

Prevalence and Multilocus Genotyping of *Giardia lamblia* in Cattle in Jiangxi Province, China: Novel Assemblage E Subtypes Identified

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Abstract: *Giardia lamblia* is a common enteric pathogen associated with diarrheal diseases. There are some reports of *G. lamblia* infection among different breeds of cattle in recent years worldwide. However, it is yet to know whether cattle in Jiangxi province, southeastern China is infected with *G. lamblia*. The objectives of the present study were to investigate the prevalence and examine the multilocus genotypes of *G. lamblia* in cattle in Jiangxi province. A total of 556 fecal samples were collected from 3 cattle breeds (dairy cattle, beef cattle, and buffalo) in Jiangxi province, and the prevalence and genotypes of *G. lamblia* were determined by the nested PCR amplification of the beta-giardin (*bg*) gene. A total of 52 samples (9.2%) were positive for *G. lamblia*. The highest prevalence of *G. lamblia* was detected in dairy cattle (20.0%), followed by that in beef cattle (6.4%), and meat buffalo (0.9%). Multilocus sequence typing of *G. lamblia* was performed based on sequences of the *bg*, triose phosphate isomerase and glutamate dehydrogenase loci, and 22, 42, and 52 samples were amplifiable, respectively, forming 15 MLGs. Moreover, one mixed *G. lamblia* infection (assemblages A and E) was found in the present study. Altogether, 6 novel assemblage E subtypes (E41*-E46*) were identified for the first time. These results not only provided baseline data for the control of *G. lamblia* infection in cattle in this southeastern province of China, but also enriched the molecular epidemiological data and genetic diversity of *G. lamblia* in cattle.

Key words: *Giardia lamblia*, cattle, prevalence, multilocus genotyping, Jiangxi Province

Giardia lamblia is a common zoonotic parasite, causing intestinal diseases such as diarrhea in humans and animals. In the life cycle of this parasite, cysts are spread directly through feces by infected humans or animals [1]. The transmission route is *via* fecal-oral or by ingesting food and water contaminated with cysts [1]. *G. lamblia* can cause giardiasis, which may lead to asymptomatic infection or abdominal pain, chronic diarrhea, and vomiting seriously impacting on health condition, resulting in low productivity in ruminants [2]. Cattle is one of the main hosts of *G. lamblia* that can excrete cysts, which is the potential threat to humans [3].

Molecular techniques have been applied for *G. lamblia* de-

tection [4]. To date, 8 assemblages (A to H) of *G. lamblia* have been identified from different hosts. Among these assemblages, assemblages A and B are considered to be zoonotic, while other assemblages are mostly host specific [1]. In addition, assemblage E is the dominant genotype of *G. lamblia* occurring in cattle [5-8], and zoonotic assemblages A and B have also been identified in cattle and humans in some studies [9-13]. However, there are some studies that have found assemblage E existing in human cases [5,14,15].

Despite the widespread presence of *G. lamblia* in cattle, the clinical significance of this organism in cattle is not fully understood. *G. lamblia* infection is associated with the occurrence of diarrhea in the cattle [8,16]. Recently, a study showed that diarrhea cattle is more likely to be infected with pathogens than non-diarrhea animals [17]. Furthermore, healthy cattle are also infected with *G. lamblia* [7,8]. Therefore, the prevention is more important than treatment. Multilocus sequence typing has been used to identify multilocus genotypes (MLGs)

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of *G. lamblia* in dairy cattle based on sequences of the beta-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) [18]. But most of previous studies have identified *G. lamblia* in cattle by a single gene locus only [19].

Jiangxi province is located on the south bank of the middle and lower reaches of the Yangtze River, with a population of more than 40 million people. It is one of the main beef producing provinces in southern China, with a large number of cattle being sold to southeast coastal cities. To date, there was no report of *G. lamblia* infection in cattle in Jiangxi province. Thus, the objectives of the present study were to investigate the prevalence and examine the MLGs of *G. lamblia* in cattle in Jiangxi Province, southeastern China.

