BMC Veterinary Research

RESEARCH Open Access

Establishment and preliminary application of PCR-RFLP genotyping method for *Giardia duodenalis* in goats

Xuanru Mu[1†], Jianchao Guo[2†], Hongcai Wang[1], Yilong Li[1], Kaijian Yuan[1], Hui Xu[1], Wenjing Zeng[1], Qiaoyu Li[1], Xingang Yu[1*] and Yang Hong[3,4*]

Abstract

Background *Giardia duodenalis* (*G. duodenalis*) is a globally distributed zoonotic protozoan that parasitizes the small intestines of humans and various mammals, such as goats and sheep. The objective of this study was to establish a convenient, accurate, and specific method based on restriction fragment length polymorphism (RFLP) for genotyping assemblages A, B and E of *G. duodenalis* in goats. The *β-giardin* gene was amplified using primer pairs *bg*F1, *bg*R1, *bg*F2 and *bg*R2 by nested PCR. The PCR products were digested with the restriction enzymes *Hinf* I and *Bgl* I. The established PCR-RFLP method was used to detect and analyze the genetic subtypes of *G. duodenalis* in 130 fecal samples from goats and compared simultaneously with microscopic examination and nucleic acid sequencing for *G. duodenalis*.

Results Genetic sequencing confirmed that the PCR-RFLP method accurately distinguished *G. duodenalis* assemblages A, B and E, as well as different combinations of mixed infections of these three assemblages. Among the 130 samples tested by PCR-RFLP, a total of 26 samples (20.00%) tested positive for *G. duodenalis*, a higher sensitivity than microscopic examination at 13.85% (18/130). Sequence alignment analysis revealed that among the 26 PCR-positive samples, two were identified as assemblage AI, while the remaining 24 were identified as assemblage E or E12.

Conclusions This study established an accurate, efficient and rapid PCR-RFLP genotyping method using the *bg* sequence of *G. duodenalis*, enabling accurate identification and effective differentiation of goat-derived *G. duodenalis* assemblages without requiring sequencing.

Keywords *Giardia Duodenalis*, Genotype, PCR-RFLP, Goat, Restriction enzymes

†Xuanru Mu and Jianchao Guo contributed equally to this work.

*Correspondence:
Xingang Yu
yuxingang4525@163.com
Yang Hong
hongyang_7@126.com
[1]School of Animal Science and Technology, Foshan University, Foshan 528231, China
[2]Agro-Tech Extension Center of Guangdong Province, Guangzhou 510500, China
[3]National Key Laboratory of Intelligent Tracking and Forecasting for Infectious Diseases, Key Laboratory of Parasite and Vector Biology, Collaborating Center for Tropical Diseases, National Institute of Parasitic Diseases, Chinese Center for Diseases Control and Prevention (Chinese Center for Tropical Diseases Research), National Health Commission of the People's Republic of China (NHC), World Health Organization (WHO), National Center for International Research on Tropical Diseases, Shanghai 200025, China
[4]Hainan Tropical Diseases Research Center (Hainan Sub-Center, Chinese Center for Tropical Diseases Research), Haikou 571199, China

Background

Giardia duodenalis (syn. *G. lamblia*, *G. intestinalis*) is an important and common zoonotic parasite that infects many mammals, including goats, cattle, cats, dogs and humans [1–3]. The life cycle of *G. duodenalis* is simple, consisting of two stages with the disease producing trophozoites and infectious cysts [4]. The cysts can infect humans and various mammals through contaminated food and water, causing giardiasis, which is characterized primarily by symptoms such as abdominal pain, diarrhea, vomiting and malabsorption [5–7]. Since the 1970s, giardiasis has been prevalent or has occurred in outbreaks worldwide, and is listed by the World Health Organization as one of the neglected diseases that endanger human health [2], with more than 280 million cases of human giardiasis are estimated to occur worldwide each year [8]. *G. duodenalis* is described as a species complex consisting of eight distinct genetic assemblages A to H, identified through genetic analysis. Assemblages A and B are found in humans and various mammals, while assemblages C to H have more distinct host specificity. Assemblages C and D are specific to dogs, assemblage E is commonly found in goats, pigs and other artiodactyls; assemblage F in cats; assemblage G in rodents and assemblage H in pinniped [9].

Direct microscopy is widely used for the clinical detection of *G. duodenalis*, but it is time-consuming, labor-intensive and prone to missed detections in practical applications [10]. As it is difficult or unable to differentiate different *G. duodenalis* assemblages based on morphological characteristics alone, accurate identification of its genotypes requires molecular methodology [11]. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) is a simple, fast and accurate molecular marker method. The fundamental principle of PCR-RFLP involves the digestion of PCR-amplified DNA fragments from target genes with specific restriction endonucleases. Pathogen genotypes are identified based on the electrophoretic patterns of the resulting digestion products, making further sequencing or sequence alignment analysis unnecessary. This significantly reduces detection time and eliminates dependence on expensive instrumentation [12]. Xu et al. [13] successfully established a PCR-RFLP genotyping method for *Balantioides coli*, that accurately distinguished genetic variants A and B of *Balantioides coli*. We previously developed a PCR-RFLP method for typing zoonotic (A, B) and host-specific (C, D) assemblages of *G. duodenalis* from dogs, which effectively distinguished the common four assemblages A, B, C and D [14].

Goats are an economically efficient type of livestock that yield high-quality meat, cashmere, and milk [15, 16]. *G. duodenalis* is widely distributed among ruminant populations, with infection rates in goats or sheep worldwide ranging from 2.9 to 42.2% [17]. The *G. duodenalis* strains infecting goats or sheep primarily belong to assemblage E, but they frequently occur as mixed infections involving assemblages A and E, A and B, A, B or E [16, 18, 19]. Goats are commonly raised in free-range systems, with their feces directly discharged into the surrounding environment without further treatment. As significant and prevalent hosts of *G. duodenalis*, goats carry zoonotic assemblages A and B, which risk zoonotic transmission through contaminated water sources to humans and other mammal [20–22].

