Molecular detection of genotypes and subtypes of C*ryptosporidium* infection in diarrheic calves, lambs, and goat kids from Turkey

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Abstract

Background: Cryptosporidium spp. are enteric protozoan parasites that infect a wide range of animals as well as humans. The studies on Cryptosporidium infections of animals in Turkey are mostly rely on microscopic observation. Few data are available regarding the distribution of Cryptosporidium genotypes and subtypes infection. The aim of this study is to analyse the zoonotic potential of Cryptosporidium oocysts shed from young ruminant livestock.

Methods: A total of 415 diarrheic fecal specimens from 333 calves, 67 lambs, and 15 goat kids were examined for the presence of Cryptosporidium oocysts by microscopy. Microscopic positive specimens were then analyzed for Cryptosporidium genotypes and subtypes detection by use of nested PCR of the small subunit ribosomal RNA (SSU rRNA) gene and the highly polymorphic 60 kDa glycoprotein (gp60) gene followed by sequence analyses.

Results: The results of this study revealed that 25.6% (106 of 415) of the specimens were positive for Cryptosporidium spp. infection by microscopic examination and molecular analysis. We identified 27.4% (91/333), 19.4% (13/67), and 13.4% (2/15) of positivity in calves, lambs and goat kids, respectively. Genotyping of the SSU rRNA indicated that almost all positive specimens were of C . parvum , except for one calf which was of C. bovis . Sequence analysis of the gp60 gene revealed the most common zoonotic subtypes (IIa and IId) of C . parvum. We detected 11 subtypes (IIaA11G2R1, IIaA11G3R1, IIaA12G3R1, IIaA13G2R1, IIaA13G4R1, IIaA14G1R1, IIaA14G3R1, IIaA15G2R1, IIdA16G1, IIdA18G1, IIdA22G1); three of them (IIaA12G3R1, IIaA11G3R1 and IIaA13G4R1) was novel subtypes found in calves and lambs. Additionally, three subtypes (IIaA11G2R1, IIaA14G3R1, and IIdA16G1) were detected in calves, lambs, and goat kid for the first time in Turkey.

Conclusions: The findings illustrate the high occurrence of Cryptosporidium infection in Turkey and suggest that calves, lambs, and goat kids are likely a major reservoir of C. parvum and a potential source of zoonotic transmission, which may have public health implications. Keywords: Calves, C. bovis, C. parvum , Cryptosporidium , Diarrhea, Goat kids, Lambs, Subtypes, Turkey.

Background

Cryptosporidiosis is a primary disease playing role in the etiology of neonatal diarrhea syndrome of ruminants and causes severe illness or death in young animals [1]. Among the 16 recognized species, Cryptosporidium parvum is of medical and veterinary importance. Cryptosporidium parvum has a number of subtypes that can be grouped into subtype families as measured by gp60 sequence analyses [2]. Healthy and diarrheic calves less than one month of age may facilitate the transmission of cryptosporidiosis in both humans and animals [3]. Other livestock are also potential reservoirs of this protozoan. In cattle herds, infected animals, particularly diarrheic calves, may act as sources of direct infection for other livestock [4]. Considering the worldwide distribution and zoonotic relevance of Cryptosporidium spp., a better understanding of transmission pathways and species distribution is

important for public health [5]. In addition, indirect transmission by cattle sheds, bedding, pasture, and soil and contaminated drinking water from environmental oocysts, has been reported as a major source of bovine transmission [6]. The clinical course of the disease in lambs and goat kids are similar to those of calves.

The distribution of Cryptosporidium species in goat kids has been reported to be similar to those of lambs, with the occurrence of mainly C. parvum, C. xiaoi, and C. ubiquitum [7–13]. However, geographic differences exist in the distribution of Cryptosporidium species in lambs, with C. parvum as the dominant species in European countries, C. ubiquitum in the Americas, C. xiaoi in developing countries, and all three species common in Australia [14]. In addition, European studies revealed that C. parvum was mostly found in clinically affected lambs, whereas C. ubiquitum and C. xiaoi were commonly observed in healthy lambs [7, 15, 16].