A total of 556 fecal samples were randomly collected from cattle in 8 farms of Jiangxi province, southeastern China, including Nanchang city (2 dairy cattle farms and 1 beef cattle farm), Gao'an city (2 beef cattle farms), Xinyu city (1 meat buffalo farm) and Ji'an city (1 beef cattle farm and 1 free-range farm for beef cattle). This study was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All cattle were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China. All samples were obtained immediately after defecation by PE gloves and were stored in separate 50 ml centrifuge tubes containing 2.5% potassium dichromate, then packed into the box with ice and sent to the laboratory. Feces were stored at 4°C until extraction of genomic DNA. The information of each fecal sample was recorded, including geographical location, age, breed and collection date.

The stool samples were washed with double distilled water to wash off potassium dichromate, then 200 mg of each stool sample was packed into a 2 ml centrifuge tube for genomic DNA extraction using a commercial E.Z.N.A.[®] Stool DNA kit (Omega Bio-Tek Inc., Norcross, Georgia, USA) according to the manufacturer's protocols. The obtained genomic DNA samples were stored in -20°C until PCR amplification.

Giardia lamblia in fecal samples were identified by PCR-based sequencing of the *bg* locus [20]. All the *bg*-positive samples were then amplified to further identify MLGs of *G. lamblia* in cattle using primers for the *gdh* and *tpi* gene loci [20,21] (Supplementary Table S1). The primary PCR reaction mixture contained genomic DNA (2 µl), 10× *Ex Taq* Buffer (Mg²⁺-free) (2.5 µl), MgCl₂ (2 mM), dNTP Mixture (0.2 mM), TaKaRa *Ex Taq* (0.625 U) (TaKaRa Shuzo Co., Ltd) and each primer (0.4

µM) in a total volume of 25 µl. The 2 µl of primary PCR product as template was amplified by the secondary PCR with the same reaction conditions. The positive (*G. lamblia* DNA) and negative (deionized water) control were added in each PCR amplification. The secondary PCR products were subsequently detected by electrophoresis in 1.5% (w/v) agarose gels with ethidium bromide.

All positive second-PCR products were sent to Xi'an Qingke Biotechnology Company for 2-directional sequencing. The Chromas v.2.6. was used to check the sequencing chromatograms. The obtained sequences were aligned with relevant sequences available in GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank>) and were analyzed by the Clustal X 1.81 [22] to identify the genotypes of *G. lamblia*. The nucleotide sequences of novel subtypes in assemblages were deposited to the National Center for Biotechnology Information GenBank database under accession numbers: MT123524 for the *bg* locus, MT123525–MT123526 for the *gdh* locus, and MT123527–MT123529 for the *tpi* locus. The phylogenetic relationships among *G. lamblia* isolates were re-constructed based on *gdh* and *tpi* sequences using a Neighbor-Joining algorithm [23] in DNASTar 5.0 [24], and the Kimura 2-parameter analysis (1,000 replicates) was selected.

The difference in *G. lamblia* prevalence among different breeds, regions and ages of cattle was assessed using the Chi-square (χ^2) analysis by IBM SPSS Statistics for Windows, Version 22.0 (IBM Armonk Corp., Armonk, New York, USA), and *P*-value of 0.05 was considered as the threshold for significant difference.

In this study, *G. lamblia* was detected in 9.2% (95% CI 6.81-11.57) of 556 cattle fecal samples from 4 cities of Jiangxi province, with the prevalence ranging from 0.9% to 16.2% (Table 1). The highest *G. lamblia* prevalence (16.2%, 95% CI 11.37-21.06) was detected in cattle in Nanchang city, which was significantly higher than that in Xinyu city (0.9%, 95% CI 0-2.73) (*P*<0.01). The different prevalence could be due to different feeding methods. Moreover, dairy cattle (20%) and beef cattle (6.4%) were more susceptible to *G. lamblia* than buffaloes (0.9%), for which the ORs were 26.75 (95% CI 3.60-198.79) and 7.27 (95% CI 0.96-55.13), respectively (Table 1). This result may be related to different management and sanitation status of different farms, which suggests that we should pay more attention to control *G. lamblia* transmission in dairy cattle and beef cattle in investigated areas. Furthermore, the highest *G. lamblia* prevalence was detected in cattle aged 1-3

