

Cryptosporidium Infection in Dairy Cattle Calves and its Public Health Significance in Central Ethiopia

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ABSTRACT

Cryptosporidium spp. are common intestinal protozoan parasites that causes diarrhoea in neonates and young calves. This longitudinal study was conducted at two large dairy cattle farms in central Ethiopia during February/2014 to June/2015 to determine the age-related distribution of *Cryptosporidium* species, to identify risk factors of the disease and to assess the public health significance of the parasite. Thirty calves born to these dairy farms were followed-up from birth to three months of age, and 270 faecal samples were collected and examined by the Modified Ziehl-Neelsen, PCR-RFLP and Sequencing. *Cryptosporidium* was detected from week 1 to 3 months of age with an overall prevalence of 14.8%. Peak of the infection was at two weeks of age when 12 of the 30 calves (40%) shedded oocysts. *Cryptosporidium parvum* and *C. andersoni* were identified in pre-weaned and post-weaned calves, respectively. Phylogenetic analysis showed clustering of the *C. parvum* isolates from this study with GenBank sequences for *C. parvum* bovine genotype IIa and IIc subtypes. This study showed the predominance of the zoonotic *C. parvum* species in pre-weaned calves and demonstrated that this age group of calves pose the greatest risk for human infection. Due attention on the management of pre-weaned calves is recommended to prevent transmission of the infection to humans and lessen contamination of the environment by oocysts.

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Introduction

Cryptosporidium infection causes important economic impact to farmers due to its high morbidity and sometimes, high mortality rates in farm animals and sheep and goats (Fayer and Xiao 2008; Kaupke *et al.*, 2017). Four species commonly affect cattle; *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni*, and *Cryptosporidium ryanae* (Feng *et al.*, 2007; Brook *et al.*, 2008). *C. parvum* is generally associated with diarrhoea in susceptible hosts causing illness and even death, particularly in neonatal calves (Plutzer and Karanis, 2009). Infected farm animals, particularly cattle, are considered sources of human infection; this concern has put pressure on researchers and farmers to identify and manage the risks associated with the spread of the zoonotic infection. In Ethiopia, studies on *Cryptosporidium* of dairy farms are scarce yet; the few studies conducted to date show high prevalence of the

infection and signify the importance of dairy farms to human infections. However, most of these studies employed point prevalence study designs and conventional microscopy techniques which are unable to show the principal age group responsible for human infection and to identify species/genotype of the parasite. Therefore, the aim of this study was to determine the age-related distribution of *Cryptosporidium* species/genotypes, its significance in zoonotic transmission and identify the risk factors in young calves in central Ethiopia.

Materials and methods

Study area and sampling

The study was conducted at two government dairy cattle farms, Holeta Agricultural Research Centre (HARC) and Holeta Cattle Genetic Improvement Centre (HCGIC), found in Holeta town. Holeta is found in West Showa Zone of Oromiya Regional State, 45 km west of Addis Ababa. It is Located at longitude of 9°4' North and latitudes 38°30'E at an altitude of 2400 meters. Thirty calves, born to these dairy farms between

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February/2014 and June/2015, were followed-up from day one till three months of age for faecal sampling. Nine samples were collected per calf; at weekly interval during the first month and at two weeks' time then after. Thin slide smears made from each sample were fixed in absolute methanol for 5 minutes; air dried and conveyed to the laboratory while aliquots of the samples were kept in stool cups containing 2.5% potassium dichromate solution for molecular analysis.

Laboratory examinations

Microscopy of faecal specimen

The Modified Ziehl-Neelsen (MZN) staining (Henriksen and Pohlenz, 1981) was used to detect and identify oocysts of *Cryptosporidium* species at 100x magnification of the microscope. A sample was considered positive if an oocyst of correct morphology, optical properties, internal structure, size and shape (4–6 mm, refract pink, spherical round to oval with a residuum and sporozoites) was detected (Fayer, 1997).

Isolation of genomic DNA

Prior to DNA extraction 200 mg or 100–200 µl (if liquid) of each sample was washed and centrifuged three times in distilled water to clear out the preservative solution. DNA was then extracted from the cleaned samples using the QIAamp DNA Stool Mini Kit (QIAGEN inc. Valencia, USA) following the manufacturer's suggested procedures. DNA was stored at -20 OC until further analysis.

