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Full-Length Research Article

Molecular Investigation of *Enterocytozoon bieneusi* in Calves in Oromia Special Zone, Central Ethiopia

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Abstract

Enterocytozoon bieneusi, the most frequently diagnosed microsporidian species in humans, is also identified in a wide range of animals. The aim of this study, therefore, was to determine the prevalence and genotypes of *E. bieneusi* in calves and to assess its public health implications in Central Ethiopia. A total of 449 fecal samples were examined by a nested PCR targeting the internal transcribed spacer (ITS) of the rRNA gene. All positive PCR products were sequenced to determine the genotypes. *Enterocytozoon bieneusi* was found in 7.1% (32/449) of the calves. Difference in the infection rate was statistically significant ($P < 0.05$) among age groups. Out of the 10 ITS genotypes, nine belonging to the known genotypes BEB8, BEB4, BEB17, I, KIN-1, Peru11, PigEBITS5, H and ET-L2; and one novel genotype (named as ET-C1) were identified. Four of the genotypes (KIN-1, Peru11, PigEBITS5, and H) were clustered to a member of a major phylogenetic group with zoonotic potential. This study constituted the first molecular characterization of *E. bieneusi* in Ethiopia, and it suggested a potential risk of zoonotic transmission. Studies in humans and further studies in animals are necessary to assess the public health significance of *E. bieneusi.*

Keywords: *Enterocytozoon bieneusi*, genotypes, internal transcribed spacer, nested PCR

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1. INTRODUCTION

Microsporidia are obligate intracellular parasites with worldwide distribution in humans and major groups of animals (Santin and Fayer, 2011). Among the seventeen microsporidian species infecting humans, *Enterocytozoon bieneusi* is the most frequently diagnosed species in humans. It has also been reported in a variety of wild, farm, and companion mammals and in birds (Santín and Fayer, 2011; Li *et al*., 2019a; Li *et al*., 2019b). *E. bieneusi* primarily infects the enterocytes of the small intestine and it is mainly associated with chronic diarrhea and wasting syndrome (Desportes *et al.*, 1985; Matos *et al.*, 2012). Enormous genetic diversity within *E. bieneusi* has been observed based on sequence polymorphism in the internal transcribed spacer (ITS) of rRNA (Santin and Fayer, 2009; Santin and Fayer, 2011) that reported ~500 ITS genotypes of the parasite in humans, animals and environment (Gui *et al.*, 2020; Tao *et al.*, 2020; Karim *et al.*, 2020). Phylogenetic analysis of the ITS genotype sequences revealed the presence of eleven genotypic groups (Li *et al.*, 2019a; Li *et al.*, 2019b). A large cluster of genetically linked ITS genotypes named Group 1 are frequently found in both humans and animals, and they are considered to be zoonotic (Li *et al.*, 2019b). Group 2, consisting of mainly ruminant specific genotypes (Thellier and Breton, 2008), also contains some genotypes that occur both in humans and animals having zoonotic potential. The remaining genotypes represent largely host-adapted groups (Groups 3 to 11) associated with specific animals and probably have no significant public health importance (Li *et al.*, 2019b).

Except few works in humans and sheep using microscopy and Polymerase Chain Reaction (PCR), *E. bienusi* has not been studied and characterized in calves in Ethiopia (Adamu *et al.*, 2005; Endeshaw *et al.*, 2006; Wegayehu *et al.*, 2020). As a result, the aim of this study was to determine the prevalence of *E. bieneusi* and the circulating genotypes in calves in Oromia Special Zone, Central Ethiopia with a perspose of assessing the public health potential.

2. MATERIALS AND METHODS

2.1. Study Area

This study was conducted in Oromia Special Zone, Central Ethiopia. The Special Zone has an estimated total area of 4,800 km^2 which accounts for 1.5% of the total area of the Oromia Regional State. The zone borders with Eastern Shewa zone in the east, North Shewa zone in the North East and South-west Shewa Zone in the South West. The mean annual temperature of the Zone is between 20-25 ºc in the low lands and 10-15 ºc in the central high lands. Based on the available meteorological data, the mean annual rainfall varies from 700mms to 1400mms in low lands and high lands, respectively. The Special Zone has six districts and eight major towns of which, this study included Holeta, Sendafa and Chancho areas because of the high population of cattle and sheep (Figure 1).