Table 1. Prevalence and factors associated with *Giardia lamblia* infection in cattle in Jiangxi Province, China

Variable	Category	No. tested	No. positive	% (95% CI)	OR (95% CI)	P-value
Age	< 1 month	5	0	0		
	1-3 months	46	9	19.6 (8.10-31.03)	3.0 (1.27-7.07)	<0.05
	4-24 months	239	23	9.6 (5.88-13.36)	1.3 (0.70-2.45)	>0.05
	>24 months	266	20	7.5 (4.35-10.69)	Reference	
Region	Nanchang	222	36	16.2 (11.37-21.06)	20.71 (2.80-153.20)	<0.05
	Gao'an	166	13	7.8 (3.74-11.92)	9.09 (1.17-70.54)	
	Xinyu	108	1	0.9 (0-2.73)	Reference	
	Ji'an	60	2	3.3 (0-7.88)	3.69 (0.33-41.56)	>0.05
breed	Dairy cattle	165	33	20.0 (13.90-26.10)	26.75 (3.60-198.79)	<0.05
	Beef cattle	283	18	6.4 (3.52-9.20)	7.27 (0.96-55.13)	
	Buffalo	108	1	0.9 (0-2.73)	Reference	
Total		566	52	9.2 (6.81-11.57)		

Table 2. Intra-assemblage substitutions in *bg*, *tpi*, and *gdh* sequences from assemblage E

Subtype (number)	Nucleotide positions and substitutions					GenBank ID
<i>bg</i>						
	54	159	261	303	402	
Ref. sequence	T	A	T	C	T	MK252651
E1 (14)	T	A	T	C	T	
E5 (9)	C	A	C	C	T	
E8 (8)	C	A	C	C	C	
E9 (8)	T	A	C	C	C	
E11 (5)	T	G	C	C	C	
E18 (2)	T	A	C	C	T	
E41* (5)	T	A	C	T	T	MT123524
<i>tpi</i>						
	92	179	251	339	476	
Ref. sequence	A	A	G	A	G	KY633482
E1 (1)	T	A	T	T	G	
E13 (1)	G	G	C	C	A	
E17 (7)	T	G	C	C	A	
E23 (2)	T	G	C	C	A	
E38 (1)	T	G	C	C	A	
E42* (5)	A	A	G	A	G	MT123527
E43* (2)	G	A	G	A	G	MT123528
E44* (1)	T	G	T	C	G	MT123529
<i>gdh</i>						
	74	137	231	311	446	
Ref. sequence	G	T	A	C	A	KY711410
E1 (7)	A	A	A	C	A	
E2 (29)	A	A	G	C	A	
E45* (4)	G	T	A	C	G	MT123525
E46* (1)	A	A	A	T	A	MT123526

*indicate that subtypes have not been reported.

months (19.6%, 95% CI 8.10-31.03), which is consistent with that of previous studies [25-27]. Cattle aged 1-3 months (19.6%) had a significantly higher *G. lamblia* prevalence compared with cattle aged more than 24 months (7.5%) ($P < 0.05$)

Table 3. Intra-assemblage substitutions in *bg*, *tpi*, and *gdh* sequences from assemblage A

Subtype (number)	Nucleotide positions and substitutions					GenBank ID
<i>bg</i>						
	60	120	180	240	360	
Ref. sequence	C	C	A	G	A	MK573339
A (1)	C	C	A	G	A	
<i>tpi</i>						
	60	120	180	240	516	
Ref. sequence	G	A	G	A	C	HQ179652
A1 (2)	G	A	G	A	G	
<i>gdh</i>						
	120	180	240	360	480	
Ref. sequence	C	C	G	C	C	EF507642
A1 (1)	C	C	G	C	C	

(Table 1). This finding illustrates that calves are more susceptible to *G. lamblia* infection.

