Occurrence and genotyping of *Giardia duodenalis* and *Cryptosporidium* in pre-weaned dairy calves in central Sichuan province, China

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**Abstract** — *Giardia duodenalis* and *Cryptosporidium* spp. are common human and animal pathogens. They have increasingly been reported in dairy calves in recent years; however, multilocus genotyping information for *G. duodenalis* and *Cryptosporidium* infecting pre-weaned dairy calves in southwestern China is limited. In the present study, the prevalence of *G. duodenalis* and *Cryptosporidium* spp. in pre-weaned dairy calves in central Sichuan province was determined and the pathogens were analyzed molecularly. Of 278 fecal samples from pre-weaned dairy calves, 26 (9.4%) were positive for *G. duodenalis* and 40 (14.4%) were positive for *Cryptosporidium* spp. *Cryptosporidium bovis* (*n* = 28), *Cryptosporidium ryanae* (*n* = 5) and *Cryptosporidium parvum* (*n* = 7) were detected. All seven *C. parvum* isolates were successfully subtyped based on the *gp60* gene sequence, and only IIdA15G1 was detected. Multilocus sequence typing of *G. duodenalis* based on beta-giardin (*bg*), triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*) genes revealed 19 different assemblage E multilocus genotypes (two known and 17 unpublished genotypes). Based on eBURST analysis, a high degree of genetic diversity within assemblage E was observed in pre-weaned dairy calves in Sichuan province. To the best of our knowledge, this is the first study using multilocus sequence typing and eBURST analysis to characterize *G. duodenalis* in pre-weaned dairy calves in southwestern China.

**Key words:** *Giardia duodenalis*, *Cryptosporidium*, Multilocus genotyping, Pre-weaned dairy calves, Sichuan province.

**Résumé** — *Giardia duodenalis* et *Cryptosporidium* spp. sont des pathogènes humains et animaux communs. Ils ont été signalés de plus en plus chez les veaux laitiers au cours des dernières années; cependant, l’information de génotypage multilocus pour *G. duodenalis* et *Cryptosporidium* infectant les veaux laitiers pré-sevrés dans le sud-ouest de la Chine est limitée. Dans la présente étude, la prévalence de *G. duodenalis* et de *Cryptosporidium* spp. chez les veaux laitiers pré-sevrés dans la province centrale du Sichuan a été déterminée et les pathogènes ont été analysés moléculairement. De 278 échantillons fécaux de veaux laitiers pré-sevrés, 26 (9.4 %) étaient positifs pour *G. duodenalis* et 40 (14.4 %) étaient positifs pour *Cryptosporidium* spp. *Cryptosporidium bovis* (*n* = 28), *Cryptosporidium ryanae* (*n* = 5) et *Cryptosporidium parvum* (*n* = 7) ont été détectés. Tous les sept isolats de *C. parvum* ont été sous-typés avec succès sur la base de la séquence du gène *gp60* et seul IIdA15G1 a été détecté. Le typage multilocus de *G. duodenalis* basé sur les gènes de bêta-giardine (*bg*), triose phosphate isomérase (*tpi*) et glutamate déshydrogénase (*gdh*) a révélé 19 différents assemblages E multilocus (deux connus et 17 non-publiés). D’après l’analyse eBURST, un degré élevé de diversité génétique au sein de l’assemblage E chez les veaux laitiers pré-sevrés de la province du Sichuan a été observé. À notre connaissance, il s’agit de la première étude utilisant le typage de séquence multilocus et l’analyse eBURST pour caractériser *G. duodenalis* chez les veaux laitiers pré-sevrés dans le sud-ouest de la Chine.

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Introduction

Protists of the genera *Giardia* and *Cryptosporidium* infect a wide range of animals as well as humans [3, 12, 19]. Typically, the infection is acquired following the ingestion of highly resilient, infective stages (oocysts or cysts) via the fecal-oral route [3, 4]. Disease is commonly associated with clinical signs including diarrhea, dehydration, fever, inappetence and anorexia. Infections are often self-limiting in immune-competent individuals [2, 31], but can be chronic and severe in infants, elderly people, and immune-compromised individuals [9, 16].