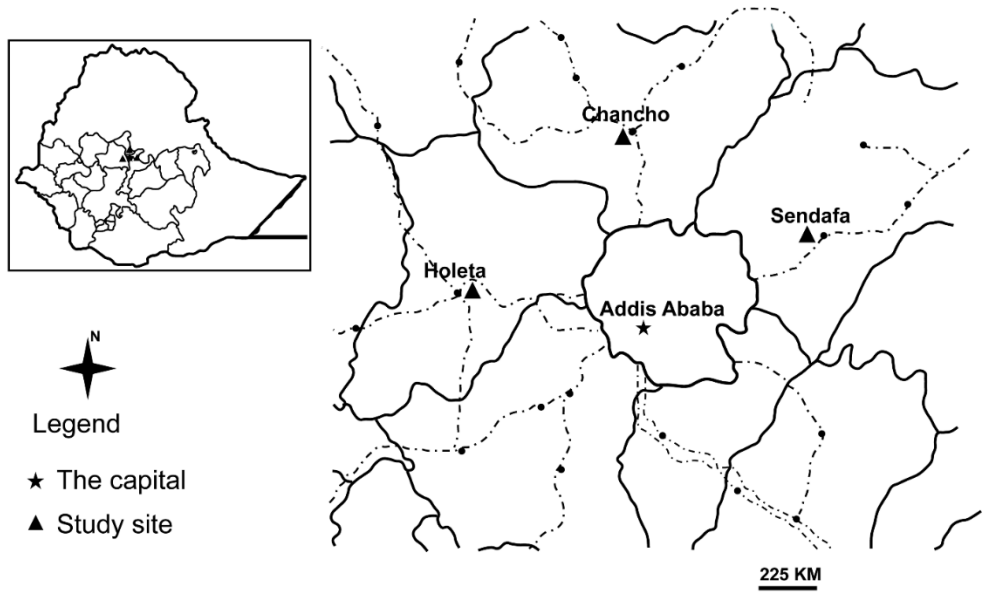

Figure 1: Locations of the study area in Oromia Special Zone, Central Ethiopia. The Special Zone has six districts and eight major towns of which, this study included Holeta, Sendafa and Chancho areas because of the high population of cattle (Adapted from Wegayehu *et al*., 2020).

2.2. Study Design and Period

A community-based cross-sectional study was conducted between January and June 2014 to evaluate the circulating genotypes of *E. bieneusi* in calves in Oromia Special Zone, Central Ethiopia.

2.3. Sample Population and Sampling

Calves younger than three months were the study population. The owners of these animals who consent to allow us to take fecal samples from their animals were included in this study. A total of 449 fresh fecal samples were collected from calves in separate and labeled stool containers. The samples were taken directly from the rectum of each animal or immediately after defecation using sterile disposable gloves. Identification number, animal species, age and sex were recorded during sample collection. The samples were preserved in 2.5% potassium dichromate solution, transported to Parasitology Laboratory of Aklilu Lemma Institute of Pathobiology in icebox and stored at 4°C before deoxyribonucleic acid (DNA) extraction.

2.4. DNA Extraction

The preserved fecal samples were washed with deionized water until the potassium dichromate was removed. Genomic DNA was extracted from each fecal sample using the E.Z.N.A.® Stool DNA kit (Omega Biotek Inc., Norcross, USA). Briefly, about

50-100 mg of fecal sample was added in a 2ml centrifuge tube containing 200 mg of glass beads and placed on ice. Following, 300µl buffer SP1 and proteinase K were added and incubated at 70ºC for 10 minutes. Subsequently, all the procedures outlined in the product manual were performed according to the manufacturer's protocol. Finally, DNA was eluted in 200 µL of elution buffer and the extract was stored at -20°C until used in PCR.