In the present study, the PCR-RFLP method was developed for the rapid detection and genotyping of goat-derived assemblages A, B and E, as well as several combinations of mixed infections involving these three assemblages, providing technical support for a molecular epidemiological survey of goat-derived *G. duodenalis*.

Methods

Source of samples

Genomic DNAs of assemblages A, B and E were provided by the Parasite Laboratory of Foshan University (Guangdong, China). In December 2022, a total of 150 fresh goat fecal samples were collected from three farms in Zhanjiang City, Guangdong Province, including 20 goats that were younger than three months and 130 goats aged four to 30 months. Each 10 g sample was individually packed in a clean, sealed bag and marked with the farm, goat age and date of collection. The samples were transported quickly to the laboratory while packed in ice, then stored at 4 ℃. The 130 fresh goat fecal samples were used to assess the accuracy and reliability of the PCR-RFLP genotyping method established in this study. Twenty fecal samples from juvenile goats that were confirmed negative by nested PCR [16], were used as controls for the sample testing.

Microscopic examination

All fecal samples were concentrated by flotation using 33.2% zinc sulfate with a specific gravity of 1.3. The method was carried out as described in previous reports with some modifications [2, 23]. Briefly, two to four grams of feces were diluted with sterile water (about 5 ~ 10 mL), passed through a 60-mesh copper sieve into a 50 mL centrifuge tube (Corning Science, Shanghai, China), and centrifuged at 200×*g* for 5 min. The supernatant was discarded and then zinc sulfate solution (about 5 ~ 10 mL) was added along the rim of the tube, followed by thorough mixing. The supernatant was carefully transferred into new 15 mL centrifuge tube (Corning Science, Shanghai, China), and a cover slip was then carefully placed on top of the tube, contacting the crescent-shaped meniscus formed by the zinc sulfate solution. The tube was subsequently centrifuged at 500×*g* for 1 min with the

Table 1 Primers for *bg* gene sequence of nested PCR

Primer	Sequence	Tm(℃)	Predicted fragment size (bp)
*bg*F1	5′-TCGACGACGACACGCGCGTGAAGA-3′	60	619
*bg*R1	5′-GAGACGACGTCCTCGAGAGTGTTG-3′		
*bg*F2	5′-AAGCGCCAGGCCTCGTTCGAG-3′	58	516
*bg*R2	5′-GATCTTGTCCTCTGCCTCCTTGCG-3′		

Table 2 Expected results of restriction endonuclease digestion of *Hinf* I and *Bgl* I

Assemblage	PCR amplicons (bp)	Cleavage site (number)	Predicted fragment size (bp)
Assemblage A	516	1	193;323
Assemblage E	516	2	66*; 193; 257
Assemblage B	516	0	516
Assemblages A, B	516	1	193;323;516
Assemblages A, E	516	3	66*;193;257;323
Assemblages B, E	516	2	66*;193;257;516
Assemblages A, B, E	516	3	66*;193;257;323;516

Note: * Indicates that it may not be displayed in the gel

cover slip in place. Following centrifugation, the cover slip was gently removed and immediately examined under the microscope.

Fecal DNA extraction and PCR amplification of *β-giardin*

Approximately 200 to 300 mg of each sample was used to extract DNA with E.Z.N.A.® Stool DNA Kit (Omega, Norcross, GA, USA) [2, 16, 23], according to the instructions of the kit. The DNA samples were stored at −20 ℃ before PCR analysis.

Two pairs of primers for the *bg* gene of *G. duodenalis* were designed in Table 1 using DNAMAN (v6.0) software. All PCRs were performed in a total volume of 25 µL, consisting of 8.5 µL ddH_2O, 2 µL of each primer pair *bg*F1/*bg*R1 (*bg*F2/*bg*R2) (10 µM), 12.5 µL 2× Taq Master Mix (Dye Plus) (Vazyme, Nanjing, China), and 2 µL DNA sample. The thermocycler program was consisted of 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 40 s, annealing at 60 ℃ for 30 s (second round at 58 ℃ for 30 s), extension at 72 ℃ for 60 s and a final extension at 72 ℃ for 10 min.

The secondary PCR was performed in a similar reaction condition as the primary PCR, except for the replacement of the template with 2 µL primary amplicon. The positive PCR products exhibited an expected band of 516 bp length, visualized using a UV transilluminator (Dublin, CA, USA) after electrophoresis on a 1.2% agarose gel.

Establishment of the PCR-RFLP method

The reference *bg* gene sequences MK610389 (assemblage E), MK610390 (assemblage E), KP687765 (assemblage A), KM926506 (assemblage A), EU626199 (assemblage B) and MT542772 (assemblage B) were analyzed using Primer premier 5.0 software (Premier Biosoft Intl., CA USA). Two restriction enzymes, *Hinf* I and *Bgl* I were then selected to digest the PCR products and the expected results are shown in Table 2. Digestion was performed in a 20 µL reaction volume containing 10 µL PCR product, 2 µL 10×M Buffer, 2 µL QuickCut^{Tm}*Hinf* I (Takara, Beijing, China) and 6 µL ddH_2O for 2 h at 37℃, followed by the addition of 1 µL *Bgl* I and 2.1 µL 10 × *Bgl* I Buffer (Takara, Beijing, China), and incubated for 3 h at 37℃. The digested PCR products were analyzed by 3% agarose gel electrophoresis stained with ethidium bromide.

At the same time, nucleic acid sequencing of *G. duodenalis* was performed, involving sequence analysis of PCR products from the *bg*, glutamate dehydrogenase (*gdh*) and triosephosphate isomerase (*tpi*) genes with multilocus genotyping (MLG). Three primer pairs (Table S1) were used following the methodology described in a previous study [16]. Positive PCR products of the *bg*, *gdh*, and *tpi* genes from *G. duodenalis* assemblages A, B and E, as well as several combinations of mixed infections involving these three assemblages, were sent to Sangon Biological Engineering Technology and Service Co., Ltd. (Songjiang, Shanghai, China) (Availability of data: The accession numbers PQ284089–PQ284142).