Ruminants are recognized as a significant reservoir of *Giardia* and *Cryptosporidium* taxa that infect animals and humans [19, 21]. Current data indicate that of the eight assemblages within *Giardia* duodenalis, assemblages A and E and the *Cryptosporidium* species *C. parvum*, *C. andersoni*, *C. ryanae*, and *C. bovis* predominate in cattle worldwide [4, 20].

Unlike in other countries (e.g. Australia, Sudan, Japan and India) [6, 14, 15, 24], where *C. parvum* is known to be the predominant species in pre-weaned calves, this does not appear to be the case everywhere in China. Some studies have shown that *C. parvum* is a major species in pre-weaned calves in some regions, whereas *C. bovis* is a major species in other regions [5, 25, 28].

According to the National Bureau of Statistics of the People’s Republic of China, in 2016, the total population of dairy cattle in Sichuan Province was 176 thousand heads. However, no information about *G. duodenalis* and *Cryptosporidium* infection of pre-weaned dairy calves was previously available in Sichuan Province. We undertook a molecular epidemiological study to obtain a preliminary snapshot of the prevalence of *G. duodenalis* assemblages and *Cryptosporidium* genotypes in pre-weaned calves in Sichuan province, China.

Materials and methods

Sample collection

We collected 278 rectal fecal samples from pre-weaned dairy calves (<1 month of age) from 10 farms with a history of bovine diarrhea in 10 regions in Sichuan province, southwestern China, between June 2016 and March 2017. Collection sites included: Chengdu (104°06’E, 30°57’N), Hongya (103°37’E, 29°91’N), Aba (102°22’E, 31°90’N), Meishan (103°84’E, 30°08’N), Mianyang (104°67’E, 31°47’N), Ziyang (104°62’E, 30°13’N), Anyue (105°33’E, 30°10’N), Qionglai (103°46’E, 30°41’N), Qingbaijiang (104°25’E, 30°88’N), and Deyang (104°39’E, 31°13’N). The 10 farms are distributed in central Sichuan Province (Fig. 1). The city-level map was provided by the National Geomatics Centre of China (National Geomatics Centre of China, Beijing, China, http://ngcc.sbsm.gov.cn/).

Of the 10 farms, six (Chengdu, Hongya, Aba, Mianyang, Ziyang, and Qionglai) are intensive feeding farms, while the other four are free-ranging. For intensive farms, there were approximately 1000-2500 cattle per farm, with more than 100 pre-weaned dairy calves, and the fecal samples were randomly collected from about 20% in each farm. For free-ranging farms, there were approximately 100-120 cattle per farm and the herd sizes of pre-weaned dairy calves were less than 50; in this case we collected fecal samples from all of the pre-weaned dairy calves at each farm (Table S1). In intensive farms, calves were bred in different calf stalls, with one hour outdoor time after eating and excretion in the morning and afternoon, respectively. Calves shared one yard during the outdoor time in intensive farms. In free-ranging farms, calves were kept in a field with a half cover and were raised together. The farms we selected had solely cattle, and no other animals.

Fecal samples were collected from the rectum using disposable gloves, transferred into disposable plastic bags, and stored in 2.5% potassium dichromate at 4 °C.

DNA extraction

Before DNA extraction, feces were washed with distilled water to remove potassium dichromate. Genomic DNA was extracted from 250 mg (approximately) of individual samples using the Power Soil DNA isolation kit (MOBI, USA), according to the manufacturer’s instructions, and frozen at −20 °C until use.