2.5. PCR and Sequence Analysis

A nested PCR targeting the internal transcribed spacer (ITS) region of the rRNA gene was used to detect *E. bieneusi* (Sulaiman *et al.*, 2003). The PCR was performed in 25µl reaction volume that includes 23µl mixes and 2µl DNA template. The PCR amplifications were performed with rTaq DNA polymerase (Takara Bio Inc, Shiga, Japan) in Applied Biosystems Thermal Cycler version 2.09. The primary PCR program was involved enzyme activation at 94ºC for 5 minutes, followed by denaturation at 94ºC for 30 seconds, annealing at 57ºC for 30 seconds and primer extension at 72ºC for 40 seconds for 35 cycles. A seven-minute final extension at 72ºC was done after 35 cycles followed by cooling at 4ºC. The second PCR was run under the same conditions as the first except the annealing temperature which was reduced from 57ºC to 55ºC for 30 seconds; and the cycle which was again reduced from 35 to 30 cycles. To validate the PCR amplification, positive and negative controls were included in each batch of PCR. The amplified products were separated by electrophoresis on 1% agarose gel and visualized under a trans-illuminator after staining with ethidiumbromide. The PCR was conducted in International Joint Research Laboratory for Zoonotic Diseases at Henan Agricultural University, China.

The secondary PCR products were purified using Montage PCR filters (Millipore, Bedford, MA) and sequenced using an ABI BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3100 automated sequencer (Applied Biosystems). The nucleotide sequences obtained were aligned with reference *E. bieneusi* sequences using ClustalX software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). The genotype determination was based on polymorphism in 243 bp ITS region of the rRNA gene as previously proposed (Santín and Fayer, 2009). The established and reputable nomenclature system was used in naming *E. bieneusi* ITS genotypes. Bayesian inference (BI) and Monte Carlo Markov chain methods were used to construct the phylogenetic trees in MrBayes program (version 3.2.6). The posterior probability values were calculated by running 1,000,000 generations. A 50% majority rule consensus tree was constructed from the final 75% of the trees generated via BI. Analyses were run three times to ensure convergence and intensity to priors. The representative nucleotide sequences of the ten *E. bieneusi* ITS genotypes obtained in the present study were deposited in the GenBank database under the accession numbers: KT922241.

2.6. Ethical Considerations

Ethical clearance was obtained from the National Health Research Ethics Review Committee, Ministry of Science and Technology. Support letters were obtained from concerned health and agricultural offices and administrative authorities at the community level. The objectives of the project were explained to the owners of the animals and informed consent was obtained before inclusion to the study.

2.7. Data Analysis

Data were entered using EpiData version 3.1 and transferred to STATA Software for analysis. Chi square test was used to verify the possible association of *E. bieneusi* infections in different study groups. Values were considered to be statistically significant when the P-value was less than 0.05.

3. RESULTS

3.1. *E. bieneusi* Infection in Calves

Of the 449 fecal samples investigated by the nested PCR at ITS region (392 bp) of the rRNA gene, *E. bieneusi* was found in 32 (7.1%) calves' fecal samples (Table 1). The prevalence of *E. bieneusi* varied across the study areas. A relatively higher prevalence of 8.5% (13/153) was found in Holeta followed by 7.5% (9/120) in Chancho and 5.7% (10/176) in Sendafa. The prevalence showed statistically no significant difference (P = 0.602) among the study sites (Table 1).

To investigate the distribution of *E. bieneusi* among calves in relation to sex, age and breed groups, data were arranged and summarized in Table 1. The prevalence of *E. bieneusi* was 7.0% (14/200) and 7.2% (18/249) in male and female calves, respectively. The difference in the prevalence of *E. bieneusi* infection was not statistically significant between male and female calves. There was an age-associated difference in the occurrence of *E. bieneusi* infections. Calves less than 5 weeks had the highest infection rate of *E. bieneusi* (12.0%, 15/125), followed by those of 5-8 weeks (6.6%, 8/121), and greater than 8 weeks (4.43%, 9/203). The age-associated difference in *E. bieneusi* infection rates was statistically significant (P = 0.034) among different age groups (Table 1). Likewise, the infection rate of *E. bieneusi* in the exotic breed (10.9%) was higher than that of the local calves (3.5%) and the difference was statistically significant (P = 0.002) (Table 1).