Nested PCR

A two-step nested PCR protocol was used to amplify a fragment of the SSU rRNA gene of *Cryptosporidium* species oocyst (830 bp) as described previously (Fayer and Xiao, 2008). In the primary PCR, a PCR product of 1325 bp was amplified using the forward and reverse primers SSU-F2 (5'-TTCTA-GAGCTAATACATGCG-3') and SSU-R2 (5'-CCCATTCCTTC-GAAACAGGA-3'), respectively. The primary, 25 µl, PCR mixture consisted of 1x PCR buffer, 6 mM MgCl₂, 200 mM of each of the four deoxyribonucleotide triphosphates (dNTPs), 10 pmol of each primer, 2.5 units of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 5 µl of template DNA and non-acetylated bovine serum albumin (BSA) (New England Biolabs, Beverly, MA, USA). The cycling conditions for the primary PCR were: an initial denaturation at 94°C for 3 min followed by 35 cycles (94°C for 45s, 55°C for 45 s, 72°C for 60s) and a final extension at 72°C for 7 minute. In the secondary PCR, a product size of 819-825 bp was amplified using the forward primer SSU-F3: 5'-GGAAGGGTTGATTTATTAGATAAAG-3' and the reverse primer SSU-R4: 5'- CTCATAAGGTGCTGAAG-GAGTA-3' (Fayer and Xiao, 2008). The secondary PCR reaction mixture consisted of 1x PCR buffer, 3 mM MgCl₂, 200 mM of each dNTPs, 10 pmol of each primer, 2.5 units of Taq DNA polymerase and 2 µl of the primary PCR product in a final volume of 25 µl. The cycling conditions for the secondary PCR were the same as the primary PCR. Secondary products of the nested PCR reactions were analyzed by 1.5% agarose gel electrophoresis and visualised on Gel red staining. All PCR reactions were run on a Thermocycler (PCR-Gene Amp PCR System 9700, Applied Biosystems) and DNA concentration was determined spectrophotometrically on a Nanodrop 2000 (Thermo Fisher Scientific Inc. USA).

Restriction fragment length polymorphism (RFLP) analysis

The secondary PCR products of the 18S rRNA gene were

purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instruction and were digested using the *SspI* and *MboII* (New England BioLabs Inc.) restriction enzymes (Xiao et al., 1999, Feng et al., 2007). Briefly, 10 µl of the purified secondary PCR product was digested with 5 units of enzyme and 2 µl of the corresponding 10x buffer in a final volume of 20 µl. All restriction digestions were carried out at 37°C overnight, fractionated on 2% agarose gel and visualised after Gel red staining.

DNA sequencing

The sequencing reaction of purified secondary nested PCR products was performed in both directions using the secondary PCR primers and the Big Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems, CA, U.S.A.) on an ABI 3730-48 Capillary Genetic Analyzer (Applied Biosystems Sequencer, Foster City, CA). The obtained sequences were analyzed using the CLC main workbench (CLC version 7.6.4, QIAGEN Aarhus) and compared with the GenBank sequences of *Cryptosporidium* using BLAST (Basic Local Alignment Search Tool, NCBI <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) to identify species and determine homology percent. Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers KX264363 to KX264365 and KX856003 to KX856004. Sequencing was performed at the Segolip unit, the Biosciences Eastern and Central Africa-International Livestock Research Institute Hub (BecA-ILRI Hub), Nairobi, Kenya.

Phylogenetic Analysis

Nineteen nucleotide sequences, 5 from this study and 14 from GeneBank, were used for analysis and sequence alignment was performed using CLUSTAL X version 2.0 Software (Larkin et al., 2007). The Neighbor-Joining tree was constructed based on the Kimura 2-parameter model (Kimura, 1980) using the MEGA version 6 software (Tamura et al., 2013). The tree was anchored by using *Eimeria tenella* (AF026388) as the out-group.

Risk factor data

Information on risk factors of *Cryptosporidium* was generated from primary follow-up data of the study calves collected during the sampling dates, secondary data on the study calves and management practices were collected from record books of the dairy farms.

Ethical clearance

Ethical clearance was obtained from the College of Veterinary medicine and Agriculture, Addis Ababa University. The aim of the study was explained and permission was obtained from farm owners and employees before collection of samples and data.

