Characteristics of Cryptosporidium Transmission in Preweaned Dairy Cattle in Henan, China

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To estimate the prevalence and public health significance of cryptosporidiosis in preweaned calves in China, 801 fecal samples from eight farms in seven areas in Henan Province were examined for Cryptosporidium oocysts. The overall infection rate of Cryptosporidium was 21.5%, with the farm in Xinxiang having the highest prevalence (40%). No significant difference in infection rates was observed between seasons. Cryptosporidium spp. were characterized by PCR-restriction fragment length polymorphism (RFLP) analysis of the small subunit (SSU) rRNA gene and DNA sequencing of the 60-kDa glycoprotein (gp60) gene. The SSU rRNA-based PCR identified four Cryptosporidium species, including Cryptosporidium parvum (54/172), C. bovis (65/172), C. ryanae (19/172), and C. andersoni (12/172), and the occurrence of infections with mixed species (22/172). The earliest detection of C. bovis was in calves of 1 week of age, showing that the prepatent period was shorter than the previously stated 10 to 12 days. Infections with C. parvum peaked in summer, whereas C. bovis dominated in autumn and winter. There was no apparent difference in the age of cattle infected with either C. parvum or C. bovis. Sequencing analysis of the gp60 gene showed all 67 C. parvum samples belonged to subtype IIdA19G1. These findings suggested that the transmission of Cryptosporidium spp. in preweaned calves in Henan, China, appeared to be different from other areas both at genotype and subtype levels. Further molecular epidemiologic studies (including samples from both calves and humans) are needed to elucidate the transmission dynamics and public significance of C. parvum in cattle in China.

Cryptosporidium spp. are important gastrointestinal agents in a wide spectrum of hosts, including humans, other mammals, birds, reptiles, amphibians, and fish. There are extensive genetic variations within the genus Cryptosporidium. In addition to more than 20 recognized species of Cryptosporidium, more than 60 Cryptosporidium genotypes with no designated species names have been described.

Cattle is the common mammalian species in which Cryptosporidium infection was detected, and preweaned calves are considered the most important reservoir for zoonotic infection. Thus far, seven Cryptosporidium species and two genotypes have been identified in cattle, including Cryptosporidium parvum, C. bovis, C. andersoni, C. ryanae, C. felis, C. hominis, C. suis, a C. suis-like genotype, and the Cryptosporidium pig genotype II (41). The former four species are mostly responsible for bovine cryptosporidiosis. Studies conducted in numerous industrialized nations suggest that there is an age-associated distribution of the four common Cryptosporidium spp. Thus, C. parvum is mostly found in preweaned calves and is a significant cause of diarrhea (41), whereas C. bovis and C. ryanae usually infect weaned calves and yearlings, with C. bovis being more commonly seen than C. ryanae and both not associated with the occurrence of diarrhea (31). In contrast, C. andersoni is commonly seen in adult cattle and has been associated with gastritis, reduced milk yield, and poor weight gain (8).

Subtype analysis based on the sequencing of the 60-kDa glycoprotein (gp60) gene has indicated that IIA is the most prevalent subtype family of C. parvum in calves worldwide. It has been widely reported in cattle in the United States, Canada, the United Kingdom, Ireland, Sweden, Germany, Belgium, the Netherlands, Italy, Spain, Portugal, Hungary, Serbia, and Montenegro, Slovenia, Japan, India, Australia, and New Zealand (7, 17, 25, 33, 34, 44). Among IIA subtypes, IIA15G2R1 is the most common subtype in calves and has also been commonly identified in human cases in these countries (44). In contrast, other subtype families such as IId and III were uncommon and were only reported in small numbers of cattle in Spain, Portugal, Belgium, the Netherlands, Sweden, Germany, Hungary, Slovenia, and Serbia, and Montenegro (33, 44). The only possible exception is Egypt, where a recent small-scale study indicated that one IId subtype (IIdA20G1) of C. parvum was prevalent on two dairy farms (3).

Dairy industry plays an important role in the agricultural economy of China. In 2007, the total dairy cattle population was 12.3 million (ranking fourth worldwide) and accounted for 5.0% of the total number of dairy cattle in the world (http://kids.fao.org/glapha/). However, only a few studies genetically analyzed small numbers of Cryptosporidium isolates. In these studies, C. andersoni (n = 29) was identified in postweaned or adult dairy cattle (21, 47) and C. bovis (n = 4) and C. ryanae

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infection rates in animals of 1, 2, 3, 4, 5, 6, 7, and 8 sporidium oocysts were first observed in animals of 7 days. The Crypto-/H9273 groups were not significant (weeks were 8.0, 21.4, 21.5, 23.7, 28.4, 28.6, 7.1, and 22.2%.