Table 1: Prevalence of *E. bieneusi* in calves by study area, sex, age and breed in Oromia Special Zone, Central Ethiopia

Demographic characteristics	No. of samples examined	No. of samples positives (%)	$\chi 2$	P-value	Genotypes (no. of isolates)
Study areas					
Holeta	153	13 (8.5)	1.0143	0.602	BEB8 (6), I (2), BEB4 (1), BEB17 (1), H (1), Peru11 (1), ET-C1 (1)
Sendafa	176	10 (5.7)			BEB8 (7), BEB4 (1), KIN-1 (1), PigEBITS5 (1)
Chancho	120	9 (7.5)			BEB8 (5), I (2), BEB17 (1), ET-L2 (1)
Sex					
Male	200	14 (7.0)	0.0088	0.925	BEB8 (10), I (1), PigEBITS5 (1), Peru11 (1), ET-L2 (1)
Female	249	18 (7.2)			BEB8 (8), I (3), BEB4 (2), BEB17 (2), KIN-1 (1), H (1), ET-C1 (1)
Age group					
< 5 weeks	125	15 (12.0)	6.758	0.034*	BEB8 (9), KIN-1 (1), H (1), Peru11 (1), ET-C1 (1), ET-L2 (1)
5-8weeks	121	8 (6.6)			BEB8 (5), I (3), BEB17 (1), PigEBITS5 (1)
> 8weeks	203	9 (4.4)			BEB8 (4), I (1), BEB4 (2), BEB17 (1)
Breed group					
Local	229	8 (3.5)	9.322	0.002*	BEB8 (5), I (1), ET-C1 (1), KIN-1 (1)
Exotic breed	220	24 (10.9)			BEB8 (13), I (3), BEB4 (2), BEB17 (2), H (1), PigEBITS5 (1), Peru11 (1), ET-L2 (1)
Total	**449**	**32 (7.13)**			BEB8 (18), I (4), BEB4 (2), BEB17 (2), KIN-1 (1), H (1), PigEBITS5 (1), Peru11 (1), ET-C1 (1), ET-L2 (1)

Key: $\chi 2$ and P- values compare the prevalence among study areas, male and female, age and breed groups in calves

* Represent statistically significant difference ($P < 0.05$).

3.2. Genotypes of *E. bineusi*

The sequence analysis revealed the presence of ten different genotypes in calves (Table 2). Among these genotypes, BEB8, BEB4, BEB17, I, KIN-1, Peru11, PigEBITS5, H and ET-L2 were previously described in cattle, humans, macaques, swine and sheep. One new genotype, named ET-C1 was identified for the first time from Ethiopia. It had a 99% sequence similarity to genotype J (Genbank accession number JF776170). Among the genotypes, BEB8 was the most prevalent genotype being detected in 18 (56.3%) isolates followed by genotype I in 4 (12.5%) isolates, and BEB4 and BEB17 each in 2 (6.3%) isolates. The other genotypes KIN-1, H, PigEBITS5, Peru11, ET-C1 and ET-L2 were identified in 1 (3.12%) isolate each.

Table 2: Distribution the genotype of *E. bieneusi* in calves in Oromia Special Zone, Central Ethiopia

Study areas	Positive samples (Number & %)	Genotypes (no. of isolates)
Holeta	13 (8.5)	BEB8 (6), I (2), BEB4 (1), BEB17 (1), H (1), Peru11 (1), ET-C1 (1)
Sendafa	10 (5.7)	BEB8 (7), BEB4 (1), KIN-1 (1), PigEBITS5 (1)
Chancho	9 (7.5)	BEB8 (5), I (2), BEB17 (1), ET-L2 (1)

3.3. Phylogenetic Analysis

The phylogenetic tree was constructed to demonstrate the genetic relationship of previously described genotypes and the new genotypes recorded. Four known genotypes (Peru11, KIN-1, H and PigEBITS5) verified from calves were clustered to the members of a major phylogenetic group with zoonotic potential (Group 1). The remaining five known genotypes (BEB8, BEB4, BEB17, I and ET-L2) and the new genotype ET-C1 identified in this study from claves were placed in the cluster with genotypes that were most commonly isolated from cattle (Figure 2).