Clinical sample testing and phylogenetic analysis

A total of 130 black goat fecal samples were collected to further evaluate the accuracy and reliability of the PCR-RFLP method established in this study. The *β-giardin* gene sequences from all positive PCR products were sent to Sangon Biological Engineering Technology and Service Co., Ltd. The obtained sequences (Availability of data: The accession numbers PQ284063–PQ284088) were compared with reference sequences retrieved from the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 July 2024). Phylogenetic analysis was performed using maximum likelihood (ML), with bootstrap values calculated by analyzing 1000 replicates and other selected default parameters in MEGA 7.0 (http://www.megasoftware.net/, accessed on 9 July 2024).

Results

PCR product of the *β-giardin* gene

The nested PCR amplification of the *β-giardin* gene from *G. duodenalis* yielded a DNA fragment approximately 516 bp in size, consistent with the expected fragment length (Fig. 1).

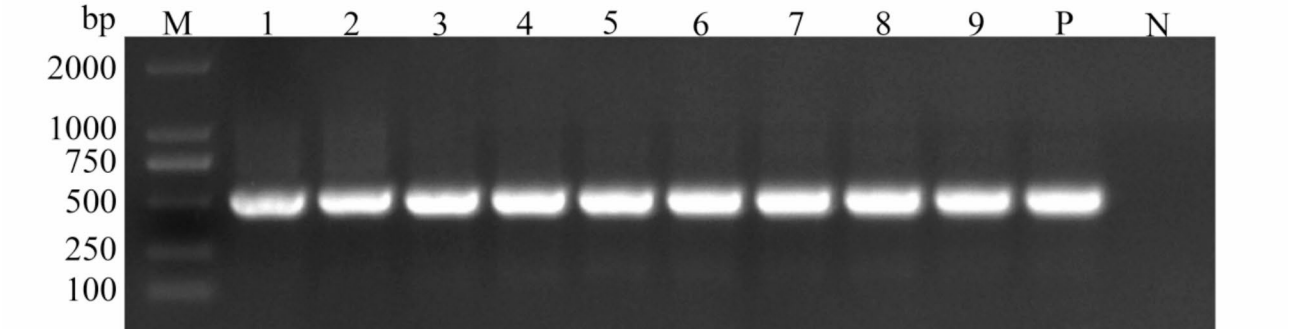

Fig. 1 PCR amplification of *bg* gene of *G. duodenalis* in partial goat-derived samples. M: DL-2000 DNA Marker; 1–9: Goat-derived samples; 10: Positive control; N: Negative control

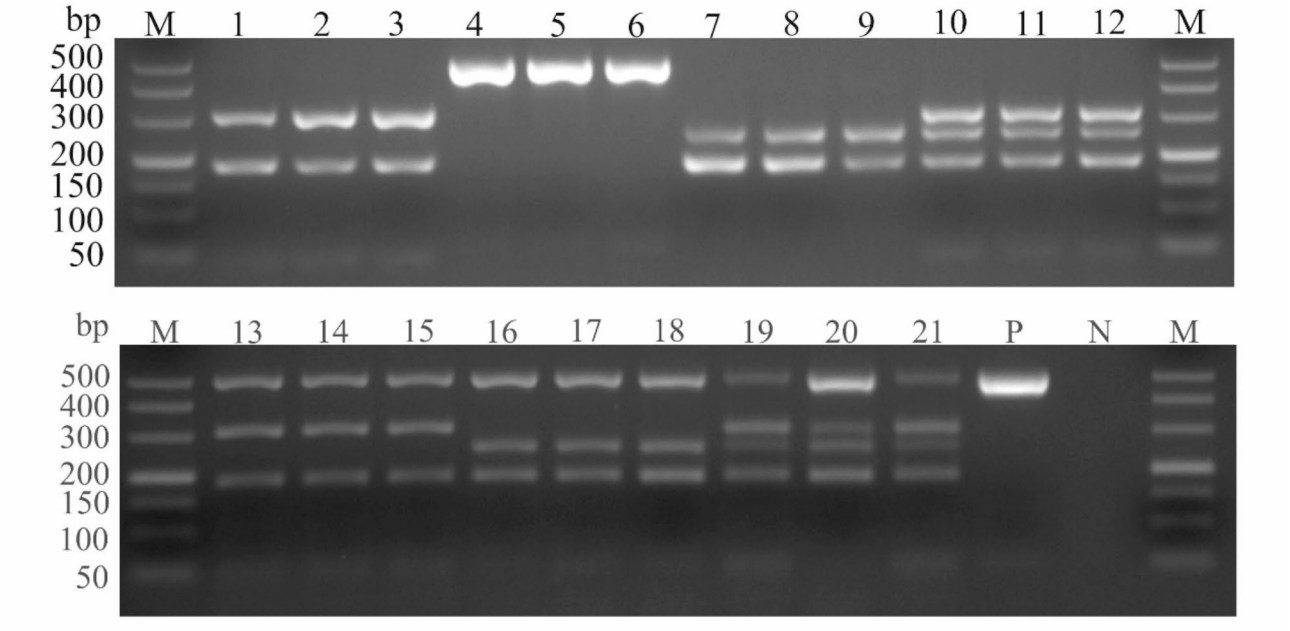

Fig. 2 Restriction enzyme digestion results of PCR products with *Hin*f I and *Bgl* I. M: DL-500 DNA Marker; 1–3: assemblage A; 4–6: assemblage B; 7–9: assemblage E; 10–12: mixed type of assemblage A, E; 13–15: mixed type of assemblage A, B; 16–18: mixed type of assemblage B, E; 19–21: mixed type of assemblage A, B, E; P: Positive control; N: Negative control

Restriction enzyme analysis

The PCR products were digested with *Hin*f I and *Bgl* I and the resulting fragments were separated using gel electrophoresis. Distinct restriction patterns were observed among isolates belonging to assemblages A, B, E, mixed types of A and B, A and E, B and E, and the combination of A, B and E (Fig. 2).