The overall *G. lamblia* prevalence (9.2%) in cattle in this study was higher than that in cattle in Zhaoqing city, Guangdong Province (2.2%) [28], and Qinghai Province, China (5.2%) [29], an arid area of central Iran (4.2%) [30], Thailand (6.0%) [31], and Urmia, northwest Iran (9.34%) [32]; but lower than that in Guangdong Province (74.2%) [6], Hubei Province (22.6%) [33] and Sichuan Province, China (9.4%) [34]; Egypt (13.3%) [35], South Korea (10.0%) [36], Ethiopia (9.6%) [37] and Vietnam (13.8%) [38]. The difference in *G. lamblia* prevalence may be related to difference in study design, breed and age of cattle studied and sampling size.

In the present study, 52 positive samples represented 2 assemblages (assemblage E and assemblage A) (Tables 2, 3), containing one novel subtype of assemblage E (named as E41*, n = 5) based on sequence analyses of the *bg* locus (Table

2). Moreover, 42 of the 52 *bg*-positive samples were tested positive at the *gdh* locus, and 22 samples were tested *tpi*-positive. Two novel subtypes (named as E45*, n=4 and E46*, n=1) and 3 novel subtypes (named as E42*, n=5; E43*, n=2 and E44*, n=1) of assemblage E were identified at the *gdh* and *tpi* loci, respectively (Table 2). Previous studies have shown that the assemblage E is the primary genotype in different animals [1,39-45]. Both assemblage A and assemblage E were also found in humans [1,5]. These findings suggested that cattle could be reservoir host of *G. lamblia* and transport this zoonotic pathogen to humans and other animals. MLG analysis can assess the genetic diversity of *G. lamblia* [46]. Among these 52 samples, 16 samples were successfully amplified and sequenced at all the 3 genetic loci (*bg*, *gdh*, and *tpi*) (Table 4), forming 15 novel MLGs (named as MLGE1-E14, MLGA1) and one mixed infection (Table 4), which revealed a high genetic diversity of *G. lamblia* assemblages.

The phylogenetic tree based on *G. lamblia tpi*-sequences and *gdh*-sequences were constructed in order to evaluate the genetic relationships of the *G. lamblia* isolates. The results showed that *G. lamblia* isolates from cattle (novel subtypes E41*-E46*) were clustered within the assemblage E (Figs. 1, 2), which contained *G. lamblia* isolates from animals and humans with a

higher confidence value, revealing closer genetic relationship (Fig. 1).

In conclusion, this study revealed a 9.2% *G. lamblia* prevalence in cattle in Jiangxi province, southeastern China, and

Table 4. Multilocus characters of *Giardia lamblia* isolates based on sequences of *bg*, *tpi*, and *gdh* genes

Isolate	Subtype			No. sequence	MLG type
	<i>bg</i>	<i>tpi</i>	<i>gdh</i>		
HX1-24, SBN4-9	E5	E17	E2	2	MLGE1
HX1-29	E8	E17	E2	1	MLGE2
XH1-11	E5	E42	E2	1	MLGE3
XH1-12	E8	E42*	E45*	1	MLGE4
XH1-20, SBN5-27	E41*	E17	E2	2	MLGE5
SBN1-3	E18	E13	E2	1	MLGE6
SBN2-1	E11	E1	E2	1	MLGE7
SBN3-1, YFX-9	E5	E43*	E2	2	MLGE8
SBN3-3	E11	E44*	E2	1	MLGE9
SBN5-20	E11	E17	E1	1	MLGE10
SBN5-28	E9	E38	E2	1	MLGE11
SBN6-28	E2	E17	E1	1	MLGE12
YFJ-8	E1	E23	E45*	1	MLGE13
YFJ-9	E1	E42*	E2	1	MLGE14
HX1-21	E1	A1	E2	1	Mixed
HX1-8	A	A1	A1	1	MLGA1

*Novel subtypes of assemblage E.

