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Comparison of three diagnostic methods in the diagnosis of cryptosporidiosis and *gp60* subtyping of *Cryptosporidium parvum* in diarrheic calves in Central Anatolia Region of Turkey

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Abstract

The aim of this study was to compare three diagnostic methods for the diagnosis of cryptosporidiosis and to detect subtypes of *Cryptosporidium parvum* by sequences analyses of *gp60* gene in diarrheic calves in several herds in Konya province located in Central Anatolia Region of Turkey. Fecal samples were collected from a total of 194 pre-weaned calves (n=158, ≤15 days old, and n=36, 15 to 40 days old), with diarrhoea. For comparative diagnosis, all samples were examined by modified Ziehl-Neelsen staining of fecal smears for the presence of oocyst, nested PCR-RFLP of SSU rRNA and TaqMan qPCR for the detection of *Cryptosporidium* DNA. A total of 92 (47.4%) and 104 (53.6%) out of the examined samples were found positive by microscopic examination and molecular tools, respectively. The diagnostic sensitivity and specificity of microscopic identification were determined as 88.5% and 100.0%, respectively compared to molecular assays. *Cryptosporidium parvum* was the only detected species in all positive samples by species-specific qPCR and nested PCR-RFLP assays. Species identifications were further confirmed by sequence analyses of the SSU rRNA PCR products. There was no statistically significant difference in *C. parvum* prevalence between early pre-weaned calves and calves older than 15 days. The sequence analyses of the *gp60* gene of *C. parvum* isolates revealed a one subtype IIaA13G2R1 belonging to zoonotic family IIa in diarrheic calves

Keywords: *Cryptosporidium parvum*, diarrheic calves, *gp60* subtype, nested PCR-RFLP, prevalence, TaqMan qPCR, Turkey

Introduction

Cryptosporidium species, the causative agents of cryptosporidiosis, are the important opportunistic intestinal pathogens that can infect various hosts including cattle and humans (1–3). The ingestion of contaminated water and foods is the main risk factor for calves and other hosts as well as humans for *Cryptosporidium* spp. infections (4,5). Cattle, especially pre-weaned calves are known as the most common hosts of *Cryptosporidium* spp. with high infection and oocyst excretion rates in their feces (6,7). *Cryptosporidium parvum* is recognized as the primary zoonotic species causing cryptosporidiosis in pre-weaned calves resulting in diarrhoea, weight loss, dehydration, delayed growth, and important economic losses related to calf morbidity and mortality (6,8). Furthermore, young calves are regarded as an important potential source of human cryptosporidiosis in various outbreaks worldwide (9).

Several techniques are used for the laboratory detection of *Cryptosporidium* in fecal samples. The oocysts of *Cryptosporidium* cannot concentrate well using standard concentration techniques but can be identified by microscopy combined with various staining methods, i.e. modified Ziehl-Neelsen of fecal smears (10). On the other hand, the diagnostic efficiency of conventional microscopic examination might be low due to sporadic oocyst shedding and the presence of few oocysts in the fecal samples. Examination of the stained fecal smears also has a disadvantage for the detection of *Cryptosporidium* because the oocysts can easily be confused with other materials (e.g fecal debris, yeast

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cells, and bacterial spores) present in the smears (11). In spite of higher costs and needs for infrastructure needs and high technical expertise, the use of molecular tools for the detection of *Cryptosporidium* has the advantages of improved sensitivity and specificity. These methods also allowed us to better understand the genetic diversity of *Cryptosporidium* species that are highly host-specific in genotype and subtype levels, transmission routes, and related impacts on public health (8,12). Currently, small-subunit rRNA (SSU rRNA) gene-based nested PCR-RFLP analysis have been developed for the detection and identification of *Cryptosporidium* species in fecal and environmental samples (13–15). A quantitative real-time PCR (qPCR) assay has also been developed to identify and quantify *Cryptosporidium* DNA in fecal and various environmental samples (16–18).