MATERIALS AND METHODS

Sample collection and examination. A total of 801 fresh fecal samples were collected between August 2008 and November 2009 from preweaned dairy cattle on eight farms in seven areas in Henan Province, China (Table 1). One of the farms (Zhengzhou A) was visited four times, and 369 samples taken in four different seasons were used to assess the seasonal variation in the prevalence. Cryptosporidium oocysts in fecal materials were concentrated by both the formalin-ethyl acetate sedimentation method and Sheather’s sugar flotation technique. Cryptosporidium oocysts concentrated by the latter method were detected by microscopy under ×400 magnification. Cryptosporidium-positive samples were stored in 2.5% potassium dichromate at 4°C prior to being used in molecular biologic characterizations.

DNA extraction. Genomic DNA was extracted from Cryptosporidium-positive feces samples by using an E.Z.N.A. Stool DNA kit (Omega Biotek, Inc., Norcross, GA) according to the manufacturer-recommended procedures.

Cryptosporidium genotyping and subtyping. Cryptosporidium species were determined by nested PCR amplification of an ~830-bp fragment of the small subunit (SSU) rRNA gene (the primary primers SSU-F2 [TTC TAG AGC TAA TAC ATG CG] and SSU-R2 [CCT ATT TTC TTC GAA ACA GGA] and the secondary primers SSU-F3 [GGA AGG GGT GTA TTT ATT AGA TAA AG] and SSU-R4 [CTC ATA AGG TGC TGA AGG AGT A] and restriction fragment length polymorphism (RFLP) analysis using restriction enzymes SspI and MboII (Fermentas, Shenzhen, China) (11). The presence of C. parvum, C. bovis, C. ryanae, and C. andersoni was confirmed by DNA sequencing of PCR product from one sample for each species. All Cryptosporidium-positive samples were also analyzed by a nested PCR targeting the gp60 gene (2). The previously established nomenclature system was used in naming C. parvum subtype families and subtypes (44).

DNA sequence analysis. Sixty-seven PCR products of gp60 gene and one SSU rRNA PCR product each of C. parvum, C. bovis, C. ryanae, and C. andersoni were sequenced on an ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA), using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product if necessary. The SSU RNA and gp60 sequences obtained in the present study were aligned with reference sequences downloaded from GenBank by using the program CLUSTAL X 1.83 (ftp://ftp-genome.toulouse.inra.fr/pub/ClustalX2). Representative nucleotide sequences have been deposited in the GenBank under accession numbers HQ009805 to HQ009880.

Statistical analysis. A chi-square test was used to compare Cryptosporidium infection rates. Differences were considered significant when P was <0.05.

RESULTS

Prevalence of Cryptosporidium spp. Microscopic analysis of 801 fecal samples showed the presence of Cryptosporidium oocysts in 172 samples (21.5%) on all eight farms (Table 1). The highest infection rate (40%) was observed on farm Xinxiang, and the lowest infection rate (10.5%) was seen on farm Luoyang (x² = 23.56; P < 0.01) (Table 1). Cryptosporidium oocysts were first observed in animals of 7 days. The Cryptosporidium infection rates in animals of 1, 2, 3, 4, 5, 6, 7, and 8 weeks were 8.0, 21.4, 21.5, 23.7, 28.4, 28.6, 7.1, and 22.2%, respectively. The differences in infection rates among age groups were not significant (x² = 14.68; P > 0.05) (Fig. 1A).