Statistical analysis

Data were analyzed by the SPSS statistical software package (SPSS ver.20.0 for Windows, SPSS Inc, Chicago, IL). The overall prevalence was calculated as the total number of *Cryptosporidium* oocyst positive samples divided by the total number of samples examined, while the prevalence at a specified sampling date was calculated as the number of oocyst positive samples divided by the total number of examined samples. The Chi-Square test was used to evaluate the association between hypothesized risk factors and the infection

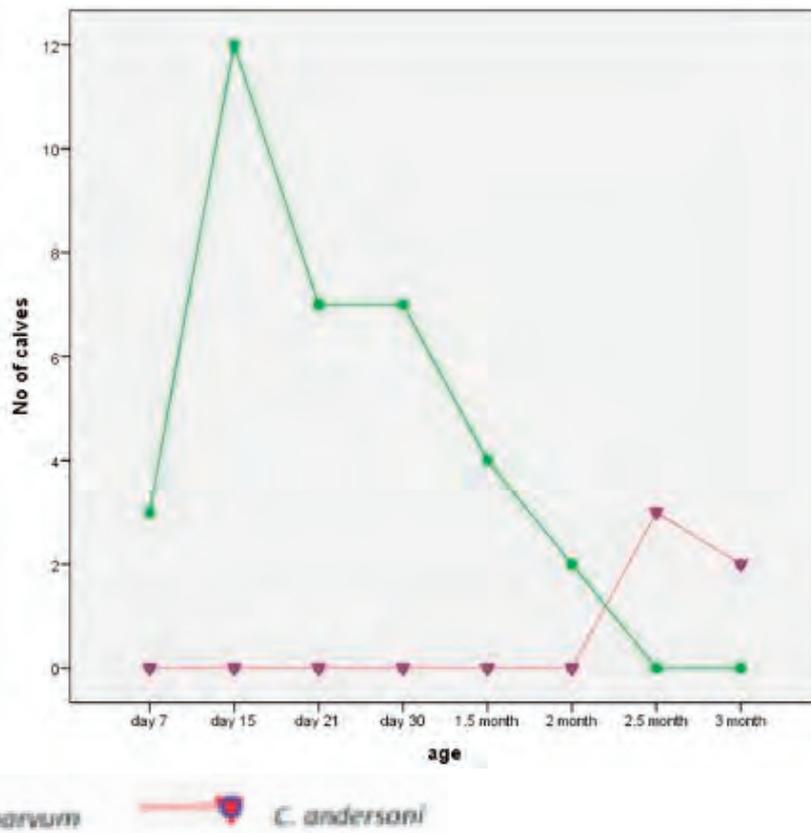


Fig. 1. Age related distribution of *Cryptosporidium* infections in calves in central Ethiopia (Feb. /2014 to June/2015)

and association of the risk factors with infection rate was estimated by the odds ratio (OR). Confidence level was held at 95% and $P < 0.05$ was set for significance level.

Results

Prevalence of *Cryptosporidium*

Two hundred seventy faecal specimens were collected and examined during the whole study period of which 40 samples (14.8%) were found *Cryptosporidium* positive. Twelve of the thirty calves (40%) shedded *Cryptosporidium* oocyst at least once during the study period. Oocysts were detected at every sampling age of the calves except at day one, the initial infection was detected at day seven of age where 10% of the calves shedded oocysts. The highest infection rate, 40%, was at two weeks of age when 12 of the 30 calves excreted oocysts followed by prevalence of 23.3% (7/30) at the age of 21 and 30 days (Fig. 1).

The overall prevalence in neonates, 19.3%, was significantly higher than the prevalence in post-neonate calves, 9.2% ($\chi^2 = 5.684$, $p = 0.022$, OR = 2.4, 95% CI = 1.1 – 5.0). The chance of infection in pre-weaned calves was 2.7 times more likely as compared to post-weaned calves ($\chi^2 = 5.818$, $p = 0.025$, OR = 2.66, 95% CI = 1.1 – 6.3).

Intensity of *Cryptosporidium* infection

Intensity of the infection was estimated through investigating 20 randomly selected microscope fields of 30 oocyst positive samples. The average number of oocysts in 13 samples was > 10 oocysts, 6 samples showed 6-10 oocysts, 7 showed 2-5 oocysts and 4 showed only 1 oocyst (Fig. 2).