PCR amplification and sequencing

*G. duodenalis* was detected by nested PCR amplification of the bgg gene. The bgg-positive samples were further characterized by amplifications of gdh and mpi. Genotyping of *Cryptosporidium* was based on amplification of the small subunit (SSU) rRNA gene by nested PCR and subsequent sequence analysis. All the *C. parvum* isolates were further characterized by amplification of the gp60 gene. The primers and amplification conditions in this study were described previously [1, 11, 23]. Positive and negative controls were included in each test. The secondary PCR products were visualized under UV light after electrophoresis on a 1% agarose gel mixed with Golden View. All positive secondary PCR products were sent to Invitrogen (Shanghai, China) and sequenced in both directions. Sequences were aligned with reference sequences from GenBank using BLAST (http://blast.ncbi.nlm.nih.gov) and ClustalX.

A previous nomenclature system was used to name subtypes at each genetic locus [29, 30]. Specimens that were successfully subtyped at all three loci were included in multilocus genotyping of *G. duodenalis*. The genetic pedigree of the assemblage E multilocus genotypes (MLGs) was assessed by using eBURST 3.0 (http://eBURST.mlst.net).

Statistical analysis

The $\chi^2$ test was used to compare the infection rates of *G. duodenalis* and *Cryptosporidium* in different feeding patterns. Differences were considered significant at $p < 0.05$.

Results and discussion

*G. duodenalis* was detected in 9.4% of 278 pre-weaned dairy calves on 6 of 10 farms, with prevalences ranging from 7.7% to 46.4% (Table 1). Its prevalence shows substantial
Figure 1. Distribution of sampling sites in Sichuan province in this study.

| Table 1. Prevalence of Cryptosporidium and G. duodenalis in pre-weaned diary calves in Sichuan province. |

| Region      | No. tested | Cryptosporidium | Cryptosporidium No. (%) of positive specimens | G. duodenalis | G. duodenalis infection rate |
|-------------|------------|-----------------|-----------------------------------------------|---------------|-----------------------------|
|             |            | C. bovis  | C. ryanae | C. parvum |                      |                           |                          |
| Chengdua    | 39         | 2       |1          |           | 3 (7.7%)                  | 3                         | 7.7%                      |
| Hongya b    | 24         | 1       |1          |           | 1 (4.2%)                  |                           |                          |
| Abab        | 20         |          |7          |           | 7 (35.0%)                 | 2                         | 10.0%                     |
| Meishanb    | 20         |          |           |           |                           |                           |                          |
| Mianyang a  | 58         | 8       |3          |           | 11 (19.0%)                |                           |                          |
| Ziyang a    | 26         | 8       |1          |           | 8 (30.8%)                 | 2                         | 7.7%                      |
| Anyue b     | 22         | 2       |1          |           | 2 (9.1%)                  | 3                         | 13.6%                     |
| Qionglai a  | 28         | 4       |1          |           | 4 (14.3%)                 | 13                        | 46.4%                     |
| Qingbaijiang b | 20   | 2       |1          |           | 2 (10.0%)                 | 3                         | 15.0%                     |
| Deyang b    | 21         | 1       |1          |           | 2 (9.5%)                  |                           |                          |
| Total       | 278        | 28      |5          |7          | 40 (14.4%)                | 26                        | 9.4%                      |

* Intensive farming;
* free-ranging.
differences, ranging from 7.1% to 60.1% in other studies in China [7, 13, 18, 26, 29]. In this study, the overall infection rate in southwestern China was close to the prevalence in northwestern (9.7% [18]), northeastern (13.3% [13]) and north China (7.1% [7]), but much lower than the infection rates in central (17.6% [26]) and southeastern (60.1% [29]) China. Prior to the present study, these results were interpreted as related to differences in geographic distribution, environmental management and cultivation scale [7, 13, 18, 26, 29]. Cattle were kept in groups or in free stalls, which might promote the transmission of *G. duodenalis* infection among animals and lead to the high infection rates [26, 29]. Furthermore, we analyzed the infection rates between intensive feeding and free-ranging farms; there was no significant difference between the two breeding patterns ($X^2 = 0.629$, df = 1, $p = 0.428$).

