Molecular Investigation of Zoonotic Intestinal Protozoa in Pet Dogs and Cats in Yunnan Province, Southwestern China

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Abstract: Giardia duodenalis, Enterocytozoon bieneusi and Cryptosporidium spp. are common enteric pathogens that reside in the intestines of humans and animals. These pathogens have a broad host range and worldwide distribution, but are mostly known for their ability to cause diarrhea. However, very limited information on prevalence and genotypes of G. duodenalis, E. bieneusi and Cryptosporidium spp. in pet dogs and cats are available in China. In the present study, a total of 433 fecal samples were collected from 262 pet dogs and 171 pet cats in Yunnan province, southwestern China, and the prevalence and the genotypes of G. duodenalis, E. bieneusi and Cryptosporidium spp. were investigated by nested PCR amplification and DNA sequencing. The prevalence of G. duodenalis, E. bieneusi and Cryptosporidium spp. was 13.7% (36/262), 8.0% (21/262), and 4.6% (12/262) in dogs, and 1.2% (2/171), 2.3% (4/171) and 0.6% (1/171) in cats, respectively. The different living conditions of dogs is a risk factor that is related with the prevalence of G. duodenalis and E. bieneusi (p < 0.05). However, there were no statistically significant difference in prevalence of three pathogens in cats. DNA sequencing and analyses showed that four E. bieneusi genotypes (PtEb IX, CD9, DgEb I and DgEb II), one Cryptosporidium spp. (C. canis) and two G. duodenalis assemblages (C and D) were identified in dogs; two E. bieneusi genotypes (Type IV and CtbEb I), one Cryptosporidium spp. (C. felis) and one G. duodenalis assemblage (F) were identified in cats. Three novel E. bieneusi genotypes (DgEb I, DgEb II and CtbEb I) were identified, and the human-pathogenic genotypes/species Type IV C. canis and C. felis were also observed in this study, indicating a potential zoonotic threat of pet dogs and cats. Our results revealed the prevalence and genetic diversity of G. duodenalis, E. bieneusi and Cryptosporidium spp. infection in pet dogs and cats in Yunnan province, southwestern China, and suggested the potential threat of pet dogs and cats to public health.

Keywords: Giardia duodenalis; Enterocytozoon bieneusi; Cryptosporidium spp.; zoonotic genotypes; pet dogs and cats; Yunnan province; China

1. Introduction

Giardia duodenalis, Cryptosporidium spp. and Enterocytozoon bieneusi are three eukaryotic unicellular protozoans, which are the causative pathogens of giardiasis, cryptosporidiosis, and microsporidiosis, respectively [1–4]. These pathogens can cause many gastrointestinal...
symptoms such as abdominal pain, nausea, vomiting, anorexia and weight loss especially acute and chronic diarrhea [5–10]. Humans and various animals can be infected by G. duodenalis, Cryptosporidium spp. and E. bieneusi through fecal-oral transmission of their cysts or spores [11,12].

At present, eight G. duodenalis assemblages (A–H) have been identified by the molecular biological detection method [13]. Among these genotypes, assemblages A and B are regarded as zoonotic assemblages which mainly infect humans and other mammals [14]. Other G. duodenalis assemblages (C–H) are commonly considered as host-specific, while assemblages C and D are usually canine-specific assemblages, and assemblage F is usually a feline-specific assemblage [15,16]. However, assemblages E and F have also been detected in humans [17,18]. In total, over 40 Cryptosporidium species have been reported, and over 21 species have been reported in humans, including C. canis and C. felis, which cause the vast majority of infections in dogs and cats, respectively [12,19]. Moreover, Cryptosporidium muris, Cryptosporidium parvum and Cryptosporidium ubiquitum have also been reported in dogs and cats [6,7,20–24]. Enterocytozoon bieneusi is the most common species causing human gut infections among nearly 1500 microsporidian species [23]. At least 500 E. bieneusi genotypes have been defined thus far, which can be divided into several genetically isolated groups, including zoonotic groups (Group 1 and Group 2) and host adapted groups (Groups 3 to 11) [23,24].