There are a few studies on Cryptosporidium infections in animals in Turkey; however, most of them rely on microscopic observation of oocysts and detection of coproantigens by the ELISA method. Cryptosporidiosis in slaughtered animals was investigated in Van province [17]. Since then, Cryptosporidium oocysts have been reported in animals with and without diarrhea from Kars [18], Erzurum [19], and Van provinces of Turkey [20]. Recently, a study mentioned C. parvum was the predominant species in pre-weaned calves whereas C. bovis and C. ryanae were mostly found in post-weaned calves and heifers in the Mediterranean and Central Anatolia regions of Turkey [21]. Another study reported the distribution of C. parvum in free-range pre-weaned diarrheic calves and goat kids in five provinces of Turkey [22]. Two studies subtyped C. parvum-positive isolates from pre-weaned calves by using gp60 sequence analysis in Kars, Turkey [23, 24].

Molecular diagnostic approaches make it possible to detect human infections deriving from agricultural animals. Accordingly, the goal of the present study was to assess the zoonotic potential of Cryptosporidium by identifying the genotype and subtype of Cryptosporidium spp. in the diarrheic calves, lambs, and goat kids in Turkey.

Methods

Specimen collection

A total of 415 diarrheic fecal specimens from 333 calves, 67 lambs, and 15 goat kids were collected between 2016 and 2018 from Konya province, Turkey. Diarrhea was observed during the time of specimen collection. All relevant data such as age and consistency of diarrhea were recorded at the time of sample collection for further analysis. Each specimen was placed into an individual sterile polystyrene tube with 2.5% potassium dichromate, labelled, and kept at 4 °C until DNA extraction.

Specimen analyses

Specimens were microscopically examined for *Cryptosporidium* spp. oocysts by use of the modified Ziehl-Neelson (MZN) method [25]. DNA was extracted directly from the specimens by using the Biomasher IV (Funakoshi Co., Ltd.) and NucleoSpin®Tissue (MACHEREY-NAGEL) kits according to the manufacturer's instructions [26]. After extraction, DNA was stored at −20°C. For genotyping and subtyping of *Cryptosporidium* detection, an approximately 830-bp fragment of the SSU rRNA gene and the gp60 gene were amplified by using KOD FX Neo (TOYOBO, Japan) with primers as described previously [27, 28]. For the primary PCR, a product of 1325 bp was amplified using the following primers: 5'- TTCTAGAGCTAATACATGCG -3' and 5'-CCCATTTCCTTCGAAACAGGA-3'. Then, a nested PCR was done using the following primers: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA -3' to amplify the ∼830-bp fragment of the *Cryptosporidium* SSU rRNA gene. *Cryptosporidium baileyi* genomic DNA and ultrapure water were used as positive and negative controls, respectively, in all PCR sets. The amplified fragments were electrophoresed in 1.5% agarose, stained with GelRed® (Biotium), and visualized on an UV transilluminator in the QIAxcel Advanced system (Qiagen, Valencia, California). The identity of each *Cryptosporidium* species was confirmed by sequence analysis of the secondary PCR products from the specimens. The gp60 gene was used to subtype *C. parvum.* The subtypes were named after sequencing of gp60 gene of these isolates based on the number of trinucleotide repeats encoding the amino acid serine (TCA, TCG, TCT, and ACATCA) sequence [29].

Sequencing and phylogenetic analyses

All secondary PCR products were sequenced in both directions by using an ABI 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan) with the secondary primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan). The sequences were aligned by using Clustal X_2 [30], and the computed sequences were edited by hand with BioEdit 7.0.5.3 [31]. All gaps were eliminated and SSU rRNA genes were used for the phylogenetic analysis. Maximum Likelihood analyses were performed by using MEGA version 7 software program [32]. Substitution models and optional parameter sets were selected according to the Akaike information criterion. To assess the reliability of the tree, bootstrap analysis was done with 1000 replicates using the same datasets. We constructed two phylogenetic trees: one for the SSU rRNA gene, in which the substitution model and optional parameters were also used on the Tamura 3-parameter model [33], and the other for the gp60 gene, in which the Hasegawa-Kishino-Yano model [34] was used, incorporating the invariable site and Gamma distribution options.