Host-specific and zoonotic subtypes have been described using sequence analysis of the 60 kDa glycoprotein (*gp60*) gene that is widely used in subtyping of *C. parvum* because of its high polymorphism (19,20). To date, at least 20 subtype families have been described within *C. parvum* (21). Subtypes IIa and IId are considered zoonotic subtypes found in both humans and ruminants. The subtype family IIa of *C. parvum* is common in calves and the IIaA15G2R1 is the most prevalent subtype in many countries (9,21,22).

Cryptosporidium parvum is known as one of the main causative agents of neonatal calf diarrhoea in herds in Turkey. However, there have been little data on the molecular characterization and subtyping of *C. parvum* in pre-weaned calves in Turkey (23–25). This study was conducted to evaluate diagnostic efficiency and usefulness of the nested PCR, Real-Time PCR assay and conventional modified Ziehl-Neelsen staining of the faecal smears comparatively, for the detection of cryptosporidiosis in calves with neonatal diarrhoea in the traditional farms of private smallholders in three districts of Konya province located in Central Anatolia Region of Turkey. *Cryptosporidium parvum* subtypes were also investigated by sequence analysis of the *gp60* gene to reveal zoonotic transmission dynamics of cryptosporidiosis in the research area.

Materials and Methods

Sampling strategy, data collection and identification of oocysts

Farms from three districts (Merkez, Karatay, and Selcuklu) were selected between 2011 and 2012 according to local veterinarians' opinion. The inclusion criterion for farms was a size of 5 to 50 calves traditionally owned by private smallholders with a history of neonatal calf diarrhoea. Based on sample size calculations (EPIDAT 3.1), a total of 20 farms were selected from a total of 208 officially recorded farms belonged to private smallholders in the research area. Environmental conditions of the farms included in the study shared common characteristics such as similar climatic conditions, traditional barns and pasture based raising of calves. A total of 194 fecal samples from early pre-weaned (n=158, ≤15 days old) and pre-weaned calves (n=36, 15 to 40 days old) with neonatal calf diarrhoea were collected from the predefined farms (5 to 15 calves per farm). Approximately 30-50 g fecal samples were taken from fresh droppings on the ground after defecation, then immediately placed into a sterile plastic container and held at 4°C until analysis. The fecal smears were prepared and stained using the modified Ziehl-Neelsen staining method for the presence of oocysts (26).

Genomic DNA extraction

200 mg of each fecal sample within a microcentrifuge tube were frozen in liquid nitrogen and subsequently heated before DNA extraction. Genomic DNA (gDNA) was extracted from disrupted samples by the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations of the extracts were measured with the Qubit Fluorometric Quantitation (Thermo Fisher Scientific, USA) to optimizing the amount used in the PCR master mix. The extracted DNAs were then preserved at -20°C until the time of molecular analysis.

Quantitative Real time PCR (qPCR)

TaqMan qPCR assay with primers and probes targeting *Cryptosporidium* at genus level and *C. parvum* was carried out according to Jothikumar et al. (27). A CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for all qPCR analysis. The reaction conditions for the qPCR were adjusted according to the protocol described by Jothikumar et al. (27) using SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA, USA) and 10-30 ng template DNA in each reaction

Nested PCR-RFLP of SSU rRNA and amplification of *gp60*

gDNA isolates were analyzed with nested PCR to amplify approximately 840 bp and 850 bp fragments of the SSU rRNA and *gp60* gene regions, respectively with specific primers following the protocols previously described (28–30). PCR products were electrophoretically resolved in 1.5 % agarose gels containing SYBR™ Safe stain (Thermo Fisher Scientific, USA) and visualized using Fusion FX Gel Documentation System (Vilber Lourmat, France). For RFLP analysis, secondary PCR products of the SSU rRNA gene were digested using FastDigest SspI and FastDigest MboII (Thermo Fisher Scientific, USA). Identification of *Cryptosporidium* species was performed according to restriction patterns described previously (31).

Sequence analysis of SSU rRNA and *gp60* subtyping

All gel-purified secondary PCR products of the two target genes (SSU rRNA and *gp60*) were sequenced (Macrogen, Netherlands) in both directions to confirm RFLP results and detect subtypes of *C. parvum*. The nucleotide sequences were assembled with Geneious Prime 11.0.2 software (http://www.geneious.com). The consensus sequences were aligned with reference sequences downloaded from GenBank by MUSCLE (32) using Geneious prime. Subtypes of *C. parvum* were named

with established for *Cryptosporidium* spp. *gp60* nomenclature system (33).