*Cryptosporidium* was detected in 14.4% of 278 fecal samples, on 9 out of 10 farms, with prevalences ranging from 4.2% to 35.0% (Table 1). The overall infection rate for *Cryptosporidium* is lower than the average prevalence of 19.5% reported previously in pre-weaned cattle in China [5], but similar to the infection rate reported in Xinjiang (15.6%) [17], and much higher than the rate in Hebei and Tianjin, China (1.0%) [7]. Prevalence of *Cryptosporidium* was significantly different ($X^2 = 4.924$, df = 1, $p = 0.026$) between intensive feeding and free-ranging farms in this study, which suggests that cultivation scale may lead to differences in infection rates with *Cryptosporidium*. Other studies also showed that geographic distribution and host health status may lead to the difference [5, 10, 17]. Three species of *Cryptosporidium* (28 *C. bovis*, 7 *C. parvum* [subtype IIdA15G1] and 5 *C. ryanae*) were identified in this study. Previous studies have shown that *C. parvum* is a major species in pre-weaned calves in Beijing [10], Xinjiang [17] and Ningxia [8], whereas *C. bovis* predominated in pre-weaned calves in this study, similar to reports from Henan [27] and Heilongjiang [32].

*Giardia duodenalis* in all 26 positive samples corresponded to assemblage E. *G. duodenalis* infection is relatively common in pre-weaned dairy calves. We further characterized the 26 *G. duodenalis* bg-positive samples at the *tpi* and *gdh* loci. Among these 26 samples, the *tpi* and *gdh* loci were successfully amplified and sequenced in 24 and 25 specimens, respectively. The *bg*, *tpi* and *gdh* loci all showed high levels of sequence polymorphism; seven subtypes were identified at each locus. Of the *bg* subtypes, E1 (MF671885), E8 (KY769093), E9 (KY769091), and E15 (KT698677) were known, and E13 (MF671880), E14 (MF671883), and E16 (MF671886) were unpublished. At the *tpi* locus, five known subtypes E1 (MF671900), E3 (KT922259), E9 (KF654690), E15 (KY432848) and E19 (KY769103) and two unpublished subtypes, E21 (MF671904) and E24 (MF671907), were found. The sequences from the *gdh* locus represented five known subtypes E1 (MF671891), E8 (KT368785), E10 (KT698971), E13 (KY432838) and two unpublished subtypes, E19 (MF671896) and E20 (MF671899).

For *G. duodenalis*, multi-locus genotyping analysis suggested a high genetic diversity of assemblage E in pre-weaned calves in Sichuan province.

### Table 2. Multilocus sequence genotypes of *G. duodenalis* in pre-weaned dairy calves in Sichuan province.

| Isolate | Geographic source | Subtype | MLG        |
|---------|-------------------|---------|------------|
| ABG3417 | A'ba              | E9      | E15        | #E19/MF671896 | #MLGE72 |
| ABG3422 |                   | E1      | E15        | #E19/MF671896 | #MLGE74 |
| AYG6943 | Anyue             | #E13/MF671880 | E1     | E10        | #MLGE70 |
| AYG6950 |                   | E14/MF671883 | E3      | E10        | #MLGE67 |
| AYG6953 |                   | E1      | E3        | E3         | #MLGE62 |
| CDG16089| Chengdu           | E9      | E3        | E1         | #MLGE61 |
| CDG16090|                   | E8      | E9        | E10        | #MLGE60 |
| CDG16100|                   | E9      | E19       | E1         | #MLGE68 |
| QBJG13  | Qingbaijiang      | #E14/MF671883 | E3     | E10        | #MLGE67 |
| QBJG17  |                   | #E16/MF671886 | E3      | E3         | #MLGE63 |
| QBJG18  |                   | E9      | E3        | E10        | MLG E3  |
| QLG5065 | Qionglai          | E1      | #E24/MF671907 | #E19/MF671896 | #MLGE75 |
| QLG5066 |                   | E9      | E3        | E10        | MLG E3  |
| QLG5067 |                   | E1      | E3        | E8         | #MLGE64 |
| QLG5070 |                   | E1      | E15       | E1         | #MLGE65 |
| QLG5071 |                   | E9      | E19       | #MF671896  | #MLGE73 |
| QLG5073 |                   | E1      |           | E10        | #MLGE65 |
| QLG5074 |                   | E1      | E15       | E1         | #MLGE65 |
| QLG5075 |                   | #E13/MF671880 | E3     | E1         | MLG E13 |
| QLG5076 |                   | #E13/MF671880 | E3     | #E20/MF671899 | #MLGE59 |
| QLG5083 |                   | E1      | E3        | E1         | MLG E66 |
| QLG5091 |                   | #E13/MF671880 | E3     | E1         | MLG E13 |
| QLG5092 |                   | #E13/MF671880 | E3     | E1         | MLG E13 |
| QLG5093 |                   | #E13    |           |           | #MLGE71 |
| ZYG6836 | Ziyang            | E9      | E1        | E1         | #MLGE71 |
| ZYG6844 |                   | E15     | #E21/MF671904 | E13        | #MLGE69 |