Distribution of Cryptosporidium species. All 172 Cryptosporidium-positive samples produced the expected PCR product of the SSU rRNA gene. RFLP analysis of the PCR products revealed the presence of four Cryptosporidium species, including C. parvum (54/172) on four farms, C. bovis (65/172) on

| Farm                      | C. parvum | C. bovis | C. ryanae | C. andersoni | Total (%)
|---------------------------|-----------|----------|-----------|--------------|-----------
| Jiaozuo                   | 75        | 9 (10.5) | 0         | 0            | 17 (23.56)
| Luoyang                   | 86        | 9 (12)   | 0         | 0            | 19 (26.2)
| Shanggu                   | 53        | 9 (17)   | 0         | 0            | 16 (21.1)
| Xingxiu                   | 33        | 4 (12.1) | 0         | 0            | 5 (14.68)
| Xingyang                  | 40        | 11 (27.5)| 0         | 0            | 12 (30)
| Zhengzhou A               | 39        | 8 (20.5) | 0         | 0            | 16 (40.5)
| Zhengzhou B               | 19        | 2 (10.5)| 0         | 0            | 3 (15.8)
| Total                      | 172       | 23 (13.5)| 0         | 0            | 48 (28.0)

a Mixed infections are indicated with a hyphen. Total percentage values are given in parentheses.

TABLE 1. Infection rates of Cryptosporidium species determined by microscopy on each farm and the distribution of Cryptosporidium species, as determined by PCR-RFLP analysis of the SSU rRNA gene, in milk samples, as identified by sequence analysis of the gp60 gene.
seven farms, C. ryanæ (19/172) on five farms, and C. andersoni (12/172) on three farms; 22 samples from five farms had concurrent infection of mixed species (Table 1). With the exception of the farm Xinmi, all of the farms had more than one Cryptosporidium species (Table 1). Cryptosporidium bovis was the dominant species on Farm Zhengzhou A ($\chi^2 = 71.95; P < 0.01$), whereas C. parvum was commonly seen on farms Zhengzhou B ($\chi^2 = 39.62; P < 0.01$) and Shangqiu ($\chi^2 = 10.89; P < 0.01$) (Table 1). DNA sequencing of the SSU rRNA gene PCR products confirmed the identification of C. parvum, C. bovis, C. ryanæ, and C. andersoni.

**Age patterns of Cryptosporidium species.** Cryptosporidium bovis was the most commonly identified Cryptosporidium, responsible for 37.8% of all Cryptosporidium infections. It was found in all weekly age groups examined in the present study (Fig. 1B). C. parvum, the second most common species, was detected in seven age groups and accounted for 31.4% of all Cryptosporidium infections (Fig. 1B). No significant difference was observed in the infection rates of C. parvum and C. bovis among the eight age groups (Fig. 1B). The initial detection of C. bovis and C. ryanæ was in calves aged 1 week and 2 weeks, respectively (Fig. 1B). In contrast, C. andersoni was first detected in animals of 5 weeks in age. The mixed infections were mostly concentrated in calves 4 to 6 weeks and 8 weeks of age, with C. parvum commonly seen in mixed infections (Fig. 1B).

**Seasonal variation in distribution of Cryptosporidium spp.** The highest infection rate (50%) was seen in summer and the lowest (17.3%) in winter ($\chi^2 = 7.17; P > 0.05$) (Fig. 2A). Except for spring, the distribution of Cryptosporidium spp. differed among seasons, with C. parvum dominating in summer and C. bovis in the autumn ($\chi^2 = 10.89; P < 0.01$) and winter ($\chi^2 = 11.41; P < 0.01$) (Fig. 2B).

**Subtypes of the C. parvum.** Sequences of the gp60 gene were successfully obtained from 54 C. parvum-positive samples and 13 samples of mixed infections (Table 1). All of them belonged to the subtype IIdA19G1 (Table 1).

**DISCUSSION**

The prevalence of cryptosporidiosis in dairy cattle varies among countries in the world. However, a general trend was observed in many studies: the prevalence of Cryptosporidium declined with increases in age (17, 18, 31, 32). In the present study, a 21.5% infection rate was seen in preweaned dairy calves, which was much higher than the 5.6% (27/485) in postweaned and adult dairy cattle reported in a recent Chinese study (21).

RFLP and sequence analyses of the SSU rRNA identified four Cryptosporidium species in the 172 positive samples, namely, C. parvum, C. bovis, C. ryanæ, and C. andersoni. Among the species detected, C. parvum and C. bovis were the two most common species, with C. bovis having a higher infection rate (37.8% versus 31.4%) (Fig. 1B). Previously, the results of most studies conducted in numerous countries suggested that C. parvum was the predominant Cryptosporidium species in preweaned calves (Table 2). The only exception was a recent study conducted in Sweden, in which 54 of 73 Cryptosporidium-positive samples from preweaned calves had C. bovis (33). In the present study, the youngest calf infected with C. bovis was 1 week old, indicating that the prepatent period of C. bovis is shorter than the previously stated 10 to 12 days (10). Thus, the distribution of Cryptosporidium spp. in preweaned dairy calves in Henan, China. (A) Infection rates of Cryptosporidium spp. in calves in different seasons. (B) Distribution of C. parvum and C. bovis in calves by season.
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<th>Cryptosporidium species (no. of positive isolates)</th>
<th>Reference</th>
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<td>16</td>
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</tbody>
</table>

* Mixed infections are indicated with a hyphen.