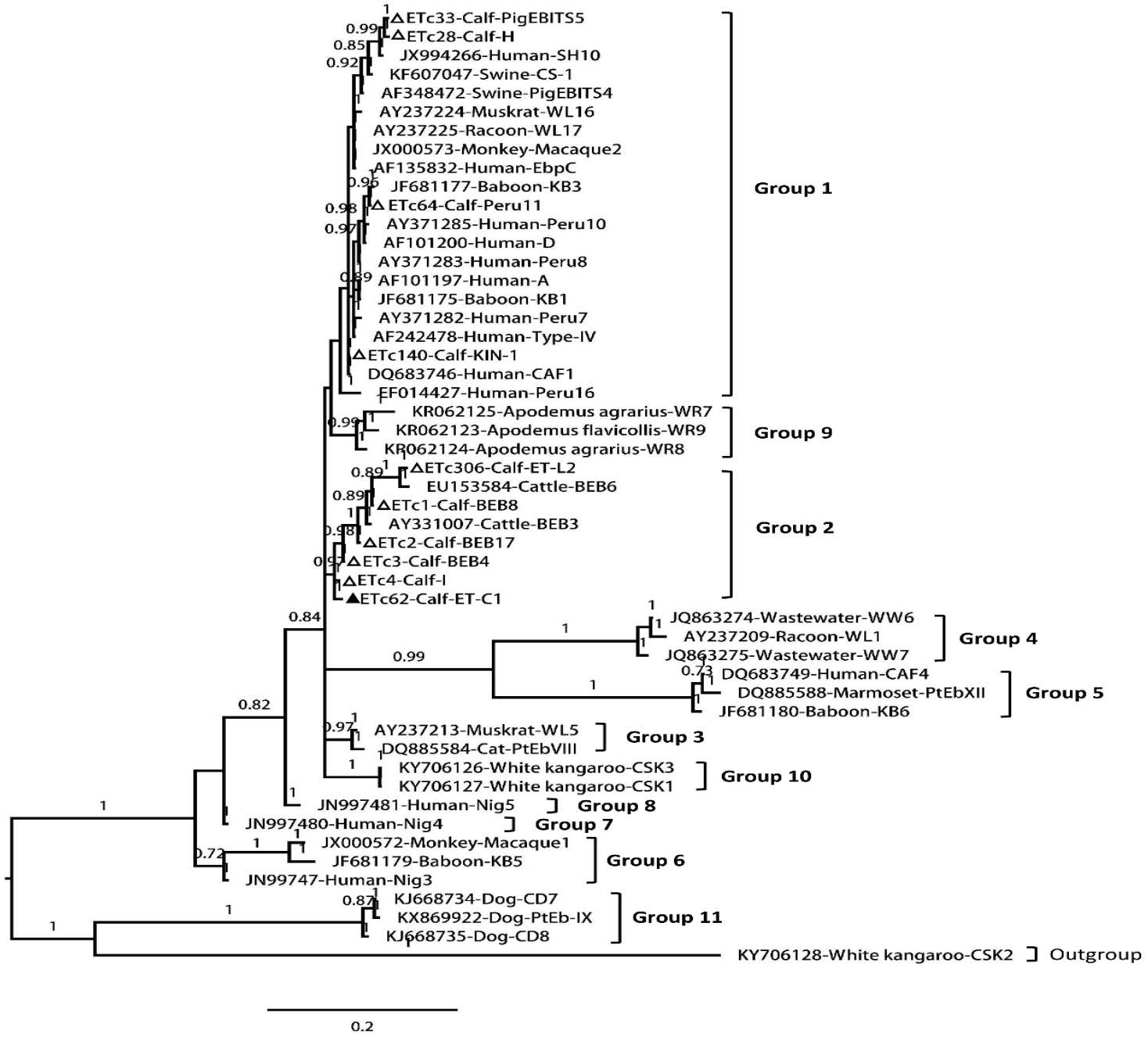

Figure 2: Phylogenetic tree based on Bayesian inference (BI) analysis of the *Enterocytozoon bieneusi* ITS sequences. Statistically significant posterior probabilities are indicated on the branches. Known and novel *E. bieneusi* ITS genotypes identified in the present study are indicated by empty and filled triangles, respectively.