The PCR products derived from assemblage A isolates were cleaved into two fragments measuring 193 bp and 323 bp in length. Those from assemblage B samples did not contain *Hin*f I and *Bgl* I restriction sites, resulting in a single fragment of 516 bp. Assemblages E isolates yielded three fragments of 66, 193 and 257 bp. Mixed type isolates of assemblages A and B produced three fragments of 193, 323 and 516 bp. Mixed type isolates of assemblages A and E yielded four fragments of 66, 193, 257 and 323 bp. Mixed type isolates of assemblages B and E produced four fragments of 66, 193, 257 and 516 bp and mixed type isolates of assemblages A, B and E resulted in five fragments of 66, 193, 257, 323 and 516 bp. These findings were consistent with the sequencing analysis, so the genotyping of goat-derived *G. duodenalis* could be rapidly distinguished using *Hin*f I and *Bgl* I restriction enzymes.

The sequencing results were compared with those obtained using the PCR-RFLP method established in this study (Table S2). The results indicated that the PCR-RFLP method effectively differentiated *G. duodenalis*

Table 3 Comparative analysis of PCR-RFLP and microscopy observation of clinical fecal samples

Method		Microscopy observation		Total	Coincidence rate (%)
		Positive	Negative		
PCR-RFLP Result	Positive	18	8	26	100
	Negative	0	104	104	92.85
	Total	18	112	130	93.84

assemblages A, B, and E, along with several combinations of mixed infections involving these assemblages, consistent with the results of genetic sequencing.

Comparison with microscopy and sequencing method

Both the PCR-RFLP method and microscopy were simultaneously used to test 130 fecal samples. The PCR-RFLP results revealed that 26 samples were positive for *G. duodenalis*, resulting in a positivity rate of 20.00%. In contrast, microscopy detected only 18 samples, resulting in a positivity rate of 13.85%. The PCR-RFLP method exhibited a positive concordance rate of 100%, a negative concordance rate of 92.85% and an overall concordance rate of 93.84% (Table 3).

Among the 26 PCR-RFLP positive samples, 24 were identified as assemblage E and 2 as assemblage A (Fig. 3). Notably, no cases of assemblage B or mixed infections were detected and the PCR-RFLP method's accuracy aligned perfectly with the sequencing results.

Phylogenetic analysis

The PCR products of the *bg* gene from positive samples were sequenced, yielding a total of 26 *G. duodenalis bg* sequences. Of these, two *bg* sequences were identified as assemblage A, while the remaining 24 were classified as assemblage E or subtype E12 (Fig. 4). The 2 assemblage AI sequences shared 100% *bg* sequence homology with KP687765 from human samples in Spain.

In contrast, the remaining 24 sequences belonged to assemblage E or its subtypes. Among these, 22 sequences of assemblage E showed 97 to 100% homology with MK452880 from sheep in Greece, while the other two sequences E12 shared over 99% homology with KY432834 from dairy cattle in China (Fig. 4).

Discussion

In developing countries, giardiasis detection rates are higher than in developed nations due to factors like socioeconomic conditions, sanitation, and exposure to contaminated water or infected individuals [17, 24]. Ingesting as few as 10 viable cysts can cause *G. duodenalis* infection in humans [25]. Assemblages A and B infect both humans and mammals via contaminated food and water, while assemblage E mainly affects artiodactyls such as goats and sheep, though human infections have also been reported [9, 26, 27]. In China, the prevalence of giardiasis in sheep and goats is around 7%, ranging from 0 to 28.93% across provinces [17]. Infections in goats and sheep are predominantly caused by assemblage E, but mixed infections involving assemblages A and E, A and B, or A, B, and E [3, 17, 18, 28–31]. Given that goat farming is often extensive with limited fecal disinfection, these assemblages pose zoonotic risks, underscoring the need for rapid molecular detection to monitor genetic diversity and control strategies.

Common genetic typing methods for *G. duodenalis* include nested PCR-based multi-locus sequence analysis

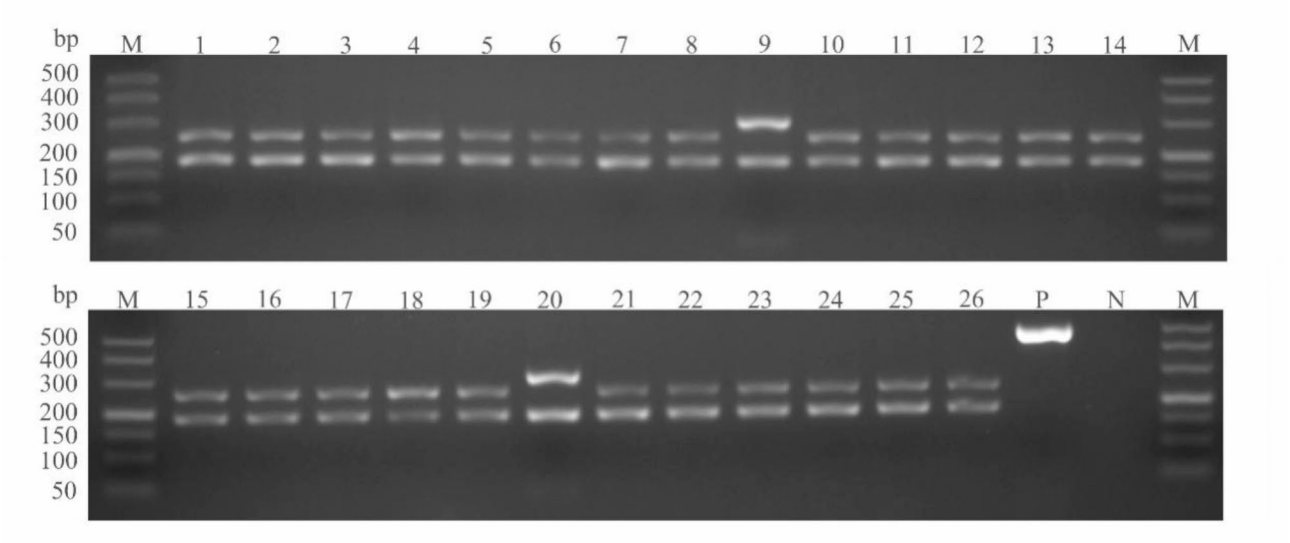

Fig. 3 PCR-RFLP identification results of goat-derived *G. duodenalis* from positive samples. M: DL-500 DNA Marker; 1–26: Samples; P: Positive control; N: Negative control

