidium ryanae (n=12), were detected in pre-weaned calves in Shaanxi Province, with C. bovis (in 12 farms) identified as the most common species on cattle farms. Two additional and previously unknown C. ryanae genotypes, CRTypes III and IV, were observed in the present study. However, the zoonotic species, Cryptosporidium parvum, was not detected in this study, which suggested a low zoonotic potential in Cryptosporidium-infected pre-weaned calves in this province.

INTRODUCTION

Cryptosporidium, an apicomplexan parasite of public health and veterinary importance, has been observed in the gastrointestinal tract of many, mostly healthy, animals in all five classes of vertebrates (Tzipori & Widmer, 2008; Petry et al., 2010; Bouzid et al., 2013). Cryptosporidiosis, caused by Cryptosporidium spp., is commonly associated with enteritis characterized by acute, watery or steatorrhoeic diarrhoea and colic. Death can result in severe cases with immunocompromised hosts, such as HIV/AIDS patients and neonatus (Jex et al., 2008; Tzipori & Widmer, 2008; Petry et al., 2010). Asymptomatic infection, or self-limiting or chronic disease can occur in immunocompetent individuals. However, there is no efficacious treatment or preventive intervention available for the control of cryptosporidiosis (Jex et al., 2008; Petry et al., 2010).

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Abbreviations: CBType, C. bovis type; CRType, C. ryanae type; MLST, multi-locus sequence typing; SSU, small subunit.

The GenBank/EMBL/DDBJ accession number for the small subunit rRNA gene sequences of Cryptosporidium spp. in this study is KJ020900–KJ020910.

Previous studies around the world have identified cattle as a common animal host of Cryptosporidium (Xiao & Feng, 2008; Xiao, 2010; Imre et al., 2011). Cryptosporidium infections frequently cause diarrhoea and malnutrition, which can result in increased/high mortality of calves (de Graaf et al., 1999). Bovine cryptosporidiosis is of great concern, due to the huge number of cattle farmed and their economic significance (Zhang et al., 2013); cattle are considered an important source of zoonotic cryptosporidiosis and is regarded as a major host of Cryptosporidium parvum (Feng et al., 2007). Humans can be infected from vegetables or other food contaminated by cattle manure (Millard et al., 1994; Quiroz et al., 2000; Blackburn et al., 2006). Such contamination may lead to food- or water-borne outbreaks (de Graaf et al., 1999; Glaberman et al., 2002; Zhang et al., 2013).

Four species, namely C. parvum, Cryptosporidium andersoni, Cryptosporidium bovis and Cryptosporidium ryanae, are commonly found in cattle, and their occurrence is usually related to the age and geographical origins of the host (Xiao, 2010). In the USA, C. parvum is mostly found in pre-weaned dairy calves, C. bovis and C. ryanae in weaned calves and C. andersoni in yearlings and adult cattle (Fayer et al., 2006, 2007; Santin et al., 2008). In China, C. andersoni was found...
in cattle of all age groups, and was the predominant species in post-weaned and adult dairy cattle in Henan Province (Wang et al., 2011a, b). C. parvum, C. bovis and C. ryanae were observed in pre-weaned dairy calves in Heilongjiang and Henan provinces (Wang et al., 2011b; Zhang et al., 2013). Cryptosporidium meleagris, an important zoonotic species that inhabits birds, was also detected in pre-weaned dairy calves in Heilongjiang Province, north-eastern China (Zhang et al., 2013). Additionally, Cryptosporidium hominis and Cryptosporidium serpents were also found in dairy cattle in some provinces of eastern China (Chen & Huang, 2012; Chen & Qiu, 2012).

Shaanxi Province in north-western China is an important cattle producer with about two million dairy cattle (Meng, 2013) and is also home to the native QinChuan beef cattle, one of five native beef breeds in China. It originates from the Guanzhong plain in Shaanxi Province and is now reared in more than 20 provinces (Lin et al., 2012). To date, a single molecular study has been carried out in cattle in this province, and, which exclusively identified the species C. andersoni (Zhao et al., 2013). To assess the public health significance of pre-weaned calf cryptosporidiosis, the infection rate was assessed by PCR detection of the small subunit rRNA (SSU rRNA) gene, which was then sequenced to determine the infecting species.

METHODS

Ethics statement. This study was carried out in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China, and our protocol was reviewed and approved by the Animal Research Ethics Committee of North-west A&F University and local government. Faecal sampling was permitted by farm owners and the animal husbandry bureau in each location. Field studies did not involve any endangered or protected species.