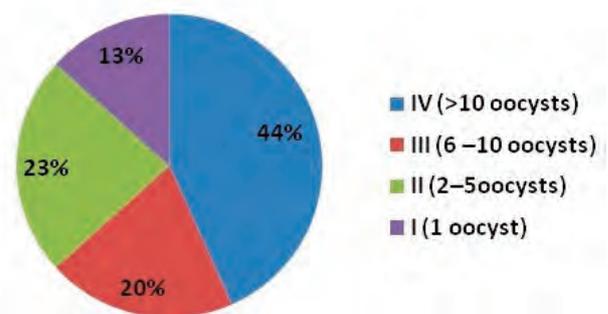


Fig. 2. Intensity of *Cryptosporidium* infection in calves in central Ethiopia (Feb./2014 to June/2015).

Risk factors of *Cryptosporidium*

Farm management practices, age, sex, genetic blood level, time of birth and parity of the dams were considered as risk factors of *Cryptosporidium* for investigation while consistency and colour of faeces as well as presence of mucus or blood in faeces were assessed for associations with the infection. The prevalence in the two study farms HARC (13.3%) and HCGIC (17.8%), as well as, the prevalence in male (15.2%) and female calves (14.6%), were statistically not different (Table 1). Calves with > 75% Holstein Friesian blood level showed prevalence of 18.1% which was higher and statistically different ($\chi^2 = 4.321$, $p=0.048$, OR=2.21, 95% CI=1.0 - 4.9) than the prevalence of 9.1% in crossbreed calves with 50-75% Holstein Friesian blood level. Calves born during night-time and calves born to cows above second parity were 2.6 and 3.6 times more

likely to acquire *Cryptosporidium* infection as compared to calves born during daytime and calves born to cows in their first or second parity (Table 1). Pre-weaned calves acquired *Cryptosporidium* infections 2.66 times more likely as compared to post weaned calves ($\chi^2 = 5.818$, $p = 0.025$, OR = 2.66, 95% CI = 1.1 – 6.3). *Cryptosporidium* infections showed significant associations with diarrheic faeces ($\chi^2 = 32.956$, $p = 0.000$, OR = 11.286, 95% CI = 4.0 – 31.9), creamy orange coloured faeces ($\chi^2 = 10.947$, $p = 0.002$, OR=3.732, 95% CI = 1.6 - 8.5) and faeces containing mucus ($\chi^2 = 13.348$, $p = 0.000$, OR = 3.610, 95% CI = 1.8-7.4) (Table 2).

Molecular results and Sequence analysis

All of the 40 microscopy positive samples were found positive by the nested PCR generating the expected product size of 830-bp (Fig. 3) and were sequenced successfully. The *SspI* and *MbolI*-RFLP restriction of the purified secondary PCR products indicated *C. parvum* in 35 (87.5%) specimens, and *C. andersoni* in 5 (12.5 %) specimens. Electrophoresis of *SspI* digested products showed restriction patterns of *C. parvum* with

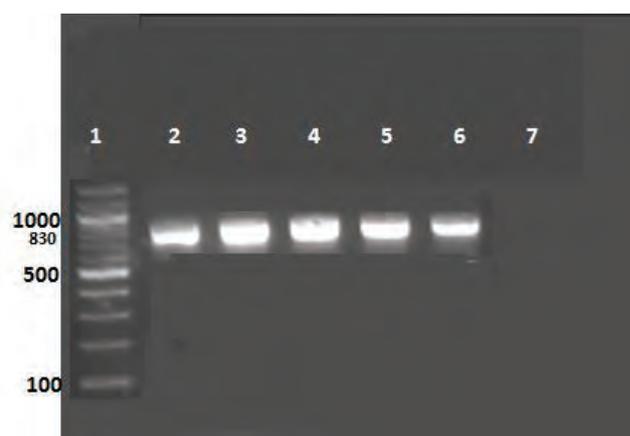


Fig. 3. Nested PCR secondary products of the SSU rRNA gene amplification of *Cryptosporidium*. Lane 1:100 bp ladder, lanes 2: positive control, lanes 3-6 positive samples, lane 7: negative control.

Table 1. Risk factors of *Cryptosporidium* in calves less than 3 month old in Central Ethiopia (Feb. /2014 to June/2015).