* Unpublished subtypes and MLGs.

*Giardia duodenalis* was detected in 14.4% of 278 fecal samples, on 9 out of 10 farms, with prevalences ranging from 4.2% to 35.0% (Table 1). The overall infection rate for *Giardia* is lower than the average prevalence of 19.5% reported previously in pre-weaned cattle in China [5, 10, 17].
dairy calves in this study. Based on the combination of bg, tpi and gdh loci, 19 MLGs of assemblage E were detected (Table 2). A high degree of nucleotide variation in assemblage E has been also detected in previous studies [18, 22, 26, 29]. Of the 19 MLGs, 17 were unpublished MLGs. The majority of MLGs were MLG-E3 and MLG-E13, which have also been detected in dairy calves in Shanghai [29]. To further analyze the evolutionary descent of the 19 assemblage E MLGs, we used eBURST analysis of the 19 assemblage E MLGs and 58 reference MLGs, which revealed two clonal complexes and seven singletons (Fig. 2). MLG-E3 is the primary founder of clonal complex 1, which is consistent with findings in a previous study in Shanghai [29]. The majority of MLGs (14/19) originated from MLG-E3. Furthermore, MLG-E60 is a variant of clonal complex 2, and MLG-E59 and MLG-E70 were singletons. The latter three MLG subtypes showed distant evolution from other assemblage E MLGs, which may indicate substantial differences in their evolutionary divergence [29].

Conclusion

This is the first study to genotype G. duodenalis and Cryptosporidium in pre-weaned dairy calves in Sichuan province. C. bovis and G. duodenalis assemblage E are the dominant species in pre-weaned dairy calves in Sichuan, and high genetic diversity of assemblage E MLGs was observed.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article. Cryptosporidium and G. duodenalis sequences were deposited in the GenBank database under accession numbers MF671870–MF671908.

Competing interests

The authors declare that they have no conflict of interest.

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Author contributions

Experiments were conceived and designed by Z.J.Z and G.N.P. R.T, Y.N.T, S.Z.C and L.H.S collected samples. Experiments were performed by Z.J.Z, J.M.D, Y.W, X.B.G and J.L.D, and the data were analyzed by Z.J.Z, S.M.Y, H.F.L and Y.G. The manuscript was written by Z.J.Z, J.M.D and G.W.Y. All authors read and approved the final manuscript.

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Figure 2. eBURST networks for G. duodenalis assemblage E. Each MLG is represented by a dot. MLG-E3 is the primary founder, and the subgroup founders are MLG-E1, MLG-E4, MLG-E33, MLG-E66, MLG-E61, MLG-E74, MLG-E30, MLG-E48, MLG-E50, MLG-E6, and MLG-E18. The variants are connected by lines.
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Supplementary Material

Table S1 is available at https://www.parasite-journal.org/

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