Faecal specimen collection and detection. A total of 258 faecal specimens of pre-weaned calves were randomly collected from 19 cattle farms in six areas (Baoji, Hanzhong, Xianyang, Tongchuan, Weinan and Yangling) in Shaanxi Province, north-western China, between April 2012 and December 2013. Samples were taken directly from the rectum of each live calf using sterile disposable gloves and placed in a clean plastic bag. All specimens were stored in 2.5% potassium dichromate in tubes at 4 °C for further study. Each faecal sample was then examined by Sheather’s sugar flotation technique and microscopy at ×400 magnification (McNabb et al., 1985). However, due to the small amount of faeces and many fat droplets in samples, we decided to investigate Cryptosporidium infection by direct DNA isolation and PCR sequencing for each faecal sample.

DNA extraction. The specimens were washed with distilled H2O to remove potassium dichromate solution prior to DNA extraction. Genomic DNA was extracted from 200 mg of each faecal sample using the E.Z.N.A. Stool DNA kit (OMEGA) according to the manufacturer’s protocol and stored at −20 °C before PCR analysis.

Determination of Cryptosporidium infection and species. To screen samples for Cryptosporidium infection, nested PCR was performed using previously described primers (Xiao et al., 1999) that targeted an 830 bp segment of the SSU rRNA gene. A 25 µl reaction mixture was used in both primary and secondary reactions, which contained 1× PCR buffer, 2 mM MgCl2, 0.2 mM deoxy-nucleoside triphosphates, 0.5 μM of each primer, 1 μl of 10 mg BSA ml−1 and 0.25 μl Taq DNA polymerase (TaqRa). One microlitre of DNA template was used in the primary reaction mixture and 1 μl of the primary PCR product was used as the template in the secondary reaction mixture. Primary amplification was carried out at 35 cycles of 94 °C for 45s, 55 °C for 45 s and 72 °C for 1 min, with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 10 min. The PCR conditions for secondary amplification were the same except the annealing temperature was 58 °C. Amplification products were examined by 1.5% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The positive PCR amplicons were sent to Shanghai Sangon Biological Engineering Biotechnology Company for direct sequencing using ABI 377 automated DNA sequencer (BigDye Terminator Chemistry) to identify the species/genotype. The sequences obtained were aligned with reference sequences in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov) and computer program CLUSTAL_X 1.83 (Thompson et al., 1997). The representative nucleotide sequences obtained in the present study were deposited in the GenBank database under the following accession numbers: KJ020900–KJ020910.

RESULTS

Prevalence of Cryptosporidium infection in pre-weaned calves

Of the 258 faecal samples examined, 52 were positive for Cryptosporidium infection, with a total prevalence of 20.2%. Fourteen of the 19 farms (73.7%) were positive for Cryptosporidium infection, with the infection rate ranging from 0 to 100% (Table 1). Both dairy (24.7%) and native beef cattle (Qinchuan cattle, 8.3%) were found to have Cryptosporidium infection, but the prevalence in dairy calves was higher than that in Qinchuan calves (Table 2).

Distribution and percentage of Cryptosporidium species

DNA sequencing of the SSU rRNA gene confirmed the presence of three Cryptosporidium species, with 26.9% (14/52) C. andersoni-positive samples on six farms, 50.0% (26/52) C. bovis on 12 farms and 23.1% (12/52) C. ryanae on seven farms (Table 3). There were one, two and three Cryptosporidium species detected in five, seven and two farms, respectively (Table 1). All three Cryptosporidium species were detected in both dairy and beef calves, with C. bovis being the most common in both breeds and most widely distributed species in pre-weaned calves in the areas investigated (Table 2).