Due to the closer relationships between humans with pet dogs and cats, many pathogens can be transmitted to humans through pets and cats, including G. duodenalis, Cryptosporidium spp. and E. bieneusi. Therefore, investigation of the prevalence and genotypes/species of G. duodenalis, Cryptosporidium spp. and E. bieneusi in pet dogs and cats will improve our understanding of the potential threat posed by these pathogens in companion animals in Yunnan province, China.

2. Results

2.1. Prevalence of G. duodenalis, E. Bieneusi and Cryptosporidium spp. in Pet Dogs and Cats

The prevalence of G. duodenalis, Cryptosporidium spp. and E. bieneusi was 13.7% (95%CI 9.6–17.9), 4.6% (95%CI 2.0–7.1), 8.0% (95%CI 4.7–11.3) in dogs; and it was 1.2% (95%CI 0–2.8), 0.6% (95%CI 0–1.7), 2.3% (95%CI 0.1–4.6) in cats, respectively (Table 1). Among three regions, the prevalence of G. duodenalis in dogs in Kunming city was significantly higher than that in Chuxiong city and Lijiang city (p < 0.05). Moreover, the prevalence of G. duodenalis in dogs in shelter dogs (27.8%, 20/72, 95%CI 17.4–38.1) was higher than that in pet markets (2.9%, 1/34, 95%CI 0–8.6) and pet hospitals (9.6%, 15/156, 95%CI 5.0–14.2), and the difference was statistically significant (p < 0.001). However, no statistically significant difference in prevalence of G. duodenalis in pet cats was observed (Table 1).

Among the different living conditions of dogs, the difference in E. bieneusi prevalence was statistically significant (p < 0.001). The prevalence of E. bieneusi in dogs aged more than 6 months was 10.3% (95%CI 6.0–14.6), which was significantly higher than that in dogs aged less than 6 months (1.5%, 95%CI 0–4.3) (Table 1). Also, the prevalence of E. bieneusi in female dogs was 10.3% (95%CI 5.5–15.1), which was higher than that in male dogs (4.7%, 95%CI 0.7–8.7), but the difference in prevalence was not statistically significant (p = 0.098). Similarly, the prevalence of E. bieneusi in female cats (3.3%, 95%CI 0–7.9) was slightly higher than that in male cats (1.8%, 95%CI 0–4.3) (Table 1).