Results

Identification and distribution of *C. parvum* according to the diagnostic methods

A total of 92 and 104 samples were found positive by utilizing microscopic examination (Figure 1a) and molecular assays, respectively (Table 1) and there was full agreement between the results of these two tests. Microscopic analysis revealed a lower number of *Cryptosporidium* positivity and its diagnostic sensitivity and specificity were determined as 88.5% and 100.0% compared to molecular tools, respectively (Table 1). 15 of samples from diarrheic pre-weaned calves were found positive in the *C. parvum* species-specific real-time PCR assay (Figure 1c) and there was no positivity at the genus level without *C. parvum*.

RFLP and sequence analysis of SSU rRNA

RFLP analysis of all SSU rRNA nested PCR products from the isolates that were identified as *C. parvum* in TaqMan qPCR revealed three (449, 267, and 108 bp) and two (771 and 76 bp) band profiles specific to *C. parvum* with the restriction enzymes SspI and MboII, respectively (Figure 1b) confirming the qPCR results. No mixed infections were found in the examined specimens with the RFLP analysis of the SSU rRNA gene. DNA sequence analysis and BLAST search of the SSU rRNA nucleotide sequences of the isolates showed species-based homology with the corresponding sequences of *C. parvum* in GenBank further confirming the results of qPCR and RFLP analysis. SSU rRNA sequences of *C. parvum* characterized in the study were deposited to the NCBI GenBank database under accession numbers KF008173-75.

C. parvum subtypes with *gp60* sequencing

Gp60 gene was successfully amplified and sequenced from isolates of all *C. parvum* positive samples. Sequence analysis identified only one subtype belonging to family IIa (Table 2).

Table 1. Comparison of the results of microscopic examination and molecular assays in the detection of *Cryptosporidium* in calves and evaluation of diagnostic sensitivity and specificity.

Method		Molecular tools		Sensitivity	Specificity
		Positive	Negative		
Microscopy	Positive	92	0	85.5% (80.7-93.9%)	100% (96.0-100.0%)
	Negative	12	90		
Total		104	90		

The distribution of *C. parvum* over the age groups of pre-weaned calves with diarrhoea based on the molecular assays is presented in Table 2. The prevalence in early pre-weaned calves (≤15 days old) (56.3%) was higher than the prevalence in pre-weaned calves (15 to 40 days old) (41.7%). However, this difference was not statistically significant ($p > 0.05$) (Table 2).

IIaA13G2R1 subtype has 13 copies of TCA (A) and, 2 copies of TCG (G) trinucleotide repeat encoding serine, and one copy of the sequence ACATCA (R1) following the trinucleotide repeat. *Gp60* sequences of the isolates were submitted to the NCBI GenBank database under accession numbers KF008176-85.

Table 2. Distribution and subtype of *C. parvum* over age groups of the examined calves based on molecular diagnostic tests used in the study.

Age group (days)	Total no. of samples	*Cryptosporidium parvum*			
		No. positive (%)	χ2	p	*gp60* subtype
Early pre-weaned calves (≤15)	158	89 (56.3)	2.535	0.139	*IIaA13G2R1*
Pre-weaned calves (15-40)	36	15 (41.7)			
Total	194	104 (53.6)			

Discussion

Cryptosporidium parvum is an important causative pathogen of neonatal calf diarrhoea and definitive and differentiative diagnosis is crucial for effective treatment and taking control