Genetic variability of DNA sequences for Cryptosporidium species at the SSU rRNA locus

Since subtypes of C. andersoni in cattle in Shaanxi Province have previously been genotyped using multi-locus sequence typing (MLST) by our group (Zhao et al., 2013), the genetic variability in C. bovis and C. ryanae was further studied.
Table 1. Infection rate and distribution of Cryptosporidium spp. in pre-weaned calves in Shaanxi Province, China, by sequence analysis of the SSU rRNA gene

<table>
<thead>
<tr>
<th>Area</th>
<th>Farm</th>
<th>No. positive (no. examined)</th>
<th>Cryptosporidium spp. (no.)</th>
<th>Genotype of C. ryanae (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baoji</td>
<td>Farm 1</td>
<td>2 (6)</td>
<td>C. bovis (2)</td>
<td>CRType I (1)</td>
</tr>
<tr>
<td></td>
<td>Farm 2</td>
<td>2 (2)</td>
<td>C. andersoni (1), C. ryanae (1)</td>
<td>CRType I (1)</td>
</tr>
<tr>
<td></td>
<td>Farm 3</td>
<td>3 (3)</td>
<td>C. bovis (2), C. ryanae (1)</td>
<td>CRType I (1)</td>
</tr>
<tr>
<td></td>
<td>Farm 4</td>
<td>2 (3)</td>
<td>C. andersoni (1), C. ryanae (1)</td>
<td>CRType I (1)</td>
</tr>
<tr>
<td></td>
<td>Farm 5</td>
<td>0 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hanzhong</td>
<td>Farm 6</td>
<td>0 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongchuan</td>
<td>Farm 7</td>
<td>5 (16)</td>
<td>C. andersoni (1), C. bovis (1), C. ryanae (3)</td>
<td>CRType I (2), CRType IV (1)</td>
</tr>
<tr>
<td></td>
<td>Farm 8</td>
<td>1 (8)</td>
<td>C. bovis (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Farm 9</td>
<td>0 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Farm 10</td>
<td>4 (8)</td>
<td>C. bovis (3), C. ryanae (1)</td>
<td>CRType II (1)</td>
</tr>
<tr>
<td>Xianyang</td>
<td>Farm 11</td>
<td>6 (12)</td>
<td>C. andersoni (5), C. bovis (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Farm 12</td>
<td>7 (21)</td>
<td>C. andersoni (5), C. bovis (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Farm 13</td>
<td>0 (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weinan</td>
<td>Farm 15</td>
<td>3 (15)</td>
<td>C. bovis (3)</td>
<td></td>
</tr>
<tr>
<td>Yangling</td>
<td>Farm 16</td>
<td>5 (18)</td>
<td>C. bovis (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Farm 17</td>
<td>5 (56)</td>
<td>C. andersoni (1), C. bovis (3), C. ryanae (1)</td>
<td>CRType I (1)</td>
</tr>
<tr>
<td></td>
<td>Farm 18</td>
<td>6 (8)</td>
<td>C. bovis (2), C. ryanae (4)</td>
<td>CRType I (4)</td>
</tr>
<tr>
<td></td>
<td>Farm 19</td>
<td>1 (16)</td>
<td>C. bovis (1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>52 (242)</td>
<td>C. andersoni (14), C. bovis (26), C. ryanae (12)</td>
<td>CRType I (9), CRType II (1), CRType III (1), CRType IV (1)</td>
</tr>
</tbody>
</table>

All 26 C. bovis isolates had 100% homogeneity with each other at the SSU rRNA locus and were identical to bovine samples in Brazil (EF493331; Thomaz et al., 2007), the USA (EU203217; Feltus et al., 2008), Iran (AB441689; Keshavarz et al., 2009), India (GQ345005; Khan et al., 2010), Poland (KC618594; Rzezutka & Kaupke, 2013) and Egypt (AB777172; Amer et al., 2013). C. bovis type I (CBType I) was the only type found in this study and in Henan Province (Wang et al., 2011b).

However, intra-species variations were observed for C. ryanae at the SSU rRNA locus. One mutation (A:T) and two indels (A/- and A/-) existed in the obtained sequences, which formed four distinct haplotypes, namely C. ryanae types (CRTypes) I–IV (Tables 1 and 4). CRTypes I (75.0%, 9/12 samples) and II (8.3%, 1/12 samples) had 100% similarity with the SSU rRNA gene sequences of cattle-derived isolates, e.g. AB777174–76, KC582857, JX198275, AB628203, JN400800, JX416367, HQ009807, HQ822137, GQ345007, EU203216, DQ871345, and KC582860, KC582855 and AB628202. CRType I was the most prevalent type in C. ryanae-positive specimens and was reported in Heilongjiang and Henan provinces (Wang et al., 2011b; Zhang et al., 2013). CRTypes III (8.3%, 1/12 samples) and IV (8.3%, 1/12 samples), with one and two base variations compared with CRTypes I and II, respectively, have not been described previously (Table 4). All four C. ryanae types were found in dairy cattle, whereas a single isolate identified in a beef calf belonged to CRType I (Table 2).