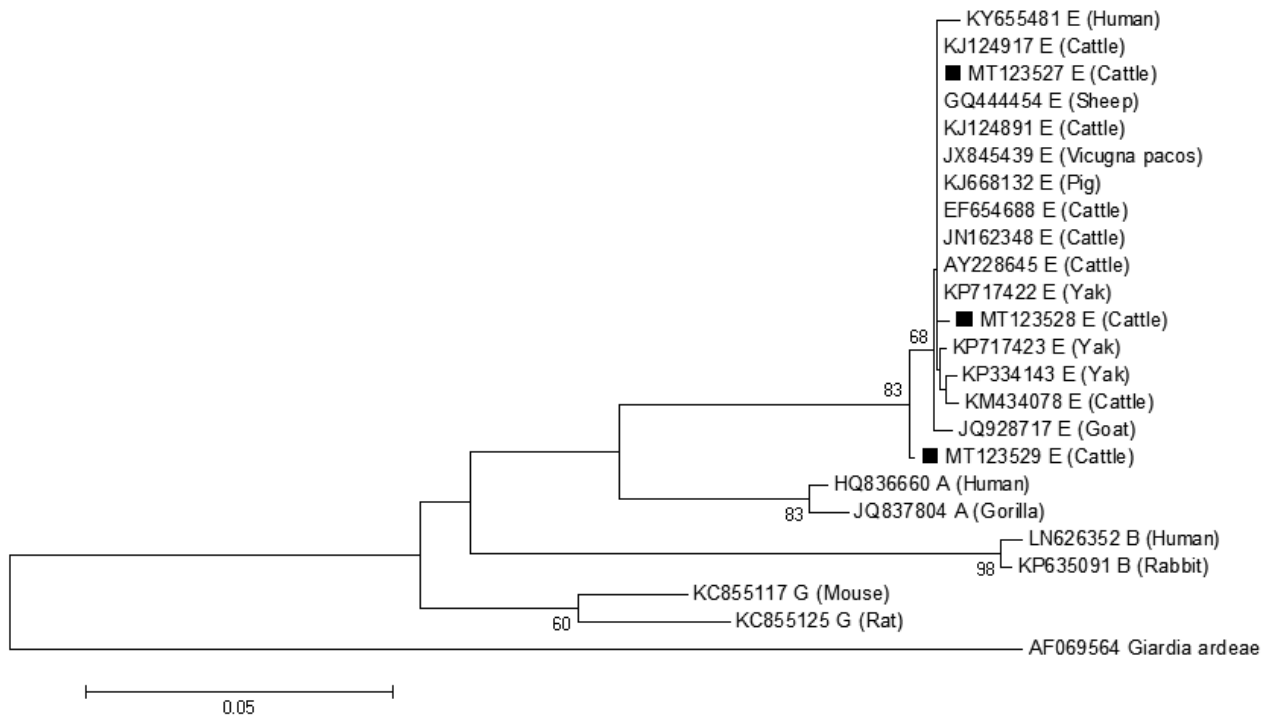


Fig. 1. A phylogenetic tree of *Giardia lamblia tpi* gene sequences. Novel subtypes of assemblage E isolates identified from cattle in this study are marked with a black square (■).