Risk factors	Label	¹ No of samp examined	² Prev (%)	³ χ^2	P value	⁴ OR	95% CI ⁵ for OR
Dairy farm	HARC	180	13.33		00		
	HCGIC	90	17.78	0.917	0.334	1.41	0.7– 2.8
Sex	Female	171	14.6		00		
	Male	99	15.2	0.014	0.906	0.96	0.5– 1.9
Exotic Blood level	50-75	99	9.1		00		
	>75	171	18.1	4.321	0.048	2.21	1.0 - 4.9
Age group	Post-weaned	90	7.78		00		
	Pre-weaned	180	18.33	5.818	0.025	2.66	1.1– 6.3
Time of birth	Day	207	11.1		00		
	Night	63	27.0	8.634	0.003	2.60	1.5–6.0
Parity of dam	2 nd parity	99	10.10		00		
	>2 nd parity	63	28.57	10.987	0.003	3.56	1.5–8.4
	1 st parity	108	11.11		0.814	1.11	0.5–2.7

¹No of samp.: Number of samples; ²Prev (%): Prevalence in percent; ³ χ^2 : Chi-square value; ⁴OR: Adjusted Odds Ratio; ⁵CI: Confidence Interval; HCGIC: Holota Cattle Genetic Improvement Centre; HARC: Holeta Agricultural Research Centre.

Table 2. Macroscopic characteristics of faecal samples in *Cryptosporidium* infected calves in Central Ethiopia (Feb. /2014 to June/2015).

Characters	Label	No of samp.	¹ Prev (%)	² χ^2	P value	OR	95% CI ⁴
Consistency of faeces	Soft	92	5.43		00		
	Liquid	61	39.34		0.000	11.286	4.0 -31.9
	Formed	117	9.40	32.956	0.290	1.806	0.6 - 5.4
Colour of faeces	Dark brown	120	7.50		00		
	Creamy yellow	99	23.23	10.947	0.002	3.732	1.6 - 8.5
Presence of mucus	Brown	51	15.69		0.109	2.295	0.8 - 6.3
	No	159	8.18		00		
Presence of blood	Yes	111	24.32	13.348	0.000	3.610	1.8 -7.4
	No	225	13.33	2.144	00		
	Yes	45	22.22		0.130	1.86	0.8 - 4.1

¹No of samp.: Number of samples; ² Prev (%): Prevalence in percent; ³ χ^2 : Chi-square value; ⁴OR: Adjusted Odds Ratio; ⁵CI: Confidence Interval.

three visible bands at the level of 449, 267 and 108 bp while *C. andersoni* generated two bands at the level of 448 and 397 bp as described previously (Feng et al., 2007). *MbolI* digested secondary PCR products showed restriction patterns with two visible bands of 771 and 76 bp for *C. parvum*, and bands of 769 and 76 bp for *C. andersoni* (Fig. 4). Sequence analysis confirmed the results obtained by the RFLP analysis. Infections by *C. parvum* species constituted 100% and 54.5% of the infections in samples from neonates and pre-weaned calves, respectively. In contrast, *C. andersoni* was detected only in samples of post-weaned calves (> 2 months) and not detected in samples from pre-weaned calves.

BLAST (Basic Local Alignment Search Tool) searches of the partial SSU rRNA gene sequences of 23 *C. parvum* positive samples showed 100% similarity with *C. parvum* nucleotide sequences AB513881, AB513858, L16997, AF108865 and EU660038, while 12 of the *C. parvum* positive samples showed 100% similarity with GeneBank sequences of *C. parvum*: AH006572.2, AB513880, KT151548, KP994662, L16996, AB089290, AY204238 and AF040725. All the five *C. andersoni* positive samples showed 100% similarity with GeneBank nucleotide sequences for *C. andersoni*:- KJ917578, AB777193, AB513856, AB089285, HM002493, FJ608606, KM199850 and KF826311.

Phylogeny

All isolates from this study grouped with their homologous GenBank retrieved sequences of *Cryptosporidium* species. The two species obtained in this study formed three clades with full statistical reliability. The first clade contained *C. andersoni* showing bootstrap values of 100%, the second and third clades contained *C. parvum* showing bootstrap values of 100% and 76 %, respectively. The *C. parvum* isolate KX856003 grouped in the first clade with GenBank retrieved isolates AB513881 and KM870599 showing 84% similarity. The *C. parvum* isolate, KX856004, grouped under the third clade with five GenBank retrieved sequences (AB089290, AF040725, AY204238, AB513880, EU660038) (Fig. 5). Genetic distance analysis using Kimura-2 Parameter confirmed the above results. GenBank isolate AB513881 showed identical genetic distance (0.000) with isolate KX856003. Isolate KX856004 from this study showed genetic distances varying from 0.000 - 0.002 with the five GenBank retrieved sequences (AB089290, AF040725, AY204238, AB513880, EU660038). In the same way,

the genetic distance between *C. andersoni* isolates from this study showed genetic distances ranging from 0.000 to 0.001 with their GenBank isolates (data not shown).