Furthermore, the prevalence of Cryptosporidium spp. in dogs in shelter (15.3%, 95%CI 7.0–23.6) was higher than that in pet markets (no detection) and pet hospitals (0.6%, 95%CI 0–1.9). Between two gender groups, the prevalence of Cryptosporidium spp. in male and female dogs was not significantly different (Table 1).
| Animals | Factors | Category | No. Sample | Giardia duodenalis | | | Enterocytozoon bieneusi | | | Cryptosporidium spp. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dogs | Age | <6 months | 68 | 8 | 11.8 (4.1–19.4) | 0.582 | 1 | 1.5 (0–4.3) | 0.021 | 1 | 1.5 (0–4.3) | 0.154 |
| | | >6 months | 194 | 28 | 14.4 (9.5–19.4) | | 20 | 10.3 (6.0–14.6) | | 11 | 5.7 (2.4–8.9) | |
| | Region | Kunming | 134 | 26 | 19.4 (12.7–26.1) | 0.013 | 18 | 13.4 (7.7–19.2) | - | 11 | 8.2 (3.6–12.9) | - |
| | | Lijiang | 90 | 9 | 10.0 (3.8–16.2) | | 0 | 0 | 1 | 1.1 (0–3.3) | 0 | 0 | - |
| | | Chuxiong | 38 | 1 | 2.6 (0–7.7) | | 3 | 7.9 (0–16.5) | | 0 | 0 | | |
| | Gender | Male | 107 | 16 | 15.0 (8.2–21.7) | 0.636 | 5 | 4.7 (0.7–8.7) | 0.098 | 5 | 4.7 (0.7–8.7) | 0.95 |
| | | Female | 155 | 20 | 12.9 (7.6–18.2) | | 16 | 10.3 (5.5–15.1) | | 7 | 4.5 (1.2–7.8) | |
| | Living condition | Pet hospital | 156 | 15 | 9.6 (5.0–14.2) | <0.001 | 1 | 0.6 (0–1.9) | <0.001 | 1 | 0.6 (0–1.9) | - |
| | | Pet market | 34 | 1 | 2.9 (0–8.6) | | 3 | 8.8 (0–18.4) | <0.001 | 0 | 0 | |
| | | Shelter | 72 | 20 | 27.8 (17.4–38.1) | | 17 | 23.6 (13.8–33.4) | | 11 | 15.3 (7.0–23.6) | |
| | | Subtotal | 262 | 36 | 13.7 (9.6–17.9) | | 21 | 8.0 (4.7–11.3) | | 12 | 4.6 (2.0–7.1) | |
| Cats | Age | < 6 months | 145 | 2 | 1.4 (0–3.3) | - | 4 | 2.8 (0.1–5.4) | - | 1 | 0.7 (0–2.0) | - |
| | | > 6 months | 26 | 0 | 0 | | 0 | 0 | | 0 | 0 | - |
| | Region | Kunming | 36 | 1 | 2.8 (13.1–42.4) | - | 0 | 0 | - | 0 | 0 | - |
| | | Lijiang | 110 | 0 | 0 | | 0 | 0 | | 1 | 0.9 (0–2.7) | - |
| | | Chuxiong | 25 | 1 | 4.0 (0–11.7) | | 4 | 16.0 (1.6–30.4) | | 0 | 0 | - |
| | Gender | Male | 111 | 2 | 1.8 (0–4.3) | - | 2 | 1.8 (0–4.3) | - | 1 | 0.9 (0–2.7) | - |
| | | Female | 60 | 0 | 0 | | 2 | 3.3 (0–7.9) | | 0 | 0 | - |
| | Living condition | Pet hospital | 154 | 2 | 1.3 (0–3.1) | - | 4 | 2.6 (0.1–5.1) | - | 1 | 2.6 (0.1–5.9) | - |
| | | Shelter | 17 | 0 | 0 | | 0 | 0 | | 0 | 0 | - |
| | | Subtotal | 171 | 2 | 1.2 (0–2.8) | | 4 | 2.3 (0.1–4.6) | | 1 | 0.6 (0–1.7) | |
| | Total | | 433 | 38 | 8.8 (6.1–11.4) | | 25 | 5.8 (3.6–8.0) | | 13 | 3.0 (1.4–4.6) | |
2.2. Assemblages and Subtypes of *G. duodenalis* in Pet Dogs and Cats

PCR amplification and DNA sequencing showed that 38 positive samples (36 from dogs and 2 from cats) of *G. duodenalis* were detected at bg locus, resulting three assemblages, namely C (4 from dogs), D (32 from dogs) and F (2 from cats). In addition, at the gdh locus, the 19 gdh-positive samples were identified as assemblage C (4 from dogs), D (13 from dogs) and F (2 from cats). Only one tpi-positive sample (1 from dogs) was identified as assemblage C.

Sequence alignment analysis revealed some single nucleotide polymorphisms at bg-sequences, gdh-sequences and tpi-sequences, respectively. At bg locus, one subtype of assemblage C, 7 subtypes of assemblage D and one subtype of assemblage F were identified, including five novel (Da4 *, Da7 *, Fa1 *) and four known sub-assemblages (Table 2). Also, at gdh gene locus, three subtypes of assemblage C, seven subtypes of assemblage D and one subtype of assemblage F were identified, including four novel (Cb3 *, Db5 * ~ Db7 *) and six known subtypes (Table 2). Only one novel subtype (Cc1 *) of assemblage C was found at tpi gene locus (Table 2). Moreover, one sample were successfully amplified and sequenced at three gene loci (bg, gdh and tpi), forming one mixed infection (Table 3).