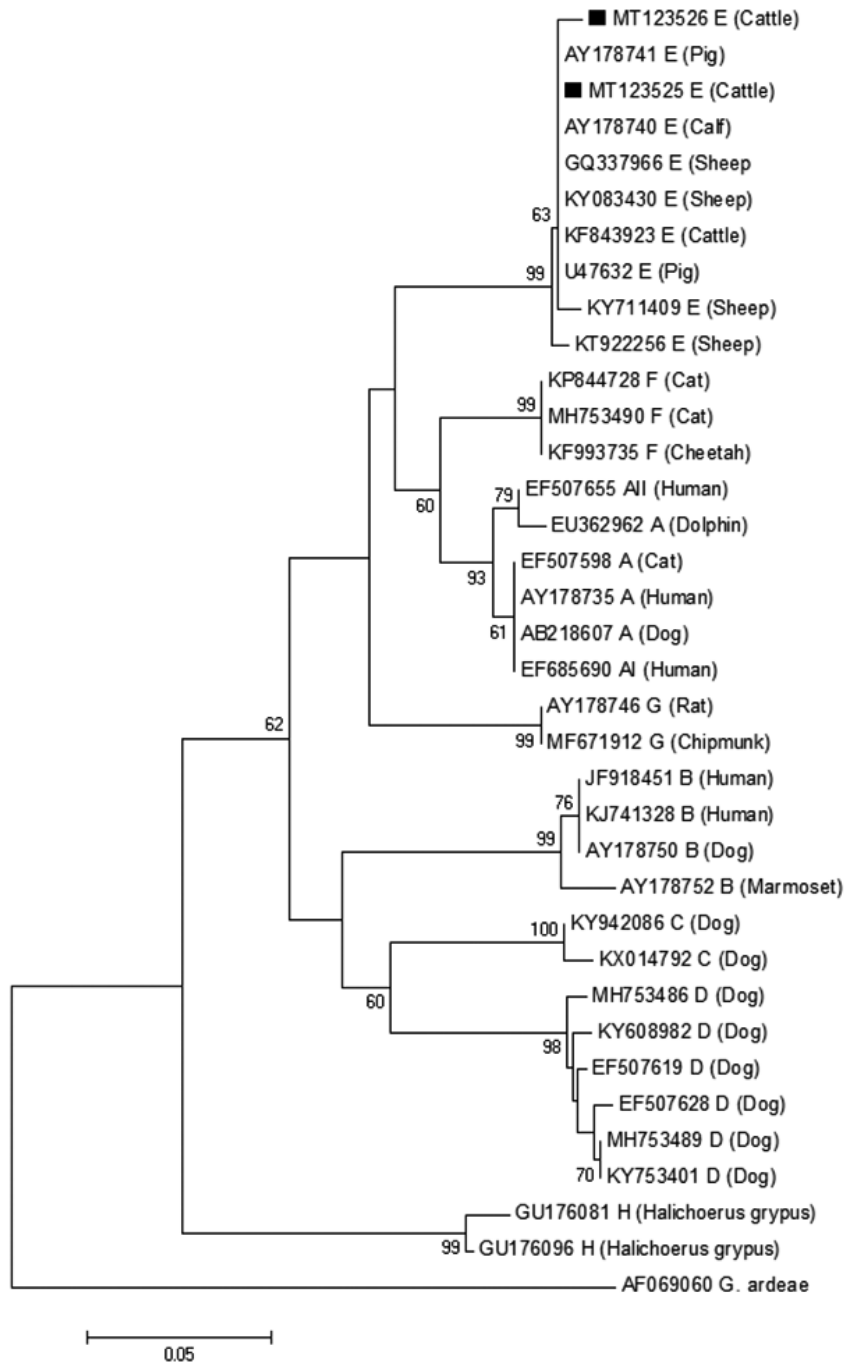


Fig. 2. A phylogenetic tree of *Giardia lamblia* on *gdh* gene sequences. The isolates marked with a black square (■) represent novel subtypes of assemblage E identified from cattle in this study.

identified 6 novel assemblage E subtypes (designated as E41*-46*) for the first time. Fourteen novel MLGs within assemblage E and one novel MLG within assemblage A were identified. These findings revealed the prevalence and MLGs of *G. lamblia* in cattle in Jiangxi province, which not only enriched

the genetic diversity of *G. lamblia*, but also provided baseline data for controlling *G. lamblia* infection in animals and humans in this southeastern province of China.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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