**DISCUSSION**

The prevalence of bovine cryptosporidiosis varies between and within countries in the world. Cryptosporidium infection has been found in over 90 countries and six continents, including China (Chen & Huang, 2007; Chen et al., 2007). In the present study, Cryptosporidium infection and species were determined using a molecular method for pre-weaned calves in Shaanxi Province, north-western China.

Table 2. Infection rate and percentage of different Cryptosporidium spp. in different breeds of pre-weaned calf in Shaanxi Province, China

<table>
<thead>
<tr>
<th>Breed</th>
<th>No. positive/no. examined (%)</th>
<th>Cryptosporidium spp. (no.)</th>
<th>Genotype of C. ryanae (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qinchuan calves</td>
<td>6/72 (8.33)</td>
<td>C. andersoni (1), C. bovis (4), C. ryanae (1)</td>
<td>CRType I (1)</td>
</tr>
<tr>
<td>Dairy calves</td>
<td>46/186 (24.73)</td>
<td>C. andersoni (13), C. bovis (22), C. ryanae (11)</td>
<td>CRType I (8), CRType II (1), CRType III (1), CRType IV (1)</td>
</tr>
</tbody>
</table>
China. *Cryptosporidium* infection was detected in five of six areas examined and 14 out of 19 (73.7 %) cattle farms. The results indicated that *Cryptosporidium* is a common parasite in pre-weaned calves in Shaanxi Province. The prevalence of *Cryptosporidium* infection was 20.2 % in pre-weaned calves, which was lower than that in pre-weaned calves in Henan and Heilongjiang provinces (Wang et al., 2011b; Zhang et al., 2013). The reason for the differences observed may be related to geographical, ecological and climatic differences. It may also have been caused by the long-term storage (>3 months) of faeces in potassium dichromate. Potassium dichromate can internalize and intercalate with the *Cryptosporidium* oocyst cell wall, thereby causing problems with downstream molecular analysis and leading to potential under-reporting of results.

Species determination is essential for evaluation of the zoonotic source of cryptosporidiosis and control of this disease. Four *Cryptosporidium* species, namely *C. bovis*, *C. andersoni*, *C. parvum* and *C. ryanae*, have been identified as the most common species in cattle in different countries, including China (Geurden et al., 2006, 2007; Starkey et al., 2006; Coklin et al., 2007; Feng et al., 2007; Langkjaer et al., 2007; Plutzer & Karanis, 2007; Thomaz et al., 2007; Thompson et al., 2007; Halim et al., 2008; Szyonyi et al., 2008; Brook et al., 2009; Chen & Huang, 2012), with *C. andersoni* reported as the most frequent species in yearlings and adult cattle. *C. ryanae* and *C. bovis* have been identified as the dominant species in cattle older than 6 months, and *C. parvum* as the species most common in pre-weaned dairy calves in the USA (Fayer et al., 2006, 2007; Santin et al., 2008). However, the succession according to the age of dairy calves is different according to geographical area and calf management system (Fayer et al., 2007; Murakoshi et al., 2012). In the present study, *C. bovis*, *C. andersoni* and *C. ryanae* were observed in pre-weaned cattle in Shaanxi Province. *C. bovis* was the most prevalent species in this province, which is consistent with results from a study in Heilongjiang Province, north-eastern China (Zhang et al., 2013), but different from those from a study in Henan Province in which *C. parvum* was the predominant species in pre-weaned calves (Wang et al., 2011b). *C. parvum*, one of the most important *Cryptosporidium* species in regard to public health (Petty et al., 2010), is a common species in pre-weaned cattle around the world (Xiao, 2010; Murakoshi et al., 2012), including some provinces of China (Wang et al., 2011b; Zhang et al., 2013). However, this species was not detected in the present study. The absence of *C. parvum* has been shown in native breeds of cattle in developing countries (Maikai et al., 2011; Feng et al., 2012; Nguyen et al., 2012). However, in industrialized nations, beef calves raised under traditional animal husbandry do not usually have *C. parvum* (Feltus et al., 2008; Fayer et al., 2010; Murakoshi et al., 2012). Two reasons may explain the absence of *C. parvum* in this study. First, the ability of PCR sequencing to detect multi-species infections is low because of the exponential copying of DNA strands. Second, the non-detection of *C. parvum* in pre-weaned calves in Shaanxi Province suggests that this province may have low zoonotic potential for *Cryptosporidium* transmission to humans. PCR-RFLP may solve this issue, and gp60 analysis can target a low level of *C. parvum* infection in addition to a predominant *C. bovis*, *C. ryanae* or *C. andersoni* infection. In addition, cloning for PCR products may confirm and/or detect mixed infection. Therefore, further loci and works will be studied in future.