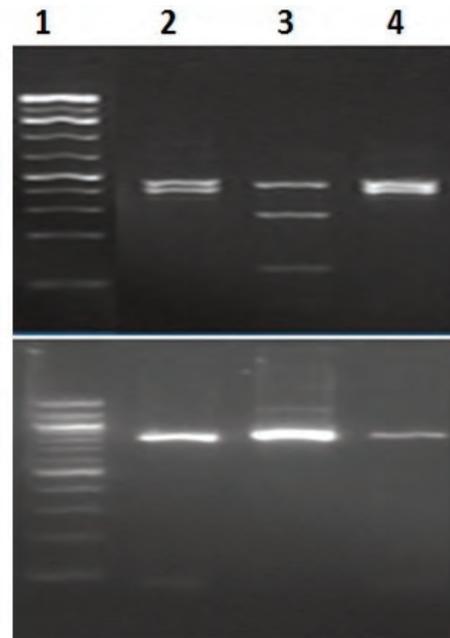


Fig. 4. Genotyping of *Cryptosporidium* isolates by RFLP analysis based on digestion of the 18s rRNA gene by *SspI* and *MbolI* enzymes.

Discussion

Results of this cohort study showed the occurrence of *Cryptosporidium* infections in calves aged between 7 to 90 days, the highest prevalence, 40%, being at two weeks of age. This finding is in agreement with a similar longitudinal study that reported the highest prevalence, 96.7%, of *Cryptosporidium* in 2 weeks old calves (Santin et al., 2008). Another study on neonatal calves reported 100% of the total infections before 14 days of age (Del Coco et al., 2008). Calfhoo infections, during the first two weeks of life, had also been reported by other studies (Castro-Hermida et al., 2002; Trotz-Williams et al., 2007). It is unclear how *Cryptosporidium* occur at such early age, since calves are segregated from their dams within hours

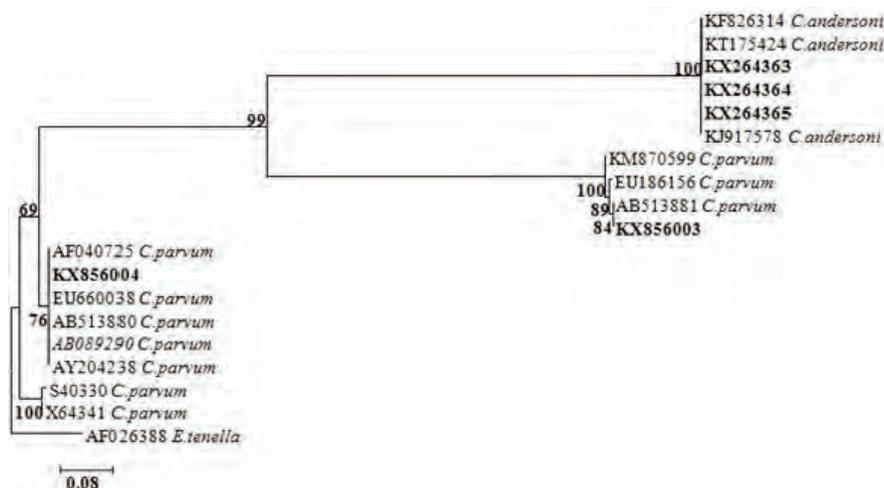


Fig. 5. Phylogenetic relationship among *Cryptosporidium* sequences retrieved from the GenBank and sequences from this study (bolded). The Neighbour-Joining tree was computed using the Kimura 2-parameter method, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale bar indicates an evolutionary distance of 0.08 nucleotides per position in the sequence.