### Table 2. Variations in nucleotide sequences of assemblages of *Giardia duodenalis* in pet dogs and cats in Yunnan province, southwestern China.

| Locus | Host (Subtypes) | Nucleotide at Position | No. Positive | Accession Number |
|-------|-----------------|------------------------|-------------|-----------------|
| bg    | (a) Variations in bg nucleotide sequences among assemblage D |
|       | Reference sequences | G A G C A | 20 | MG873354 |
|       | Dog (Da1) | A | MN734349 |
|       | Dog (Da2) | T | MN734350 |
|       | Dog (Da3) | A | MN734353 |
|       | Dog (Da4 *) | A T | 1 | MN734351 |
|       | Dog (Da5 *) | A A | 1 | MN734354 |
|       | Dog (Da6 *) | C A | 1 | MN734352 |
|       | Dog (Da7 *) | A A G | 1 | MN734355 |
|     | (b) Variations in bg nucleotide sequences among assemblage F |
| Cat (Fa1 *) | T | MN734356 |
|     | (c) Variations in bg nucleotide sequences among assemblage C |
| Dog (Ca1) | T | MN734348 |
| gdh   | (a) Variations in gdh nucleotide sequences among assemblage C |
| Reference sequences | A G A T G | 2 | MF990016 |
| Dog (Cb1) | G A T G | MN734358 |
| Dog (Cb2) | G A T G | MN734359 |
| Dog (Cb3 *) | G | MN734357 |
|     | (b) Variations in gdh nucleotide sequences among assemblage D |
| Reference sequences | C A T A C A | 1 | MF990017 |
| Dog (Db1) | T G G | MN734366 |
| Dog (Db2) | T G | MN734362 |
| Dog (Db3) | T G | MN734364 |
| Dog (Db4) | T G | MN734363 |
| Dog (Db5 *) | G | MN734361 |
| Dog (Db6 *) | G | MN734360 |
|     | (c) Variations in gdh nucleotide sequences among assemblage F |
| Cat (Fb1) | T G T | MN734367 |
| tpi   | Variations in tpi nucleotide sequences among assemblage C |
| Reference sequences | G T | MN734368 |
| Dog (Cc1 *) | T C | MN734368 |

* means novel subtypes of assemblage.
Table 3. Multilocus characterization of *Giardia duodenalis* isolates based on the bg, tpi and gdh genes.

| Isolate | Assemblage No. | Sequences | MLG Type |
|---------|----------------|-----------|----------|
| XSQG34  | D C C 1        | 1         | Mixed    |

2.3. Genotypes of *Enterocytozoon bieneusi* and *Cryptosporidium* spp. in Pet Dogs and Cats

Based on the ITS sequence, a total of four genotypes, including two known genotypes PtEb IX \((n = 18), \) CD9 \((n = 1)\) in dogs and two novel genotypes DgEb I \((n = 1)\) and DgEb II \((n = 1)\) were identified in pet dogs, and one known genotype Type IV \((n = 3)\) and one novel genotype CtEb I \((n = 1)\) were identified in pet cats (Table 4). The phylogenetic tree showed that genotypes DgEb I, DgEb II, PtEb IX and CD9 all belonged to the dog-specific group. However, genotypes Type IV and CtEb I belonged to the zoonotic Group 1 (Figure 1). Moreover, mixed infections with more than one genotype of *E. bieneusi* in dogs and cats were not detected.

Two *Cryptosporidium* species were identified among the 13 *Cryptosporidium*-positive samples, including 12 samples of *C. canis* in dogs and one sample of *C. canis* in cats (Table 4). Five nucleotide sequences of *C. canis* obtained in this study had 100% similarity to those deposited sequences in GenBank under accession numbers MN696800. Other sequences of *C. canis* had 99% similarity to those deposited sequences in GenBank under accession number KR999984 and KT749818, respectively (Table 4). Moreover, only one *C. canis* sequence had 97% similarity to those deposited sequences in GenBank under accession number KM977642 (Table 4).

Table 4. Species or genotypes of *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in pet dogs and cats in Yunnan province, southwestern China.

| Hosts | *Enterocytozoon bieneusi* Genotype (No.) | GenBank Accession Number in This Study |
|-------|-----------------------------------------|---------------------------------------|
| Dog   | DgEb I *(1)*                            | MZ542370                              |
| Dog   | CD9 *(1)*                               | MZ542369                              |
| Dog   | DgEb II *(1)*                           | MZ542373                              |
| Dog   | PtEb IX *(1)*                           | MZ542371                              |
| Dog   | PtEb IX *(17)*                          | MZ542372                              |
| Cat   | Type IV *(3)*                           | MZ542374                              |
| Cat   | CtEb I *(1)*                            | MZ542375                              |

| Hosts | *Cryptosporidium* spp. Genotype (No.) | Reference Sequences GenBank Accession Number | Similarity |
|-------|---------------------------------------|---------------------------------------------|------------|
| Dog   | *C. canis* *(5)*                       | MN696800                                   | 100%       |
| Dog   | *C. canis* *(4)*                       | KR999984                                   | 99%        |
| Dog   | *C. canis* *(3)*                       | KT749818                                   | 99%        |
| Cat   | *C. felis* *(1)*                        | KM977642                                   | 97%        |