Results

Genotype detection

Of the 415 fecal specimens investigated, 106 (25.6%) were confirmed by the MZN technique to be infected with *Cryptosporidium* spp. oocysts. All microscopically positive fecal specimens were also nested PCR positive. Two *Cryptosporidium* species, namely *C. parvum* and *C. bovis* were identified in calves using SSU rRNA gene analyses, accounting for 27.1%, and 0.3%, respectively. Only *Cryptosporidium parvum* was identified in lambs and goat kids using SSU rRNA gene analyses, accounting for 19.4%, and 13.4%, respectively (Table 1).

Specimen analyses

Of the 415 fecal specimens, *Cryptosporidium* was found more frequently in 3–7-day-old calves (30.8%) than in 8–15-day-old calves (26.3%) and 16–30-day-old calves (17.8%). *Cryptosporidium* prevalence was also higher in younger lambs: 23.1% in 3–7-day-old lambs compared with 14.2% in both 8–15-day-old lambs and 16–30-day-old lambs. However, in goat kids, 12.5% of specimens from 3–7-day-old animals were *Cryptosporidium*-positive compared with 16.7% of specimens from 8–15-day-old animals (Table 2). Most of the specimen contained watery consistency diarrhea, and *Cryptosporidium* was found predominantly in the watery consistency diarrhea of the calves, lambs, and goat kids (Additional file 1: Table S1).

Phylogenetic analyses

All PCR-positive specimens were directly sequenced; analyses of the nucleotide sequences of the SSU rRNA genes revealed the presence of *C. parvum* in calves, lambs, and goat kids, and the presence of *C. bovis* in one calf in Turkey. Fragments of the SSU rRNA gene sequences of *Cryptosporidium* spp. acquired in this study were deposited in GenBank (MN918153-MN918257 and MN918118). Based on a blast search, all of the sequences detected in this study were identified as *Cryptosporidium.* The nucleotide sequence of *C. parvum* had 99.5% to 100% genetic identity with the reference sequences previously published in GenBank (JX298604, KF533079, JQ313985, GQ983351, and MK731971). Among the *Cryptosporidium* partial SSU rRNA gene sequences, only one sample had 100% identity with the *C. bovis* reference sequences in GenBank (AB777173, AB628204, EU408317, MK880573) (Fig. 1).

All of the *C. parvum*-positive specimens generated the expected gp60 PCR product. However, only 82 of the 105 isolates the gp60 gene were successfully sequenced and deposited in GenBank (MN962650-MN962718, MN998529-MN998541). Three subtypes (IIaA13G4R1, IIaA12G3R1, and IIaA11G3R1) differed from reference sequences regarding the amount of TCA and/or TCG repeats and were considered novel *C. parvum* subtypes. Eight subtypes were identified among the calf *C. parvum* specimens, including two novel (IIaA12G3R1 and IIaA11G3R1) subtypes and three other IIa subtype families (IIaA13G2R1, IIaA15G2R1, and IIaA11G2R1) and three IId subtype families (IIdA16G1, IIdA18G1, and IIdA22G1). Moreover, six subtypes were identified among the lamb *C. parvum* specimens, including two novel

subtypes (IIaA13G4R1 and IIaA12G3R1) and four other IIa subtype family (IIaA15G2R1, IIaA13G2R1, IIaA14G3R1, and IIaA11G2R1). In addition, we identified two subtypes (IIaA14G1R1 and IIaA13G2R1) in the goat kid *C. parvum* specimens (Fig. 2 and 3).

Discussion

In the present study, the prevalence of Cryptosporidium was found to be 25.6%, which is consistent with previous studies conducted in Turkey. Except for one calf which was infected with C. bovis, all of the Cryptosporidium from 90 calves, 13 lambs and 2 goat kids were of C. parvum. In Turkey, there is only one study reporting C. bovis in calves in the Mediterranean and Central Anatolia regions [21]. In an another report C. bovis was detected in environmental water in Turkey [35]. Cryptosporidium parvum was detected in a small number of calves and cattle in northeastern Turkey [23, 36]. C. parvum infections commonly cause profuse watery diarrhea which sometimes contains mucus or blood, dehydration, abdominal pain, loss of appetite, and weight loss. The disease causes severe illness and death in young ruminants. Therefore, Cryptosporidium infections lead to substantial economic losses [1]. Cryptosporidium parvum is not host-specific; accordingly, an environment contaminated with oocysts during an outbreak in calves can give rise to infection in lambs and goat kids that subsequently use the same grazing area.

