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Research paper

Prevalence and molecular characterization of *Cryptosporidium* spp. and *Giardia duodenalis* in dairy cattle in Beijing, China

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ABSTRACT

822 fecal samples from cattle in six areas of Beijing were examined with microscopy for *Cryptosporidium* oocysts and *Giardia* cysts. The overall infection rates for *Cryptosporidium* spp. and *Giardia duodenalis* were 2.55% and 1.09%, respectively. *Cryptosporidium* was only detected in calves and heifers, whereas *G. duodenalis* was found in all age groups. *Cryptosporidium* spp. were characterized with a PCR-restriction fragment length polymorphism analysis and DNA sequence analysis of the small subunit (SSU) rRNA gene. Two *Cryptosporidium* species were identified: *Cryptosporidium parvum* ($n=12$) and *Cryptosporidium andersoni* ($n=9$). Six *C. parvum* isolates were successfully subtyped with the *gp60* gene and three subtypes were detected: IIdA19G1 ($n=1$), IIdA17G1 ($n=1$), and IIdA15G1 ($n=4$). Subtype IIdA17G1 is reported for the first time in cattle worldwide. Nine *G. duodenalis* isolates were analyzed by sequencing the triosephosphate isomerase (*tpi*) gene, and only *G. duodenalis* assemblage E was identified. Therefore, the predominance of *C. parvum* detected in calves was identical to that found in the Xinjiang Uyghur and Ningxia Hui Autonomous Regions, but differed considerably from that in Henan, Heilongjiang, and Shannxi Provinces. In contrast, the predominance of *G. duodenalis* assemblage E was more or less similar to its predominance in other areas of China or countries. Our findings confirm the unique character of the *C. parvum* IId subtypes in China. More systematic studies are required to better understand the transmission of *Cryptosporidium* and *G. duodenalis* in cattle in China.

1. Introduction

Cryptosporidium and *Giardia* are important gastrointestinal protists that can infect humans, livestock, companion animals, and wildlife. Infection occurs by the fecal–oral route after the ingestion of infective oocysts or cysts, by either direct contact or the ingestion of contaminated food or water (Feng and Xiao, 2011).

Cattle are commonly infected with *Cryptosporidium*, and preweaned calves are considered the most important reservoir for zoonotic infections (Wang et al., 2011b). Many studies have suggested that *Cryptosporidium parvum*, *Corynebacterium bovis*, *Cryptosporidium andersoni*, and *Cryptosporidium ryanae* are the most common species infecting cattle, although *Ctenocephalides felis*, *Cryptosporidium hominis*, *Chlamydia suis*, *Cryptosporidium scrofarum*, and *C. suis*-like genotype have also been detected (Trout and Santín, 2008). The four common *Cryptosporidium* species have age-associated distributions. *C. parvum* is usually found in preweaned calves and is a significant cause of diarrhea. However, *C. bovis* and *C. ryanae* usually infect post-weaned calves and yearlings, and *C. bovis* is detected more frequently than *C. ryanae*, although neither is associated with diarrhea (Santín et al., 2008). In contrast, *C. andersoni* is commonly seen in adult cattle and is associated with gastritis, reduced milk yield, and poor weight gain (Esteban and Anderson, 1995).

Giardia duodenalis is a species complex comprising eight distinct 'assemblages' or genotypes, A–H (Feng and Xiao, 2011), which can infect humans and most other mammals (Adam, 2001). Most of the assemblages (C–H) seem to be host specific for nonhuman species: assemblages C and D are specific for dogs, E for hoofed livestock, F for cats, G for rats, and H for seals. In cattle, the prevalence of *G. duodenalis* ranges from 2.2% to 50.7% worldwide, and assemblages A, B, and E have been detected, with assemblage E the predominant

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genotype in most countries (Xiao and Fayer, 2008; Feng and Xiao, 2011).

In China, *Cryptosporidium* and *Giardia* infections in cattle have been found in several areas, and differences in the *Cryptosporidium* distributions have been noted in different regions (Liu et al., 2009, 2012; Wang et al., 2011a,b; Zhang et al., 2013, 2015; Cui et al., 2014; Huang et al., 2014; Ma et al., 2015; Qi et al., 2015a,b). For example, *C. bovis* rather than *C. parvum* is the predominant species in preweaned calves in Henan (Wang et al., 2011b), Shaanxi (Qi et al., 2015b), Shandong (Ma et al., 2015), Gansu, and the Ningxia Hui Autonomous Region (Zhang et al., 2015), whereas *C. andersoni* is the most common species in cattle in Heilongjiang Province (Liu et al., 2009). Thus, the distributions of *Cryptosporidium* spp. and the *C. parvum* subtypes (IIdA19G1 and IIdA15G1) in dairy cattle differ more or less from those in other countries (Wang et al., 2011b; Liu et al., 2012; Huang et al., 2014; Zhang et al., 2015). In contrast, there have a limited number of molecular epidemiological studies of *G. duodenalis* in cattle (Liu et al., 2012, 2015a; Wang et al., 2014). The objective of this study was to identify the species of *Cryptosporidium* and *Giardia* present in dairy cattle in Beijing (the capital of the People's Republic of China, and China's political, economic, and cultural center), to assess the zoonotic potential of *Cryptosporidium* and *G. duodenalis* genotypes/subtypes for humans in this area.