Note: * represent novel genotype.
Cat Type IV (3) MZ542374
Cat CtEb I * (1) MZ542375

Hosts Cryptosporidium spp. Genotype (No.) Reference Sequences GenBank Accession Number Similarity

Dog C. canis (5) MN696800 100%
Dog C. canis (4) KR999984 99%
Dog C. canis (3) KT749818 99%
Cat C. felis (1) KM977642 97%

Note: * represent novel genotype.

Figure 1. Phylogenetic relationship based on ITS sequences of Enterocytozoon bieneusi in pet dogs and cats in Yunnan province, southwestern China. (Note: The samples in this study are indicated by triangles).

3. Discussion

Dogs and cats, as domestic animals, share a common environment with humans and other animals, and can infect them with various unicellular zoonotic pathogens. Thus far, many studies about the infection of G. duodenalis, Cryptosporidium spp. and E. bieneusi in dogs and cats have been recorded worldwide, such as Asia, Europe and Latin America, although only a few have been reported in Africa (Table 5) [6,7,16,20,25–53]. According to the studies in China, the prevalence of G. duodenalis ranges from 4.5–26.2% in dogs and 1.9–13.1% in cats [6,7,25,26]; the prevalence of Cryptosporidium spp. ranges from 3.1–7.5% in dogs and 5.6–5.8% in cats [6,7,46,47]; and the prevalence of E. bieneusi ranges from 6.0–13.9% in dogs and 1.4–11.5% in cats [6,7,33–35], respectively (Table 5).
Table 5. Prevalence of Giardia duodenalis, Enterocytozoon bieneusi and Cryptosporidium spp. in dogs and cats in different regions of the world.

| Regions | Hosts | Prevalence (%) | Hosts | Prevalence (%) | Reference |
|---------|-------|----------------|-------|----------------|-----------|
| (a) Prevalence of *Giardia duodenalis* in dogs and cats in different regions of the world. | | | | | |
| China | Dogs | Dogs 26.2% | Cats | 13.1% | [7] |
| Guangdong | Dogs | 10.8% | Cats | 5.8% | [25] |
| Heilongjiang | Dogs | 4.5% | Cats | 1.9% | [6] |
| Sichuan | Dogs | 11.3% | - | - | [26] |
| Henan | Dogs | 14.3% | - | - | [27] |
| Hangzhou | - | - | Cats | 1.2% | [28] |
| Yunnan | Dogs | 13.7% | Cats | 1.2% | Present study |
| Other countries | | | | | |
| Australia | Dogs | 6.3% | Cats | 2.0% | [20] |
| Greece | Dogs | 25.2% | Cats | 20.5% | [29] |
| Spain | Dogs | 33% | Cats | 9.2% | [30] |
| Ontario | Dogs | 64.0% | Cats | 87.0% | [31] |
| Brazil | Dogs | 19.6% | - | - | [32] |
| (b) Prevalence of *Enterocytozoon bieneusi* in dogs and cats in different regions of the world. | | | | | |
| China | Dogs | Dogs 6.0% | Cats | 5.6% | [7] |
| Heilongjiang | Dogs | 6.7% | Cats | 5.8% | [6] |
| Henan | Dogs | 13.9% | Cats | 11.5% | [33] |
| Eastern China | Dogs | 8.6% | Cats | 1.4% | [34] |
| Changchun | Dogs | 7.8% | - | - | [35] |
| Yunnan | Dogs | 8.0% | Cats | 2.3% | Present study |
| Other countries | | | | | |
| Colombia | Dogs | Dogs 15.0% | Cats | 17.4% | [36,37] |
| Egypt | Dogs | Dogs 13.0% | Cats | 12.5% | [38] |
| Germany | Dogs | 0.0% | Cats | 5.0% | [39] |
| Spain | Dogs | 0.8% | Cats | 3.0% | [40] |
| Japan | Dogs | 2.5% | Cats | 14.3% | [41] |
| Poland | Dogs | 4.9% | Cats | 9.1% | [42] |
| Thailand | Dogs | 0.0% | Cats | 31.3% | [43] |
| Portugal | Dogs | 100.0% | Cats | 100.0% | [44] |
| Iran | Dogs | 25.8% | Cats | 7.5% | [45] |
| (c) Prevalence of *Cryptosporidium* spp. in dogs and cats in different regions of the world. | | | | | |
| China | Dogs | Dogs 6.0% | Cats | 5.6% | [7] |
| Heilongjiang | Dogs | 6.7% | Cats | 5.8% | [6] |
| Zhengzhou | Dogs | 3.1% | - | - | [46] |
| Ya’an | Dogs | 7.5% | - | - | [47] |
| Yunnan | Dogs | 4.6% | Cats | 0.6% | Present study |
| Other countries | | | | | |
| Japan | - | - | Cats | 1.4% | [48] |
| Spain | Dogs | Dogs 5.5% | Cats | 8.8% | [16] |
| Germany | Dogs | 1.2% | Cats | 5.3% | [49] |
| Greece | Dogs | 5.9% | Cats | 6.8% | [29] |
| Thailand | Dogs | 2.1% | Cats | 2.5% | [50] |
| Brasil | Dogs | 24.5% | Cats | 11.1% | [51] |
| Italy | Dogs | 1.7% | - | - | [52] |
| Netherlands | Dogs | 8.7% | Cats | 4.6% | [53] |