In Turkey, the prevalence of Cryptosporidium infection in diarrheic calves, lambs, and goat kids has previously been reported to range from 7.5–79.1% depending on the geographical region or province [17–21, 24, 36]. However, most of these studies relied on microscopic examination of fecal specimens. Cryptosporidium infection rates in Europe have been reported as follows: 38.8% of calves in Italy [3], 37% and 12% of dairy and beef calves, respectively, in Belgium [37], 36.7% of calves in Sweden [38], 22.5% of calves in Poland [39], 19.2% of lambs and 37.1% of goats in Poland [12], 74.4% of lambs and 93.8% of goat kids in Spain [11], 13.1% of lambs and 9.5% of goat kids in Belgium [40], and 5.1% of lambs and 7.1% of goat kids in Greece [10]. From these studies, it is clear that cryptosporidial infections are widespread in calves, lambs, and goat kids in Europe, including Turkey.

In this study, the results of gp60 sequence analysis revealed significant genetic diversity with the presence of two novel subtypes (IIaA12G3R1 and IIaA11G3R1) of the IIa subtype family of C. parvum in calves. Most of the calves were found to be infected with subtype IIaA13G2R1, which was common subtype in all three hosts and the subtype found firstly in lambs in Turkey. Subtype IIaA13G2R1 was previously identified in free-range pre-weaned diarrheic dairy calves and goat kids in Turkey [22]. Moreover, IIaA13G2R1 was found the most common subtype in pre-weaned diarrheic calves in the Central Anatolia region of Turkey [21]. This uncommon zoonotic subtype was also reported in Algerian young calves, lambs, and goat kids [41, 42]. Moreover, there are only a few reports of this uncommon subtype in a small number of calves in Belgium, Canada, and the Netherlands [37, 43, 44]. This subtype was also detected in a HIV-positive individual in Malaysia [45]. The second-most common subtype identified in this study, IIaA15G2R1 which is the most common subtype found worldwide, has been reported previously in cattle in Kars province of Turkey [24]. This subtype is dominant in humans in Scotland [46]. The high prevalence of subtype IIaA15G2R1 in calves and lambs worldwide and its detection in humans suggest

that it is easily spread among animal populations and readily transmitted to humans as well. Subtype IIaA11G2R1 has not previously been reported in Turkey; however, it has been reported in humans in Slovenia and Slovakia [47, 48].

The IId subtype family (IIdA16G1, IIdA18G1 and IIdA22G1) of C. parvum was also identified in calves in this study. Subtypes IIdA18G1 and IIdA22G1 have previously been detected in calves and goat kids in Turkey [22]. In a study conducted in Greece, IIdA16G1 subtype was identified in diarrheic lambs and goat kids [13]. Within the subtype IId family, IIdA16G1, IIdA18G1 and IIdA22G1 subtypes have previously been reported in calves, lambs, and goat kids in Spain [7]. C. parvum IId subtypes were found at low frequencies in calves in many European countries [37, 38, 49, 50]. IIdA22G1 and IIdA16G1 detected in this study has been identified in humans in Europe [2, 51, 52].

It was elucidated that C. parvum was the dominant Cryptosporidium species among the calves, lambs and goat kids in the present study. Two novel subtypes (IIaA13G4R1 and IIaA12G3R1) were identified among the lambs. IIa was the dominant subtype family of C. parvum in both lambs and goat kids in this study. IIa and IId subtypes were found previously in diarrheic goat kids in Turkey [22]. However, the IId subtype was dominant in lambs and goat kids in northeastern Spain [7]. Interestingly, subtype IIaA14G3R1, identified in lambs in this study was previously found in fresh molluscan shellfish in Italy [53].

Cryptosporidium parvum has been identified in pregnant women, children, and an infant in Turkey, however subtype data are not available [54–56]. Common subtype families of C. parvum are IIa, IIc, IId and IIe. The IIa is the predominant family in animals and humans worldwide, whereas IId is another major zoonotic subtype family in Europe, Asia, North Africa and Australia [57]. The C. parvum subtypes found in this study were previously detected in humans and probably have public health risk.

