Research paper

# Prevalence and multilocus genotyping of *Cryptosporidium andersoni* in dairy cattle and He cattle in Xinjiang, China

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## A B S T R A C T

*Cryptosporidium andersoni* is the predominant species in post-weaned and adult cattle in China. The aim of this study was to determine the prevalence and understand the transmission of cattle cryptosporidiosis in the Xinjiang Uyghur Autonomous Region, China, a total of 1827 fecal samples (436 from He cattle and 1391 from dairy cattle) were examined for the presence of *C. andersoni*-like oocysts by microscopy after Sheather's sugar flotation technique. The overall prevalence of *C. andersoni*-like was 3.8% (70/1827) and all the *C. andersoni*-like isolates were identified as *C. andersoni* at the SSU rRNA locus. Among the *C. andersoni* isolates, a total of 60 isolates were successfully characterized into eight multilocus sequence typing (MLST) subtypes using MLST analysis at the four microsatellite/minisatellite loci (MS1, MS2, MS3 and MS16), and three new subtypes were identified. The MLST subtype A4,A4,A4,A1 showed a predominance and a wide distribution among the eight MLST subtypes obtained in the investigated areas. The MLST subtypes A2,A4,A2,A1 and A4,A5,A2,A1 showed a unique distribution in the investigated areas. A linkage disequilibrium analysis showed the presence of an epidemic population genetic structure of *C. andersoni* isolated from dairy and He cattle in Xinjiang. These findings provide new insights into the genetic structure of *C. andersoni* isolates and are also helpful to explore the infection source of *C. andersoni* in cattle in Xinjiang, China.

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## 1. Introduction

*Cryptosporidium* spp. are important gastrointestinal parasites in both humans and animals worldwide, causing mild to severe diarrhea in immunocompromised and immunocompetent individuals (Xiao, 2010). Currently, 31 valid *Cryptosporidium* species have been recognized (Ryan et al., 2014; Kváč et al., 2016). Of these, *C. parvum*, *C. andersoni*, *Cr. bovis*, and *C. ryanae* are most commonly responsible for infections in cattle (Xiao, 2010). Previous studies have shown that *C. andersoni* is primarily a parasite of cattle, but has also been detected in other animals such as golden takins, Bactrian camels, yaks, sheep, goats, hamsters, giant pandas, lesser pandas, horses, and American minks (Lv et al., 2009; Wang et al., 2012; Stuart et al., 2013; Liu et al., 2014; Ma et al., 2014; Liu et al., 2015; Wang et al., 2015a, 2015b; Zhao et al., 2015). *Cryptosporidium andersoni* has also been isolated in outpatients with diarrhea in Jiangsu Province, China (Jiang et al., 2014). In China, *C. andersoni* has been identified as the predominant species responsible for cattle infection (Wang et al., 2011; Ma et al., 2015; Qi et al., 2015).

Multilocus sequence typing (MLST) is a high-resolution typing tool that can accurately describe the genetic diversity of parasites (Gatei et al., 2007). Based on length polymorphism and single nucleotide polymorphism (SNP), a MLST tool for the genetic characterization and population structure of *C. muris* and *C. andersoni* (two most common gastric species of *Cryptosporidium*) has been described by Feng et al. (2011). To date, 19 MLST subtypes of *C. andersoni* have been identified in different hosts using this tool (Feng et al., 2011; Wang et al., 2012; Zhao et al., 2013; Zhao et al., 2014). Recent studies subtyped *C. andersoni* isolates from cattle using MLST and showed that *C. andersoni* MLST subtypes in China (A4,A4,A4,A1, A1,A4,A4,A1, A2,A4,A4,A1, A2,A4,A2,A1, A5,A4,A4,A1, A4,A4,A2,A1, A3,A4,A4,A1, A1,A2,A4,A1, A2,A1,A2,A1, A2,A1,A3,A1) (Wang et al., 2012; Zhao et al., 2013; Zhao et al., 2014) differed from those found in cattle from the United States (A2,A3,A2,A1, A2,A3,A1,A1 and A2,A3,A4,A1), Canada (A2,A3,A4,A1), and Czech Republic (A2,A3,A4,A1 and A1,A3,A4,A1) (Feng et al., 2011).