In the present study, the prevalence of *G. duodenalis* in dogs is higher than that in Heilongjiang (4.5%) [6], Guangdong (10.8%) [25] and Sichuan (11.3%) [26] provinces, China, and is also higher than other zoonotic pathogens in dogs, such as 10.3% for *Babesia canis*, 9.1% for *Anaplasma* spp., 4.5% for *Leishmania infantum*, 1.7% for *Borrelia burgdorferi*, 0.4% for *Ehrlichia* spp. and 1.7% for *Dirofilaria immitis* in Italy [54], but is lower than that in Henan province (14.3%) [27], Shanghai city (26.2%) in China [7] and other countries (Table 5).
Similarly, the G. duodenalis prevalence in pet cats is consistent with that in Hangzhou city (1.2%) [28], China; but is lower than that in Heilongjiang (1.9%) [6] and Guangdong (5.8%) provinces [25] and Shanghai city (13.1%) [7] in China and other countries (Table 5), and is also lower than L. infantum (3.0%) in Greece and Italy; Rickettsia felis (10.8%), Rickettsia typhi (4.2%), Anaplasma phagocytophilum (2.4%) and Ehrlichia canis (2.4%) in cats in Italy [55,56]. The reason is complicated among different studies because many factors could affect the prevalences such as sample sizes, sample sources, environments, animal welfare, hygiene conditions, age and sex of samples, and the sensitivity of tested methods. Moreover, the living condition is a risk factor \( (p < 0.05) \) that is significantly related to the prevalence of G. duodenalis in pet dogs in this study. We suspect that the poor sanitation of shelters contributes significantly to nosocomial transmission, adding to the prevalence of G. duodenalis in pet dogs. Furthermore, the higher prevalence of G. duodenalis was detected in pet dogs in Kunming city \( (p < 0.05) \) (Table 1), which suggests that the region is also a risk factor significantly associated with G. duodenalis infection in this study. In addition, the prevalence of G. duodenalis in male dogs was higher than that in female dogs in the present study, which is consistent with observations in other previous studies [2,57], although the difference was not statistically significant \( (p > 0.05) \). Compared with dogs, cats seem to be less susceptible to infection with G. duodenalis (Table 1). This might be explained by the different living habits of these two animals.