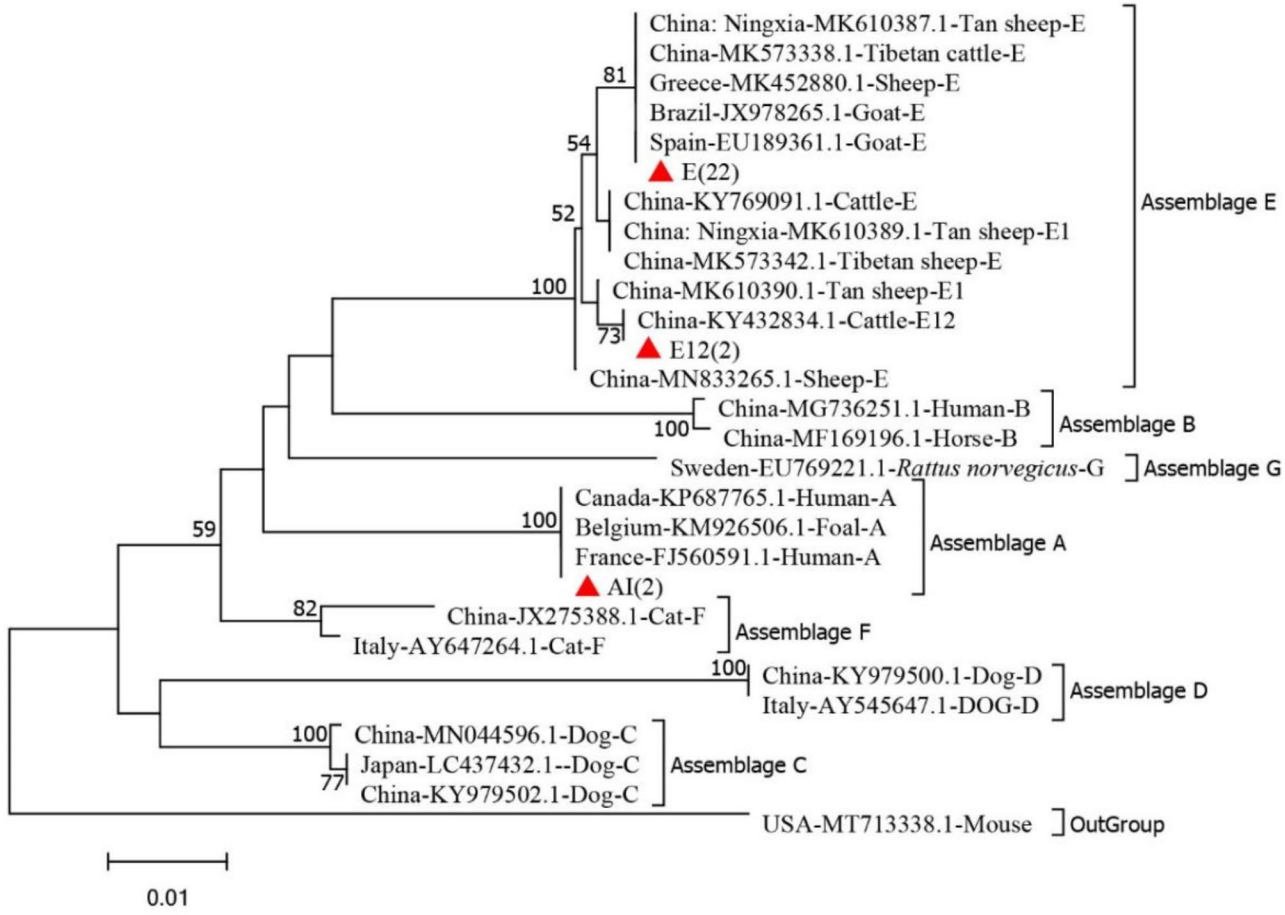

Fig. 4 Phylogenetic tree of *G. duodenalis* in black goats based on *bg* gene sequences. The Tamura-Nei model method was used with bootstrap evaluation of 1000 replicates. All the genotypes identified in this study are marked by red solid triangles. Bootstrap values are shown when > 50%

[14, 16], oligonucleotide microarray [32], and high-resolution melting (HRM) [33]. Each technique has specific advantages and drawbacks: multi-locus sequence analysis is accurate but time-consuming and expensive [14]; microarray is rapid but costly and requires extensive probe synthesis; HRM is fast and specific but requires specialized equipment. PCR-RFLP, on the other hand, is cost-effective, rapid, and user-friendly, requiring only basic equipment for gel electrophoresis of enzyme-digested products [13, 14]. The small subunit ribosomal RNA (*SSU-rRNA*), glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*) and *bg* genes are among the most commonly used loci to identify multiple variants of *G. duodenalis* in different host species [34]. Among these, *bg* is a specific gene unique to *G. duodenalis* and using the *bg* gene for detection yielded highly sensitive results [16], so *bg* was selected as the target gene for method establishment in this study.

Sequence analysis of the *bg* gene using Snapgene and Premier 5.0 software revealed that assemblages A and E shared a common *Bgl* I restriction site at gene loci 187–197. Assemblage E possesses a *Hinf* I restriction site at loci 450–455, whereas assemblage B does not have the *Bgl* I and *Hinf* I restriction sites. Comparative analysis of multiple previously published gene sequences identified positions 187–197 and 450–455 as highly conserved regions within the amplified sequences, indicating robust stability. Finally, *Hinf* I and *Bgl* I were used to digest the PCR products. The results shown that assemblage A contained two fragments of 193 and 323 bp; assemblage B had only one fragments of 516 bp; assemblage E had three fragments of 66, 193 and 257 bp; assemblages A and B had three fragments of 193, 323 and 516 bp; assemblages A and E shared four fragments of 66, 193, 257 and 323 bp; assemblages B and E shared four fragments of 66, 193, 257 and 516 bp; and assemblages A, B and E shared five fragments of 66, 193, 257, 323 and 516 bp (Fig. 2). The number and size of the bands can be easily distinguished in agarose gel electrophoresis, making it more economical and efficient than sequencing analysis.