In 2013, our group published a paper identifying *C. andersoni* as the only species found in cattle in Shaanxi Province (Zhao et al., 2013), which is contrary to the findings of the present study. This controversy may be due to sample numbers and the detection procedure. All faecal samples in the present study were collected from cattle younger than 3 months of age, but only 14 samples from this age group were obtained in the earlier study.

### Table 3. Infection rate of different *Cryptosporidium* spp. on farms in Shaanxi Province, China

<table>
<thead>
<tr>
<th>Cryptosporidium spp.</th>
<th>No. of Cryptosporidium spp. (%)</th>
<th>No. of areas infected</th>
<th>Not contaminated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. andersoni</em></td>
<td>14 (26.92)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td><em>C. bovis</em></td>
<td>26 (50.00)</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td><em>C. ryanae</em></td>
<td>12 (23.08)</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

### Table 4. Intra-species variations in the SSU rRNA nucleotide sequences of *C. ryanae*

<table>
<thead>
<tr>
<th>Type</th>
<th>No. of isolates (%)</th>
<th>Nucleotide at position</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>57 58 475 476 665</td>
<td></td>
</tr>
<tr>
<td>Reference sequence</td>
<td>–</td>
<td>T – G T – A A</td>
<td>AB777176</td>
</tr>
<tr>
<td>CRType I</td>
<td>9 (75.00)</td>
<td>T – G T – A A</td>
<td>KJ020906</td>
</tr>
<tr>
<td>CRType II</td>
<td>1 (8.33)</td>
<td>T – G T A A A</td>
<td>KJ020908</td>
</tr>
<tr>
<td>CRType III</td>
<td>1 (8.33)</td>
<td>T – G T – A A</td>
<td>KJ020907</td>
</tr>
<tr>
<td>CRType IV</td>
<td>1 (8.33)</td>
<td>T A G T – A A</td>
<td>KJ020909</td>
</tr>
</tbody>
</table>
Additionally, in our previous study (Zhao et al., 2013), the faecal samples were first examined by microscopy and positive samples were used to determine Cryptosporidium species by molecular markers. However, microscopic observation can be affected by fat drops in faeces and low oocyst number. In the present study, PCR amplification and sequencing were used directly to detect the prevalence and species in all samples, which can overcome the limitations of micro-examination and improve detection accuracy.

Intra-species variation is common in Cryptosporidium spp. Four different types of C. bovis have been identified in cattle in Heilongjiang Province based on nucleotide differences in the SSU RNA gene, with CBTType I as the most prevalent type in this province. CBType I was also identified as the only type in our study and in Henan Province (Wang et al., 2011b), which indicated the prevalence of this type in Cryptosporidium infection. In the present study, four types of C. ryanae were observed. CRType I was found in both dairy and beef cattle, and has been reported as the only type in pre-weaned dairy cattle in Heilongjiang and Henan provinces (Wang et al., 2011b; Zhang et al., 2013). CRType II has been reported in dairy calves younger than 4 months in Poland and post-weaned beef cattle in Japan (Murakoshi et al., 2012; Rzeżutka & Kaupke, 2013). CRTypes III and IV are new types found only in pre-weaned dairy cattle in Shaanxi Province. To further ascertain the existence of these two new types in beef cattle, further faecal samples from beef cattle in this province, as well as from other regions of China, will be investigated in future studies.

CONCLUSIONS

Cryptosporidium infection was found in five of the six areas examined and in 14 of the 19 cattle farms investigated. Three Cryptosporidium species, namely C. bovis, C. andersoni and C. ryanae, were identified in both dairy and native beef calves, with C. bovis as the most prevalent species. Four types of C. ryanae were observed, with two new types found in dairy calves. However, the zoonotic species C. parvum was not present in samples from this study. These results may indicate a low zoonotic potential in Cryptosporidium-infected pre-weaned calves in this province, and may contribute to a better understanding of the epidemiology and control of cryptosporidiosis in China, as well as in the rest of the world.

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