Similar to G. duodenalis, the prevalences of E. bieneusi in pet dogs and cats in different regions are different (Table 5). This is probably because the route and source of infection for dogs or cats in each region may be different. In addition, other factors can also affect the prevalence of E. bieneusi in dogs and cats. Furthermore, statistical analysis showed that a significant difference was observed among pet dogs in shelters, pet markets and pet hospitals (Table 1), which indicates that dogs living in shelters are more easily infected with E. bieneusi than those dogs in pet hospitals and markets. The reason may be the poorer hygiene conditions in shelters compared with pet markets and pet hospitals. Dogs aged more than 6 months seemed to be more susceptible to infection with E. bieneusi \( (p < 0.05) \) (Table 1), suggesting that further relevant research should pay more attention to the adult dogs. Additionally, only cats in Chuxiong city were found to be infected by E. bieneusi (Table 1); thus, we speculate that the regional factors may have a significant effect on the prevalence of E. bieneusi in cats. But this hypothesis needs to be tested. Additionally, there was no significant difference in the prevalence of Cryptosporidium spp. rate in pet dogs or cats (Table 1).

Up to now, six assemblages (assemblage A, B, C, D, E and F) have been identified in dogs and cats in previous studies [6,7,25–27,31], and canine-specific and feline-specific assemblages C, D and F are also found in other animals [11]. These findings indicate that both dogs and cats are a reservoir of G. duodenalis, which has risk of transmission among different animals. In the present study, only two assemblages (C and D) were identified in pet dogs, which is similar to previous studies [26,27]. Furthermore, a previous work demonstrated that the assemblages C and D are more sensitive than assemblage A in pet dogs [58]. Moreover, we found nine subtypes of assemblage (at bg locus, \( n = 4 \), at gdh locus, \( n = 4 \) and at tpi locus, \( n = 1 \) in dogs and one subtype of assemblage (at bg locus, \( n = 1 \) in cats (Table 2). The assemblage of G. duodenalis in dogs in the current study seems to more likely to mutate, thus further studies need to examine the genetic structure of these subtypes. Also, one mixed genotype of G. duodenalis was found in dogs in this study (Table 3), revealing the diversity of G. duodenalis in our investigation area.

Early studies have reported that genotypes of E. bieneusi CD1 to CD8, D, O, PigEBITSS, EbpA, CMI, Peru8 and EbpC are identified in dogs, and genotypes D, BEB6, I, CC1, CC2, CC3, CC4 are identified in cats in other provinces of China [33,59]. In the present study, the dominant genotype of E. bieneusi PtEb IX (18/21) is a common dog-specific E. bieneusi genotype identified in dogs (Table 4). Additionally, two novel genotypes (DgEb I and DgEb II) were also identified in dogs in our study, which enrich the genotype variety of E. bieneusi in dogs. E. bieneusi genotype Type IV and novel genotype CtEb I in pet cats.
belonged to Group 1 of zoonotic potential (Figure 1), which imply that pet cats may be a potential source of human infection with *E. bieneusi* in Yunnan province, China.

According to previous studies, *C. ubiquitum* and *C. canis* are commonly found in dogs, and *C. parvum* and *C. felis* are commonly found in cats in Heilongjiang, Shanghai and other cities or provinces of China [6,7]. In the present study, we only identified *C. canis* and *C. felis* in pet dogs and cats, respectively (Table 4). By contrast with the current study, the *C. parvum* and *C. muris* have been found in dogs or cats in other countries [20–22,36,60]. Despite our results revealing the presence of host-specific *Cryptosporidium* spp. species (*C. canis* and *C. felis*) in pet dogs and cats, these two species have been reported in humans and mainly in developing countries [6]. This finding suggests that people still need to take further precautions when they are in close contact with their pets. In addition, some nucleotide sequences of *Cryptosporidium* spp. obtained in pet dogs and cats have mutations in this study (Table 4).

4. Materials and Methods

4.1. Study Sites

The fecal samples of pet dogs and cats were collected in Kunming city, Lijiang city and Chuxiong city in Yunnan province (Location: 21°8′ N to 29°15′ N and 97°31′ E to 106°11′ E), southwestern China, which covers more than 390,000 square kilometers and has a population of approximately 48 million.