4. DISCUSSION

The results of the present study showed that *E. bieneusi* is a common enteric parasite in claves in the study area. The prevalence of *E. bieneusi* infection in calves (7.1%) in the present study was lower than 18.0% prevalence found in calves in South Africa (Samra *et al.*, 2012) and most published studies conducted elsewhere in dairy cattle (Wang *et al.*, 2019; Zhang *et al.*, 2019; Tao *et al.*, 2020). Although a significant difference was not recorded among the study areas, *E. bieneusi* infection was slightly higher in Holeta site than the other study areas among calves (8.5%). The possible reason for the absence of statistically significant difference among the three study areas is that, in addition to the similarity in environmental conditions, there are relatively similar animal management systems. The prevalence of *E. bineusi* varied with the age of the calves. The lower age group, especially less than 5 weeks, had a significantly higher prevalence of infection than the other age groups. This contrasts with a longitudinal study reported by Santin and Fayer (2009) in dairy cattle in which post-weaned calves had the highest prevalence of infection than the pre-weaned calves. This might be because as the age of cattle increases, immunity might play a role in reducing the burden of *E. bieneusi*. No sex-associated prevalence was observed in the present study in calves. The possible reasons for the absence of sex-related differences in the prevalence among the calves could be explained by lack of sex-related resistance to infection of *E. bineusi*. In this study, a higher prevalence of *E. bieneusi* was detected in exotic breeds than native calves. This might be due to differences in management systems. It might be associated with host immunity that exotic is more susceptible than the native ones. Based on the sequence data from the ITS region, 88% of *E. bieneusi* isolates found in calves in this study appear to be so-called cattle-specific genotypes. This finding is consistent with the situation in South Africa as reported by Samra *et al.* (2012) where the cattle-specific genotypic infection was 89%. The result was also comparable to previous observations in the Eastern United States where 98% of *E. bieneusi* genotypes were identified as cattle-specific (J, I, BEB3 and BEB4) (Santin *et al.*, 2005). Sulaiman *et al.* (2004) also found a high proportion (96.5%) of cattle-specific *E. bieneusi* isolates (J, I, BEB3 and -BEB4) from seven states in North America and Portugal. In South Korea, cattle-specific genotypes of *E. bieneusi* were isolated in 90% of the positive animals: CEbA, I, J, and CEbF (with 99% similarity to J) (Lee, 2007). In China also, so-called cattle-specific genotypes have predominantly been detected in cattle fecal samples (Wang *et al.*, 2019; Zhang *et al.*, 2019; Tao *et al.*, 2020). Of the ten genotypes identified in calves in this study, genotype BEB8 was the most prevalent followed by genotype I. However, some of the cattle-specific genotypes, such as BEB4, I, and J have also been reported in other animals and humans, indicating their low host specificity and zoonotic significance (Li *et al*., 2019b).

The new genotype ET-C1 found in a calf was clustered to a member of genotypes isolated from cattle in Group 2. The genotype differed from genotype J (JF776170) at nucleotide positions 87 (G-to-A substitution) and 188 (G-to-T substitution). *E. bieneusi* has been identified in water sources as well as in wild, domestic, and food-producing farm animals, raising concerns of water-

borne, food-borne, and zoonotic transmission. The identification of four common human pathogenic genotypes of *E. bieneusi* (Peru11, KIN-1, H and PigEBITS5) (Li *et al*., 2019a; Li et al., 2019b) in this study highlights the possible role of calves as a source of environmental contamination with genotypes of zoonotic potential. In addition, the identification of the two most common genotypes of cattle (I and BEB4) (Thellier and Breton, 2008), now found in humans, changes the paradigm from genotypes thought to be cattle-specific to genotypes with zoonotic potential (Li *et al*., 2019b). This indicates the public health importance of *E. bieneusi*.

5. CONCLUSION

the results of this study suggest that *E. bieneusi* is prevalent in calves in Central Ethiopia and there is extensive genetic diversity, as was reported in other parts of the world. Although most of the genotypes recorded in this study were found to be cattle-specific, four genotypes were clustered to members of a major phylogenetic group with zoonotic potential. To the best of our knowledge, this constitutes the first molecular characterization of *E. bieneusi* in Ethiopia. Therefore, studies in humans and further studies in animals are necessary using molecular techniques to assess the genetic diversity and the public health significance of *E. bieneusi.*

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Author Contributions

LZ and TW conceived the idea for this study. TW, MRK and JL conducted the experiments and analysed the data. TW, MRK, JL and LZ wrote and revised the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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