2. Materials and methods

2.1. Sample collection and examination

A fresh fecal sample was collected from each animal using a sterile disposal latex glove immediately after its defecation onto the ground, and placed individually into a disposable plastic bag, with the age of each animal being recorded. In total, 822 fecal samples were collected between January 2014 and December 2015 from 12 dairy cattle farms in Beijing, China (Table 1). The *Cryptosporidium* oocysts in the 25 g fecal materials were concentrated with Sheather's sugar flotation technique, with a further formalin-ethyl acetate sedimentation step included for the samples from preweaned calves (Wang et al., 2011b). *Giardia* cysts were detected with Lugol's iodine staining. The *Cryptosporidium*- or *Giardia*-positive fecal samples were stored in 2.5% potassium dichromate at 4 °C until DNA extraction.

2.2. DNA extraction

The 100 mg of *Cryptosporidium*- or *Giardia*-positive fecal samples were washed three times with distilled water, and the genomic DNA was extracted from the fecal pellets with the E.Z.N.A.® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA), according to the manufacturer's recommendations.

2.3. Cryptosporidium/Giardia *genotyping and subtyping*

The *Cryptosporidium* species were identified with a PCR-restriction fragment length polymorphism (RFLP) analysis and DNA sequence analysis of the small subunit (SSU) rRNA gene (Feng et al., 2007). *Cryptosporidium parvum* was subtyped with nested PCR targeting the *gp60* gene, and the previously established nomenclature was used to name the *C. parvum* subtype families and subtypes (Sulaiman et al., 2005; Xiao, 2010). *G. duodenalis* was identified by sequencing the triosephosphate isomerase (*tpi*) gene (Caccio et al., 2008), and the genotype/subtype identities of the *G. duodenalis* samples were established by direct comparison of the sequences with reference sequences downloaded from the GenBank database.

2.4. DNA sequence analysis

The PCR products were sequenced on an ABI Prism™ 3730 XL DNA Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). Sequence accuracy was confirmed with two-directional sequencing and by sequencing a new PCR product if necessary. The sequences were aligned with the ClustalX 1.83 program. Representative nucleotide sequences have been deposited in GenBank under accession numbers KT964796–KT964801.

2.5. Statistical analysis

The χ^2 test was used to compare the *Cryptosporidium* infection rates. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Prevalence of Cryptosporidium and Giardia

Cryptosporidium oocysts were found by microscopic analysis in 21 samples (2.55%) from six farms, and the highest infection rate was 12.5% on farm 5 (Table 1). The infection rates of *Cryptosporidium* spp. were 3.47%, 3.24%, and 0% in calves, heifers, and adult cattle, respectively ($\chi^2 = 7.05$, $0.01 < P < 0.05$). Fourteen samples from four farms were positive for *Giardia*, with an average infection rate of 1.09% (Table 1). The highest infection rate for *Giardia* was 4.38% on farm 4. The infection rates for *Giardia* were 2.72%, 0.93%, and 0.50% in calves, heifers, and adult cattle, respectively ($\chi^2 = 5.05$, $P > 0.05$).

3.2. Distribution of Cryptosporidium species/subtypes and G. duodenalis assemblage

The SSU rRNA genes of the *Cryptosporidium* spp. in all 21 microscopy-positive samples were successfully amplified with nested PCR. RFLP and DNA sequence analyses of the SSU rRNA gene fragments revealed the presence of two *Cryptosporidium* species: *C. parvum* ($n = 12$) on four farms and *C. andersoni* ($n = 9$) on two farms (Table 1). Only one *Cryptosporidium* species was detected on each of the *Cryptosporidium*-positive farms (Table 1). The six *C. parvum* isolates successfully subtyped with the *gp60* sequencing analysis belonged to subtypes IIdA19G1 ($n = 1$), IIdA17G1 ($n = 1$), and IIdA15G1 ($n = 4$).

The sequencing analyses of the *tpi* gene of *G. duodenalis* identified assemblage E ($n = 14$) on four farms (Table 1).

3.3. Age distributions of Cryptosporidium and G. duodenalis

C. parvum was the most commonly identified *Cryptosporidium* species in calves, whereas *C. andersoni* was the dominant *Cryptosporidium* species in heifers (Table 2). In contrast, no *Cryptosporidium*-positive sample was identified in adult cattle. *G. duodenalis* was detected in all age groups, but calves had the highest infection rate. Only *G. duodenalis* assemblage E was found in the different age groups (Table 2).

4. Discussion

The overall infection rate for *Cryptosporidium* spp. was 2.55%, which is lower than the rate of 9.68% (18/186) reported previously in Beijing (Jiang et al., 1989), and the rates reported in Henan (13.0%, 276/2116) (Wang et al., 2011a,b), Heilongjiang (15.0%, 99/658) (Liu et al., 2009; Zhang et al., 2013), Shannxi (3.4%, 70/2071) (Zhao et al., 2013), Anhui (14.9%, 52/350), Jiangsu (20.7%, 251/1215), Shanghai (12.5%, 55/440) (Chen and Huang, 2012), Gansu and Ningxia (5.09%,