* Cryptosporidium-positive samples were collected from animals younger than 10 weeks of age.

* The 134 samples were positive for oocysts by microscopic analysis.

* This isolate was from a <3-month-old calf.

* The mean age of cattle was 13 days (range, 2 to 125 days), with only 7% of cattle older than 3 weeks.

Cryptosporidium species in preweaned dairy calves in Henan, China appears to be different from that seen in most other countries. The reason for the high occurrence of C. bovis in this and the Swedish studies is not clear. Feng et al. suggested that in areas where C. parvum is endemic the high infection rate and shedding intensity of C. parvum in preweaned calves probably had masked the concurrent infection of these animals by C. bovis or C. ryaue (11).

Sequence analysis of the gp60 gene has been used extensively in characterizing the molecular epidemiology of cryptosporidiosis in calves and humans (45). In recent years, data generated from numerous studies suggested that Ha was the
predominant subtype family of \( C. \) parvum in calves. Within the Ia family, the subtype IaA15G2R1 was shown to be the most prevalent \( C. \) parvum subtype in preweaned dairy calves in the United States, Canada, Belgium, Netherlands, Spain, Portugal, Slovenia, Germany, and Japan (Table 2). Several other \( C. \) parvum Ia subtypes were predominant in other countries (Table 2). In the present study, all 67 gp60 PCR positive samples belonged to one single subtype (IIdA19G1), which was detected previously in one case in Hungary (28). There was no nucleotide difference between the IIdA19G1 isolates of two sources. The source of \( C. \) parvum in calves in Henan is unclear. Generally, IId is not as common as the major zoonotic subtype family Ia (44). Thus, only for Hungary (IIdA19G1 and IIdA20G1) and Serbia and Montenegro (IIdA16G1c), Germany (IIdA22G1), Egypt (IIdA20G1), and Serbia and Montenegro (IIdA18G1b), members of this subtype family were recorded in small numbers of dairy calves (3, 4, 23, 30, 33, 44). Previously, the IId subtype family of \( C. \) parvum was known mostly as a parasite of sheep and goats in southern Europe (29). The only exception is a recent study in Egypt, in which it was shown that 23 of 24 of \( C. \) parvum-infected preweaned dairy calves had excreted IIdA20G1 (3). In China, 10 \( C. \) parvum isolates from pet Siberian chipmunks and hamsters in Henan were identified as IIdA15G1 (22). Despite its rare occurrence in dairy cattle, the IId subtype family is common in humans in the Middle East (14, 37), and has also been reported in a few human cases in Portugal, Ireland, the United Kingdom, Belgium, the Netherlands, and Australia (44). Thus, parasites of the subtype IId may be responsible for zoonotic transmission of cryptosporidiosis in some areas.

The results of the present study suggested that there was no significant seasonal difference in Cryptosporidium infection in preweaned calves. However, there appeared to be a seasonal shift in the dominant Cryptosporidium species in preweaned calves, with \( C. \) parvum peaking in summer and \( C. \) bovis peaking in autumn and winter. This finding is somewhat different from the recent observation of \( C. \) bovis dominance in summer and \( C. \) parvum dominance in spring and winter in dairy cattle in New York (38). More large studies in different areas are needed to determine whether these differences are attributable to difference in animal management.

In conclusion, our findings suggest that the transmission of Cryptosporidium spp. in preweaned calves in China is probably different from that in other countries at both the species and subtype levels. Although \( C. \) parvum is common in preweaned dairy calves, the public health significance of \( C. \) parvum identified here is still unclear, since no \( C. \) parvum infection has been seen in humans in China (27, 42). In other countries the Ia subtype family has been a more important zoonotic pathogen than the IId family prevalent in the present study. Therefore, additional molecular epidemiology studies in cattle and humans are needed to understand the transmission dynamics of Cryptosporidium spp. in China and the public health significance of \( C. \) parvum in cattle.

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