Twenty-six out of 130 goat fecal samples tested positive for *G. duodenalis* using PCR-RFLP, yielding an overall prevalence of 20.00%, whereas microscopy detected only 18 positive samples, resulting in a positivity rate of

13.85%. The PCR-RFLP demonstrated higher detection sensitivity compared to microscopy, which is not only limited by its lower sensitivity but also time-consuming and labor-intensive. Furthermore, microscopic examination cannot accurately distinguish between different *Giardia* assemblages. This limitation is particularly pronounced in diarrheal feces, where *G. duodenalis* typically exists as trophozoites, which are easily disintegrated by external environmental factors (such as high temperature and dryness), often leading to missed or misdiagnoses. Based on PCR-RFLP genotyping of the *bg* gene sequence, only two assemblages of *G. duodenalis* were identified. Two samples were identified as assemblage A and 24 samples were identified as assemblage E. No assemblage B or mixed infections involving different assemblages were observed (Fig. 3). The phylogenetic analysis results indicated that two of these *bg* sequences were identified as assemblage AI, while the remaining 24 sequences belonged to assemblage E or E12. The results were consistent with the PCR-RFLP genotyping results obtained in this study, confirming their accuracy and reliability. In the comparison of 130 clinical samples, neither PCR-RFLP genotype nor PCR sequencing genotype methods detected mixed infections of *G. duodenalis*. This may be attributed to the actual prevalence of *G. duodenalis* complexes in the goat population studied or to sample size limitations. The current set of 130 samples used only for comparative validation purposes might not encompass all potential infection scenarios, so the absence of mixed infections was not detected.

Given the considerable diversity of *G. duodenalis* assemblages (A, B, E, A and E, A and B, B and E or A, B, and E) in goats, the method developed in this study was primarily designed to enable the rapid and accurate differentiation of the three major *G. duodenalis* assemblages. It does not extend to the more refined differentiation of sub-assemblages (e.g., AI, AII, BIII, BIV) or genotypes (e.g., E12). A significant concern is that including additional genetic subtypes in the enzyme restriction analysis could lead to overlapping band sizes, making it difficult to distinguish these variants both accurately and quickly. This would compromise the primary advantage of the method—its accuracy and convenience. Additionally, during the initial stages of experimental design, we noted that several existing studies have established PCR-RFLP genotyping methods for *G. duodenalis* [14, 35, 36], such as GDH-based assay [35], for distinguishing most different *G. duodenalis* assemblages (AI, AII, BIII, BIV, C, D and E). However, in clinical mixed infection feces, particularly those assemblages found in goats (such as AI, AII, and E), the size disparities following GDH gene digestion are often ≤30 bp (e.g., 70, 80, 90, 100, 120, 150) [35]. Such small differences in size can be easily influenced by variations in agarose gel concentration and electrophoretic conditions, making it difficult to rapidly distinguish these variants and increasing the risk of misinterpretation. In contrast, the method described in this study results in band size differences of ≥64 bp following enzyme digestion, allowing for a clearer visual distinction of the bands on gel electrophoresis. However, the present method does not address finer distinctions among sub-assemblages. This limitation is particularly relevant given reports suggesting that *G. duodenalis* sub-assemblage AII may represent a distinct species and call for a taxonomic revision of *G. duodenalis* [37, 38]. Future research will aim to refine this methodology to facilitate the differentiation of sub-assemblages derived from goats. Efforts will also focus on overcoming current technical limitations by optimizing enzyme restriction profiles, thus enabling finer genetic distinctions while maintaining the method's efficiency.

As an enteric protozoan capable of waterborne transmission, *G. duodenalis* can infect humans and various mammals through contaminated drinking water, vegetables and irrigation water. Assemblages A and B and mixed infections (mixed types of A and B, A and E, B and E, and the combination of A, B, and E) of *G. duodenalis* are prevalent in goats, posing a potential threat of interspecies transmission between humans and animals, especially for those working long-term in sheep farms or slaughterhouses. It is therefore necessary to conduct further epidemiological investigations and genotyping of *Giardia* among nearby populations and water sources.

Conclusion

This study developed an accurate, efficient, and rapid PCR-RFLP genotyping method using the *bg* sequence of *G. duodenalis*, which facilitates accurate identification and effective differentiation of goat-derived *G. duodenalis* assemblages without sequencing. It serves as an effective tool for the detection and control of both goats and human giardiasis, as well as for conducting molecular epidemiological investigations.

Abbreviations

G. duodenalis	Giardia duodenalis
SSU rRNA	Small subunit rRNA
Bg	β-giardin
RFLP	Restriction Fragment Length Polymorphism
Bp	Base Pair
PCR	Polymerase Chain Reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04386-0.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements
The authors would like to thank the Farm staff for helping to collect clinical samples.

Author contributions
MXR: Formal analysis, Investigation, Writing–original draft. GJC: Methodology, Formal analysis, Investigation, Writing–original draft. WHC: Methodology, Formal analysis, Investigation. LYL: Formal analysis, Investigation. YKJ: Formal analysis, Investigation. XH: Visualization, sampling, statistical analysis. ZWJ: Visualization, sampling, statistical analysis. QYL: Visualization, sampling, statistical analysis. YXG: Conceptualization, funding acquisition, writing–review and editing. HY: Conceptualization, writing–review and editing. All authors have read and agreed to the published version of the manuscript.

Funding
This work was funded by Basic and Applied Basic Research Foundation of Guangdong Province (Grant no. 2022A1515110474), Guangdong Provincial Department of Agriculture and Rural Affairs - Guangdong Agricultural Technical Service "Light Cavalry" Project 2024 (Grant no. NITG20240253), Guizhou Provincial Scientific and Technological Program (Qian Ke He (2023) General 183).