Conclusions

The presence of zoonotic C. parvum subtype families (IIa, IId) in this study suggests that calves, lambs, and goat kids are likely to be a major reservoir of C. parvum. Animals infected with these subtypes probably poses a threat to human health in Turkey. Our study showed the diversity of molecularly characterized C. parvum isolates isolated in Turkey; C. bovis was identified in a calf and three novel subtypes (IIaA13G4R1, IIaA11G3R1, and IIaA12G3R1) were found. Further molecular studies in calves, lambs, and goat kids from other provinces are required to better understand the epidemiology of cryptosporidiosis in Turkey.

Abbreviations

SSU rRNA
Small subunit ribosomal ribonucleic acid; gp60:60 kDa glycoprotein; MZN:Modified Ziehl-Neelsen.

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this article. The nucleotide sequences generated in this study were deposited in the GenBank database under accession numbers MN918153-MN918257, MN918118 and MN962650-MN962718, MN998529-MN998541.

Authors' contributions

KK, FS and XX were involved in planning the concept. MHBK wrote the manuscript with support from all authors. MHBK and OC manufactured the specimens, performed the experiments and processed the experimental data. MHBK, OC, CC, AAS and HB performed the analysis, designed the figures, organized and sort out all data. MHBK, OC and MIE contributed to the interpretation of the results. KK, FS and XX supervised the findings of this work. All authors read and approved the final manuscript.

Ethics approval

The research protocol was reviewed and approved by the Research Ethics Committee of Obihiro University of Agricultural and Veterinary Medicine. Permission was obtained from all animal owners before the fecal specimens were collected.

Consent for publication

Not applicable.

Competing of interests

The authors have no competing of interests to declare.