Previous studies have shown that the *C. parvum* IId subtypes in dairy cattle in the Xinjiang Uyghur Autonomous Region (Central Asia) differ considerably from those in other areas in China (Qi et al., 2015). He cattle is a famous native beef cattle breed in Xinjiang, which originated and mainly distributed in Xinjiang. However, the introduction of dairy cattle in Xinjiang only occurred in the last 20 years and the main breed is Holstein cattle from Australia and New Zealand. Therefore, to determine

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the prevalence and understand the transmission of cattle cryptosporidiosis, as well as compare the population genetics of *C. andersoni*, fecal samples from dairy and He cattle from Xinjiang were collected, and the population genetic structure was characterized using MLST.

## 2. Materials and methods

### 2.1. Ethics statement

This study was performed according to the recommendations of the Chinese Laboratory Animal Administration Act of 1988. The research protocol was reviewed and approved by the Research Ethics Committee of Henan Agricultural University. Prior to fecal samples collection, permission was obtained from the animal owners when possible. The field studies did not involve endangered or protected species.

### 2.2. Study area and sample collection

From August to September 2013, a total of 1827 fecal samples (1391 from dairy cattle and 436 from He cattle) were randomly collected from 21 intensively reared dairy cattle farms and 13 grazing He cattle herds in the cities of or near Wujiaqu, Changji, Urumqi, Kuerle, Yumin, Tacheng, Altay, Zhaosu, and Aksu in the Xinjiang Uyghur Autonomous Region of northwest China (73°40′E–96°18′E, 34°25′N–48°10′N; hereafter referred to as Xinjiang) (Tables 1 and 2) (Fig. 1). Dairy cattle were divided by age into pre-weaned (0–60 days) and post-weaned (61–180 days) calves, heifers (181–450 days) and adult cattle (>450 days). Fecal samples were collected directly from the rectum using disposable gloves and plastic containers. All grazing He cattle were adult and kept in pastures, and fresh feces were collected from the ground if the animal was observed to defecate, with care taken to avoid environmental contamination by sampling only those portions of fecal material that had not been in contact with the ground. The samples were examined using Sheather's sugar flotation technique and microscopy at ×400 magnification. All fecal samples positive for *C. andersoni*-like oocysts by microscopy were maintained in 2.5% potassium dichromate solution at 4 °C for subsequent molecular characterization.

### 2.3. DNA extraction and PCR amplification

Approximately 1 g of fecal material from each positive *C. andersoni*-like sample by microscopy was washed three times with distilled water and then centrifuged at 3000 ×g for 10 min to remove potassium dichromate. Genomic DNA was extracted from approximately 200 mg of each fecal sample using the E.Z.N.A.R® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA) according to the manufacturer's instructions. Extracted DNA was stored at −20 °C until analysis.

*Cryptosporidium* species were determined by nested PCR amplification of the small subunit ribosomal RNA (SSU rRNA) locus from each sample as described previously (Xiao et al., 1999), with the inclusion of positive and negative controls in each run. Then, DNA from all *C. andersoni* samples that demonstrated positivity at the SSU rRNA locus was subjected to further PCR analysis of four minisatellite/microsatellite targets, including MS1 (encoding a hypothetical protein), MS2 (encoding a 90-kDa heat shock protein), MS3 (encoding a hypothetical protein), and MS16 (encoding a leucine-rich repeat family protein), according to the previously described nested PCR protocols (Feng et al., 2011; Wang et al., 2012). Each sample was analyzed twice using 2 μL of extracted DNA per PCR performed. KOD-Plus amplification enzyme (Toyobo Co. Ltd, Osaka, Japan) was used for PCR amplification. The secondary PCR products were examined by agarose gel electrophoresis and visualized after GelRed™ staining (Biotium Inc., Hayward, CA, USA).

### 2.4. Sequence analysis

All secondary PCR amplicons were bi-directionally sequenced on an ABI PRISM™ 3730 XL DNA Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were identified by alignment with reference sequences downloaded from GenBank (http://www.ncbi.nlm.nih.gov) using the MEGA 5 software program (http://www.megasoftware.net/). The *C. andersoni* subtypes were named according to the repeat characteristics of microsatellite/minisatellite repeats in four genetic loci by Feng et al. (2011). Nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers KU961850–KU961858.