of birth, housed in individual cages and have no direct contact with other calves till their weaning age. However, reports suggest that fecally contaminated fields, pens, water supplies, buildings, tools, animal handlers and birds could act as mechanical transmitters (Conn et al., 2007). Infections by *C. parvum* and *C. andersoni* in this study showed a strictly age-related pattern, calves aged one week to two months harboured only *C. parvum*, while older calves, 2.5 to 3 months, were exclusively infected by *C. andersoni*. This finding is in partial agreement with a similar study in Maryland whereby *C. parvum* was reported in calves up to 8 weeks of age while *C. andersoni* was detected only in heifers, 12–24 months of age (Santín et al., 2008). In support to our finding, studies on calves aged 12–24 weeks and 2–6 months in Nigeria and Vietnam reported absence of the zoonotic *C. parvum* species and suggested that these age groups are unlikely to contribute to human cryptosporidiosis (Ayinmode et al., 2010; Nguyen et al., 2012).

Our result showed that 100% of the *C. parvum* infections were in pre-weaned calves which is in accord with findings of Santín et al. (2008) that reported 97% of the *C. parvum* infections in pre-weaned calves, and with findings of Díaz-Lee et al. (2011) that stated *C. parvum* as the major parasitic disease agent of neonatal calves in the Metropolitan Region (Santiago) of Chile. Contrary to the present finding Castro-Hermida et al. (2007) reported only *C. parvum* in cows and Liu et al. (2009) reported *C. andersoni* and *C. ryanae* in dairy calves less than two months of age.

In this study, calves infected with *Cryptosporidium* showed yellowish mucoid diarrhoea without blood as compared to non-infected calves. Comparable findings demonstrating association of *Cryptosporidium* with diarrhoea and mucoid faeces had been reported (Del Coco et al., 2008; Díaz-Lee et al., 2011). The episode of mucoid diarrhoea in *Cryptosporidium* infected calves is due to invasion and colonisation of the intestinal epithelial surface by the parasite which results in loss of epithelial cells and microvillus brush border, increased epithelial permeability and osmotic diarrhoea (Chen et al., 2002; Robinson et al., 2003). Enemark et al. (2002) stated that clinical diarrhoea was restricted to calves younger than two months in which the highest number of oocyst was detected while Fayer et al. (1998) described the diarrhoea in 5 days to 1 month calves as a malabsorptive and secretory type which is watery or mucoid and pale to yellowish in colour. However absence of association between diarrhoea and shedding was reported from France (Rieux et al., 2013), Crossbred calves possessing more Holstein Friesian blood level showed higher prevalence of *Cryptosporidium* compared to crossbreds with lower Holstein Friesian blood. This might be due to more vulnerability of exotic breeds to *Cryptosporidium* compared to the local zebu. Calves born during night time showed higher prevalence compared to calves born during daytime. This could be due to prolonged contact of night born calves with their dams increasing the chance of exposure to the infection. This result is in support of the findings by Del Coco et al. (2008) which explained prolonged exposure in maternity pen as the cause of higher infection rates in newborn calves that stayed with their dams. Calves born to cows above second parity stage showed higher infection rate compared to calves born to cows at their first or second parity. This outcome could be due to higher probability of infection in older cows resulting in higher possibility of the infection in newborns.

Phylogenetic analysis showed that all *C. andersoni* isolates from this study clustered with analogous GeneBank isolates demonstrating that the *C. andersoni* species circulating in the area have very close similarity. Conversely, the *C. parvum* isolates grouped under two taxa suggesting concurrent circulation of multiple *C. parvum* subtypes in the study area. Our

result agrees with findings of a recent study in Ethiopia that reported occurrence of the zoonotic *C. parvum* subtype IIa and IIc infections in HIV/AIDS Patients (Adamu et al., 2014). The findings are also in agreement with findings of Xiao et al. (2001) that reported simultaneous circulation of four *C. parvum* subgenotypes in calves in central Ohio. Furthermore, these authors reported the occurrence of four different subgenotypes of *C. parvum* human genotype in eight specimens from AIDS patients in New Orleans.

Conclusion

Results of the present study showed that two species of *Cryptosporidium* affect dairy cattle calves up to three months of age in central Ethiopia with higher prevalence of the infection in pre-weaned than post-weaned calves. It is remarkable that *C. parvum* was the dominant species in neonates and pre-weaned calves, in contrast to post-weaned calves which were solely infected by *C. andersoni*. Findings of the study signify the importance of pre-weaned calves as sources of human infection. Thus, special emphasis should be given to the management of this age group to prevent spread of the infection in dairy farms, prevent transmission of the infection to humans and to control environmental contamination.

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