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Figures

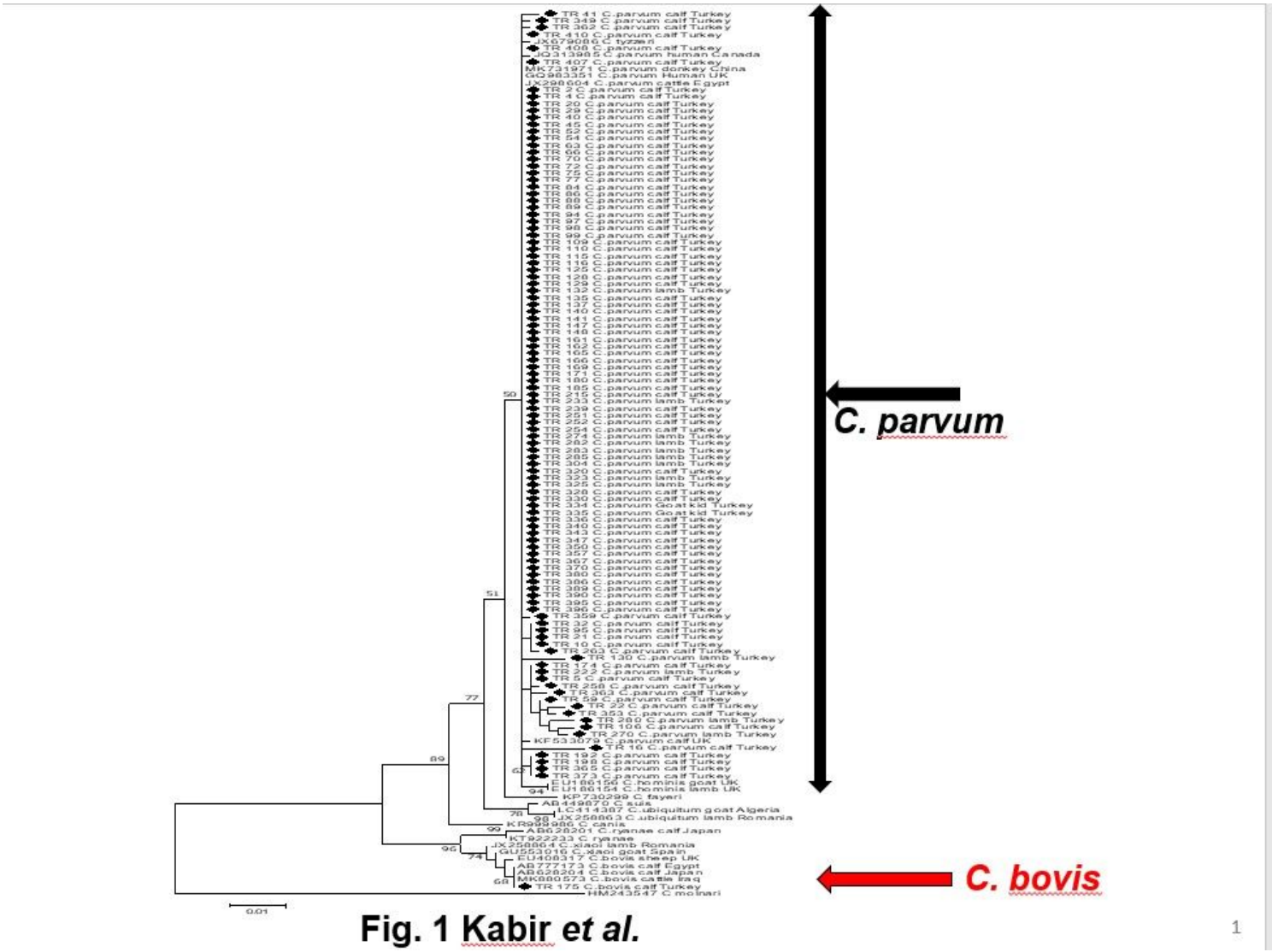

Fig. 1 Kabir *et al.*

Figure 1

Phylogenetic tree based on partial sequences of the SSU rRNA genes for Cryptosporidium spp. The phylogenetic tree was constructed without nucleotide gaps by using a Maximum Likelihood analysis with 1000 replicates based on the T92+G model. Cryptosporidium molnari sequence was used as the out-group. Only bootstrap values >50% from 1000 replicates are shown at the nodes. Black filled bold indicate sequences generated in the present study