Data availability
All data used or analyzed in this study are available upon reasonable request from the corresponding author. Representative nucleic acid sequences reported in this paper have been submitted to NCBI GenBank under accession numbers PQ284089–PQ284142 and PQ284063–PQ284088.

Declarations

Ethics approval and consent to participate
This study was conducted under the approval and instructions of the ethics committee of Foshan University and the animal ethics requirements of the People's Republic of China [No. FS2022107]. Permissions was obtained from animal owners before stool sampling, and none of the animals were injured during the specimen collection.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 3 October 2024 / Accepted: 15 November 2024
Published online: 26 November 2024

References

1. Fakhri Y, Daraei H, Ghaffari HR, Rezapour-Nasrabad R, Soleimani-Ahmadi M, Khedher KM, et al. The risk factors for intestinal *Giardia* spp infection: global systematic review and meta-analysis and meta-regression. Acta Trop. 2021;220:105968. https://doi.org/10.1016/j.actatropica.2021.105968.
2. Symeonidou I, Gelasakis AI, Miliotou AN, Angelou A, Arsenopoulos KV, Loukeri S, et al. Rapid on-site diagnosis of canine giardiosis: time versus performance. Parasites Vectors. 2020;13:544. https://doi.org/10.1186/s13071-020-04422-6.
3. Wang P, Zheng L, Liu L, Yu F, Jian Y, Wang R et al. Genotyping of *Cryptosporidium* spp., *Giardia duodenalis* and *Enterocytozoon bieneusi* from sheep and goats in China. BMC Vet Res. 2022;18:361. https://doi.org/10.1186/s12917-022-03447-6
4. Yu XG, Yuan KJ, Mu XR, Wang HC, et al. Research progress on the formation of cyst wall and cyst wall proteins of *Giardia*. Chin Anim Husb Vet Med. 2023;50:2061–8. https://doi.org/10.16431/j.cnki.1671-7236.2023.05.033. https://link.cnki.net/doi/.
5. Dos Reis LL, Lima DCDS, Da Silva TRR, Braga FCDO, Nava AFD, Vicente ACP. Circulation of *Giardia Duodenalis* in domestic and wild animals from Amazon region: a systematic review. Acta Trop. 2023;237:106708. https://doi.org/10.1016/j.actatropica.2022.106708.
6. Einarsson E, Ma'ayeh S, Svärd SG. An up-date on *Giardia* and giardiasis. Curr Opin Microbiol. 2016;34:47–52. https://doi.org/10.1016/j.mib.2016.07.019.
7. Hunter PR, Thompson RCA. The zoonotic transmission of *Giardia* and *Cryptosporidium*. Int J Parasitol. 2005;35:1181–90. https://doi.org/10.1016/j.ijpara.2005.07.009.
8. Cai W, Ryan U, Xiao L, Feng Y. Zoonotic giardiasis: an update. Parasitol Res. 2021;120:4199–218. https://doi.org/10.1007/s00436-021-07325-2.
9. Cacciò SM, Lalle M, Svärd SG. Host specificity in the *Giardia duodenalis* species complex. Infect Genet Evol. 2018;66:335–45. https://doi.org/10.1016/j.meegid.2017.12.001.
10. Yılmaz A, Uslu H. Examination of *Giardia Intestinalis* with Direct Microscopy and Direct fluorescent antibody in patients with Diarrhea. Turkiye Parazitol Derg. 2020;44:187–90. https://doi.org/10.4274/tpd.galenos.2020.6876.
11. Pomari E, Piubelli C, Perandin F, Bisoffi Z. Digital PCR: a new technology for diagnosis of parasitic infections. Clin Microbiol Infect. 2019;25:1510–6. https://doi.org/10.1016/j.cmi.2019.06.009.
12. Ruggeri P, Naldoni J, Hartikainen H, Okamura B. PCR-RFLP: a targeted method to reveal host specific malacosporean infection profiles (Cnidaria: Myxozoa: Malacosporea). Dis Aquat Org. 2020;141:91–101. https://doi.org/10.3354/dao03525.
13. Xu AH, Feng CC, Feng SW, Zhao LZ, Qi WX, Zhang W et al. Establishment of PCR-RFLP typing method for *Balantioides coli*. Chin. J. Zoonoses. 2024;1–7. https://link.cnki.net/urlid/35.1284.R.20240511.1839.002
14. Tan L, Wu S, Abdullahi AY, Yu X, Hu W, Song M, et al. PCR-RFLP method to detect zoonotic and host-specific *Giardia duodenalis* assemblages in dog fecal samples. Parasitol Res. 2016;115:2045–50. https://doi.org/10.1007/s00436-016-4948-y.
15. Pulina G, Milán MJ, Lavín MP, Theodoridis A, Morin E, Capote J, et al. Invited review: current production trends, farm structures, and economics of the dairy sheep and goat sectors. J Dairy Sci. 2018;101:6715–29. https://doi.org/10.3168/jds.2017-14015.
16. Yu X, Wang H, Li Y, Mu X, Yuan K, Wu A et al. Occurrence and Genotypic Identification of *Blastocystis* spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* in Leizhou Black Goats in Zhanjiang City, Guangdong Province, China. Animals (Basel). 2023;13:2777. https://doi.org/10.3390/ani13172777
17. Geng H-L, Yan W-L, Wang J-M, Meng J-X, Zhang M, Zhao J-X, et al. Meta-analysis of the prevalence of *Giardia duodenalis* in sheep and goats in China. Microb Pathog. 2023;179:106097. https://doi.org/10.1016/j.micpath.2023.106097.
18. Feng Y, Xiao L. Zoonotic potential and Molecular Epidemiology of *Giardia* Species and Giardiasis. Clin Microbiol Rev. 2011;24:110–40. https://doi.org/10.1128/CMR.00033-10.
19. Zhang K, Zheng S, Wang Y, Wang K, Wang Y, Gazizova A, et al. Occurrence and molecular characterization of *Cryptosporidium* spp., *Giardia Duodenalis*, *Enterocytozoon Bieneusi*, and *Blastocystis* sp. in captive wild animals in zoos in Henan, China. BMC Vet Res. 2021;17:332. https://doi.org/10.1186/s12917-021-03035-0.
20. Bourli P, Eslahi AV, Tzoraki O, Karanis P. Waterborne transmission of protozoan parasites: a review of worldwide outbreaks – an update 2017–2022. J Water Health. 2023;21:1421–47. https://doi.org/10.2166/wh.2023.094.
21. Fan Y, Wang X, Yang R, Zhao W, Li N, Guo Y et al. Molecular characterization of the waterborne pathogens *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Cyclospora cayetanensis* and *Eimeria* spp. in wastewater and sewage in Guangzhou, China. Parasites Vectors. 2021;14:66. https://doi.org/10.1186/s13071-020-04566-5
22. Li W, Wang K, Gu Y. Detection and genotyping study of *Enterocytozoon Bieneusi* in Sheep and goats in East-central China. Acta Parasitol. 2019;64:44–50. https://doi.org/10.2478/s11686-018-00006-8.
23. Truant AL, Elliott SH, Kelly MT, Smith JH. Comparison of formalin-ethyl ether sedimentation, formalin-ethyl acetate sedimentation, and zinc sulfate flotation techniques for detection of intestinal parasites. J Clin Microbiol. 1981;13:882–4. https://doi.org/10.1128/jcm.13.5.882-884.1981.
24. Asghari A, Mahdavi F, Karimi K, Mohammadi MR, Shamsi L, Asgari Q, et al. Molecular epidemiology and multilocus genotyping of *Giardia Duodenalis* in individuals attending major public hospitals in Shiraz, southwestern Iran: a public health concern. Parasite Epidemiol Control. 2024;25:e00354. https://doi.org/10.1016/j.parepi.2024.e00354.
25. Xu N, Yin JH, Shen YJ, Liu H, Cao JP. Advances in molecular epidemiology of Cryptosporidium and Giardia Lamblia. Chin J Parasitol Parasit Dis. 2018;36:661–5. https://link.cnki.net/urlid/31.1248.R.20181208.1606.002. 672.
26. Garcia-R JC, Ogbuigwe P, Pita AB, Velathanthiri N, Knox MA, Biggs PJ, et al. First report of novel assemblages and mixed infections of *Giardia Duodenalis* in human isolates from New Zealand. Acta Trop. 2021;220:105969. https://doi.org/10.1016/j.actatropica.2021.105969.