4.2. Sampling

During August to September 2018, a total of 433 fresh fecal samples were collected from pet dogs and cats in three cities of Yunnan province, including Kunming city (134 dogs and 36 cats), Lijiang city (90 dogs and 110 cats) and Chuxiong city (38 dogs and 25 cats). The Kunming, Lijiang and Chuxiong cities have more numbers of pet dogs and cats than other cities of Yunnan province, and all the samples of the cats and dogs were randomly collected from the biggest pet hospital, pet market and shelter in each city (i.e., Kunming city, Lijiang city and Chuxiong city), respectively. Moreover, the information regarding regions, ages, genders and living conditions were recorded. All the fecal samples were saved into 15 mL centrifuge tube with 2.5% potassium dichromate, and then were stored at 4 °C until for DNA extraction.

4.3. Genomic DNA Extraction and PCR Amplification

Each fecal sample was washed three times with distilled water by centrifuging at 13,000 g for 5 min to remove potassium dichromate, and 300 mg of the precipitated samples were used for DNA extraction using the E.Z.N.A. Stool DNA kit (OMEGA, Biotek Inc. USA) according to the manufacturer’s instructions. The genomic DNA was stored at −20 °C before PCR amplification. The *G. duodenalis* identification was performed by nested PCR amplification of bg, gdh and tpi gene loci according to previous reports [25,61], *Cryptosporidium* spp. identification was conducted by nested PCR amplification of the 18S ribosomal RNA [62], and *E. bieneusi* identification was carried out by nested PCR amplification of ITS rDNA sequences as previously described [63]. The positive and negative controls were included in each PCR reaction. All the secondary PCR products were checked by 2% (w/v) agarose gel electrophoresis after ethidium bromide staining and visualized under UV light.

4.4. Sequence Analysis

The PCR-positive products were sent to Tsingke Biological Technology Company (Xi’an, China) for two-directional sequencing. The obtained sequences were spliced together after initial collation with their DNA peak form graph by Chromas v.2.6. The genotypes/species of *G. duodenalis*, *Cryptosporidium* spp. and *E. bieneusi* were identified by aligning the obtained sequences with corresponding sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/, accessed on 11 July 2021). The phylogenetic
tree was established by neighbor-joining method (NJ) with Kimura 2-parameter model in MEGA 7.0 (http://www.megasoftware.net/, accessed on 11 July 2021). The novel genotypes of *E. bieneusi* were decided by the ~243-bp ITS region [64,65].

### 4.5. Statistical Analysis

Prevalence of *G. duodenalis*, *Cryptosporidium* spp. and *E. bieneusi* in age, regio, gender and living conditions groups were analyzed using Chi-square test in SPSS 24.0 (SPSS Inc., Chicago, IL, USA). The 95% confidence intervals (CIs) were estimated. The difference was considered statistically significant when *p*-value < 0.05.

### 5. Conclusions

The present investigation revealed the prevalence and assemblages/genotypes/species of *G. duodenalis*, *E. bieneusi* and *Cryptosporidium* spp. in pet dogs and cats in Yunnan province, China. The infection with *G. duodenalis*, *E. bieneusi* and *Cryptosporidium* spp. in dogs and cats suggests that we should take measures to prevent and control those pathogens from being transmitted to other animals and humans. Our data provided the valuable information for a better understanding of the epidemiology and public health threat of *Giardiasis*, *E. bieneusi* and *Cryptosporidium* spp. in pet dogs and cats in southwestern China.

**Author Contributions:** F.-C.Z., Y.Z. and X.-Q.Z. conceived and designed the study. Y.-G.W. performed the experiments, analyzed the data and drafted the manuscript. Y.Z., Z.-Z.Y., D.C., B.-Z.G., J.-F.Y. and G.-H.L. participated in the implementation of the study. X.-Q.Z., F.-C.Z., Y.Z. and Y.-G.W. critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study protocol has been reviewed and approved by the institutional animal ethical committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The approval code: AECLVRI-2018-003.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data that support the figures within this paper and other findings of this study are available from the corresponding authors upon reasonable request. All of the obtained representative *G. duodenalis* bg, gdh and tpi nucleotide sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/ accessed on 26 November 2019) under the accession numbers MN734348- MN734356, MN734357-MN734367 and MN734368, respectively. The nucleotide sequences of *Cryptosporidium* spp. and *E. bieneusi* were deposited in GenBank under accession numbers MZ540366-MZ540371 and MZ542369-MZ542375, respectively.

**Conflicts of Interest:** The authors declare no conflict of interest.

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