**Table 1**
The prevalence of *C. andersoni* in dairy cattle and the subtype identity at the four selected microsatellite and minisatellite loci.

| Farm | Positive no./examined no. (%) | Subtyped at four loci (Amplified no.)[a] | | | | MLST subtypes (n)[b] |
|---|---|---|---|---|---|---|
| | | MS1 | MS2 | MS3 | MS4 | |
| WujiaquA | 3/87 (3.4) | A4 (3) | A4 (3) | A4 (3) | A1 (3) | A4, A4, A4, A1 (3) |
| WujiaquB | 0/81 | | | | | |
| WujiaquC | 1/18 (5.6) | A4 (1) | A4 (1) | A4 (1) | A1 (1) | A4, A4, A4, A1 (1) |
| WujiaquD | 0/20 | | | | | |
| Changji | 4/54 (7.4) | A2 (4) | A4 (3) | A2 (3) | A1 (3) | A2, A4, A2, A1 (3) |
| UrumqiA | 0/24 | | | | | |
| UrumqiB | 1/51 (2.0) | A4 (1) | A4 (1) | A4 (1) | | |
| UrumqiC | 1/59 (1.7) | A4 (1) | A4 (1) | A4 (1) | A1 (1) | A4, A4, A4, A1 (1) |
| KorlaA | 0/35 | | | | | |
| KorlaB | 0/44 | | | | | |
| KorlaC | 1/19 (5.3) | A4 (1) | A5 (1) | A4 (1) | A1 (1) | A4, A5, A4, A1 (1) |
| KorlaD | 2/34 (5.9) | A5 (2) | A4 (2) | A4 (2) | A1 (2) | A5, A4, A4, A1 (2) |
| KorlaE | 5/79 (6.3) | A4 (5) | A4 (4) | A4 (4) | A1 (4) | A4, A4, A4, A1 (4) |
| KorlaF | 0/124 | | | | | |
| Tacheng | 3/29 (10.3) | A4 (2) | A2 (1), A4 (2) | A4 (2) | A1 (3) | A4, A4, A4, A1 (2) |
| ZhaosuA | 0/8 | | | | | |
| ZhaosuB | 1/35 (2.9) | A4 (1) | A4 (1) | A2 (1) | A1 (1) | A4, A4, A2, A1 (1) |
| AksuA | 14/106 (13.2) | A2 (11), A7 (2) | A4 (12), A5 (1) | A2 (12), A4 (1) | A1 (11) | A2, A4, A2, A1 (8); A2, A4, A4, A1 (1); A1, A4, A2, A1 (2) |
| AksuB | 16/127 (12.6) | A2 (1), A4 (15) | A4 (1), A5 (13) | A2 (14) | A1 (16) | A2, A4, A2, A1 (1); A4, A5, A2, A1 (13) |
| AksuC | 5/177 (2.8) | A2 (2), A4 (2) | A4 (4) | A2 (2), A4 (2) | A1 (4) | A2, A4, A2, A1 (2); A4, A4, A4, A1 (2) |
| AksuD | 3/180 (1.7) | A2 (3) | A4 (3) | A2 (3) | A1 (3) | A2, A4, A2, A1 (3) |
| Total | 60/1391 (4.3) | A1 (2), A2 (26), A4 (27), A5 (2) | A2 (1), A4 (38), A5 (15), | A2 (35), A4 (18), | A1 (53) | A1, A4, A2, A1 (2); A2, A4, A2, A1 (17); A2, A4, A4, A1 (1); A4, A4, A2, A1 (1); A4, A4, A4, A1 (13); A4, A5, A2, A1 (13); A4, A5, A4, A1 (1); A5, A4, A4, A1 (2); |

[a] Amplified no. indicates the number of *C. andersoni* isolates that were successfully analyzed by PCR at each of the four loci (MS1, MS2, MS3 and MS16), and primers were used as Feng et al. (2011) described previously.
[b] Haplotypes are arranged in accordance with the order of the loci amplified, MS1, MS2, MS3 and MS16.