27. Liu H, Shen Y, Yin J, Yuan Z, Jiang Y, Xu Y, et al. Prevalence and genetic characterization of *Cryptosporidium*, *Enterocytozoon*, *Giardia* and *Cyclospora* in diarrheal outpatients in China. BMC Infect Dis. 2014;14:25. https://doi.org/10.1186/1471-2334-14-25.
28. Jafari H, Jalali MHR, Shapouri MSA, Hajikolaii MRH. Determination of *Giardia duodenalis* genotypes in sheep and goat from Iran. J Parasit Dis. 2014;38:81–4. https://doi.org/10.1007/s12639-012-0199-8.
29. Zhang W, Zhang X, Wang R, Liu A, Shen Y, Ling H, et al. Genetic characterizations of *Giardia Duodenalis* in Sheep and goats in Heilongjiang Province, China and possibility of zoonotic transmission. PLoS Negl Trop Dis. 2012;6:e1826. https://doi.org/10.1371/journal.pntd.0001826.
30. Lim YAL, Mahdy MAK, Tan TK, Goh XT, Jex AR, Nolan MJ, et al. First molecular characterization of *Giardia duodenalis* from goats in Malaysia. Mol Cell Probes. 2013;27:28–31. https://doi.org/10.1016/j.mcp.2012.08.006.
31. Akinkuotu OA, Takeet MI, Otesile EB, Olufemi F, Greenwood SJ, McClure JT. Multi-locus genotyping and phylogenetic analyses of *Giardia Intestinalis* isolates from indigenous goats in Ogun State, Nigeria. Acta Trop. 2019;195:15–22. https://doi.org/10.1016/j.actatropica.2019.04.009.
32. Wang Z, Vora GJ, Stenger DA. Detection and genotyping of *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, and *Cryptosporidium parvum* by Oligonucleotide Microarray. J Clin Microbiol. 2004;42:3262–71. https://doi.org/10.1128/JCM.42.7.3262-3271.2004.
33. Tan L, Yu X, Abdullahi AY, Wu S, Zheng G, Hu W, et al. Development of a rapid HRM genotyping method for detection of dog-derived *Giardia lamblia*. Parasitol Res. 2015;114:4081–6. https://doi.org/10.1007/s00436-015-4636-3.
34. Hashemi-Hafshejani S, Meamar AR, Moradi M, Hemmati N, Solaymani-Mohammadi S, Razmjou E. Multilocus sequence typing of *Giardia duodenalis* genotypes circulating in humans in a major metropolitan area. Front Med (Lausanne). 2022;9:976956. https://doi.org/10.3389/fmed.2022.976956.
35. Read CM, Monis PT, Thompson RCA. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect Genet Evol. 2004;4(2):125–30. https://doi.org/10.1016/j.meegid.2004.02.001.
36. Malekifard F, Ahmadpour M. Molecular detection and identification of *Giardia Duodenalis* in cattle of Urmia, northwest of Iran. Vet Res Forum. 2018;9(1):81–5. PMCID: PMC5913565.
37. Seabolt MH, Roellig DM, Konstantinidis KT. Genomic comparisons confirm *Giardia Duodenalis* sub-assemblage AII as a unique species. Front Cell Infect Microbiol. 2022;17:121010244. https://doi.org/10.3389/fcimb.2022.1010244.
38. Wielinga C, Williams A, Monis P, Thompson RC, Andrew. Proposed taxonomic revision of *Giardia Duodenalis*. Infect Genet Evol. 2023;111:105430. https://doi.org/10.1016/j.meegid.2023.105430.

Publisher's note