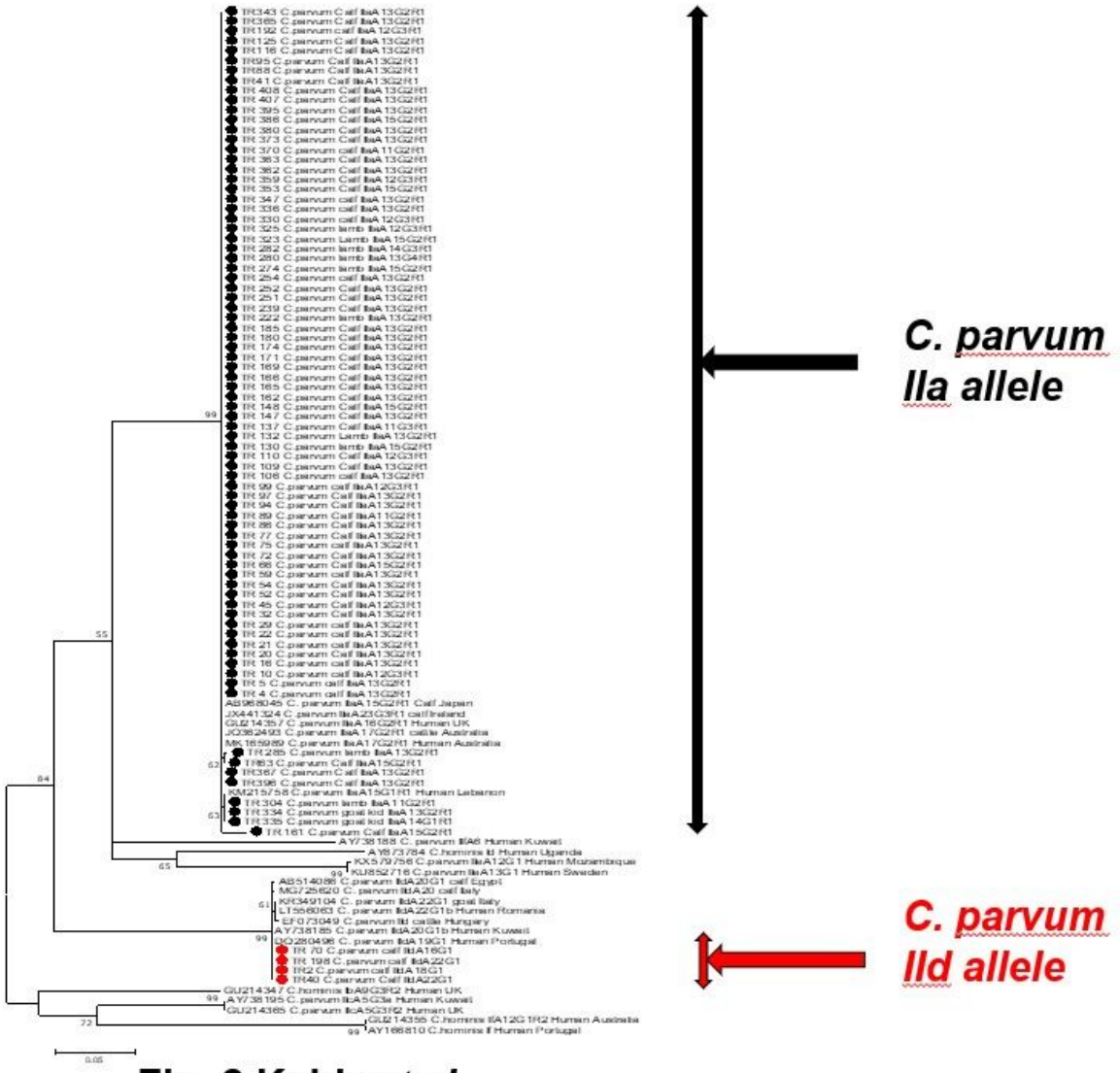

Fig. 2 Kabir *et al.*

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Figure 2

Phylogenetic tree based on partial sequences of the gp60 genes for Cryptosporidium parvum. The phylogenetic tree was constructed without nucleotide gaps by using a Maximum Likelihood analysis with 1000 replicates based on the Hasegawa-Kishino-Yano model. Only bootstrap values >50% from 1000 replicates are shown at the nodes. Black and Red filled circles represent sequences in two different subtype families generated in this study

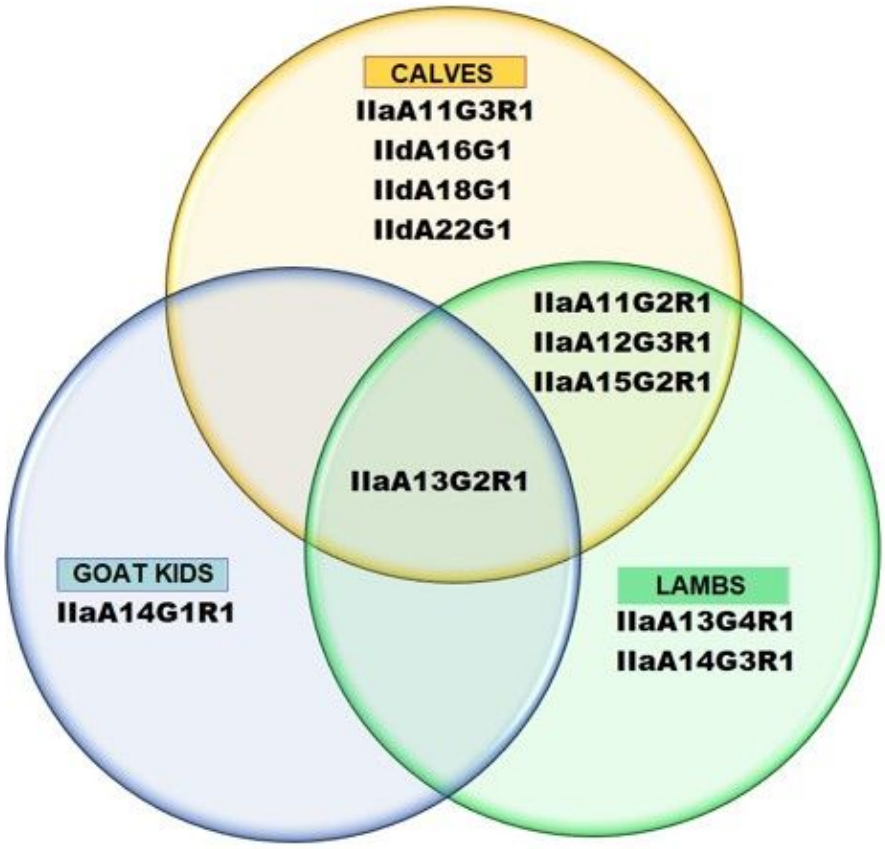

Fig. 3 Kabir *et al.*

Figure 3

Distribution of subtypes in the diarrheic calves, lambs and goat kids

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