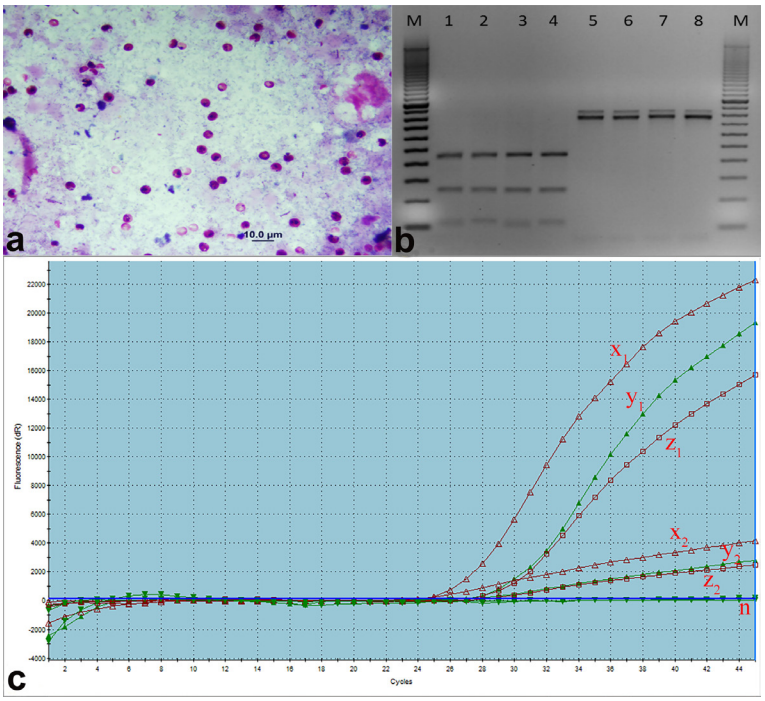

Figure 1. a: *Cryptosporidium* oocysts in fecal smears stained with the Ziehl-Neelsen by microscopic examination (×1000); **b:** Electrophoretic profiles of the SSU rRNA PCR products of *C. parvum* isolates digested with the endonucleases *SspI* (lanes 1 to 4) and *MboII* (lanes 5 to 8). M: Molecular size marker (100bp); **c:** Amplification curves with TaqMan real-time PCR analyses. x1, y1 and z1: *C. parvum* species specific qPCR curves; x2, y2 and z2: *Cryptosporidium* genus specific qPCR curves belonging to the same isolates; n: No DNA control and negative samples

measurements against the disease (9). Therefore, the diagnosis of cryptosporidiosis needs to be highly specific and sensitive. Even though microscopy is currently the common and practicable method in the diagnosis of cryptosporidiosis it has the disadvantages of poor sensitivity and specificity, especially in the case of sporadic shedding of oocyst and also low oocysts in fecal samples. Besides, the oocysts can easily be confused with other particles present in the smears, especially in the absence of considerable training and expertise. Furthermore, it is difficult to distinguish *Cryptosporidium* species (34). In our study lower positivity of *Cryptosporidium* was detected using conventional microscopic examination of the fecal smears compared with molecular assays resulting in a decreased sensitivity of 88.5%. However, the specificity was determined as 100.0%. This result is by the previous studies that used various molecular techniques to detect *Cryptosporidium* species in human and animal fecal and environmental samples (9,35). Nested PCR-RFLP and qPCR assays determined the same samples as positive for *Cryptosporidium* revealing the high sensitivity and specificity of both techniques in our study. Despite their high accuracy in the diagnoses, these molecular assays have the disadvantage of being time-consuming and having many steps in the diagnostic procedure. Furthermore, they are also expensive due to the complex equipment needed to run assays.

SSU rRNA-based PCR is the most commonly used assay in many epidemiological studies to detect and identify *Cryptosporidium* species. However, this technique requires further analysis involving RFLP or DNA sequencing to distinguish species (14,29,31,36,37). Recently, a quantitative real-time PCR assay has been developed for the identification of *Cryptosporidium* species and *C. parvum* strains in clinical samples and water matrices (14,16). qPCR has several advantages including the elimination of post-amplification handling, easier automation, reducing contamination risk and assay times, and processing of high numbers of samples (27,38,39). Jothikumar et al. (27) reported that the duplex TaqMan qPCR approach can specifically detect all *Cryptosporidium* species at the genus level and *C. parvum* in a single reaction. Our results agree with the findings of Jothikumar et al. (27) and also sequence analysis of the SSU rRNA gene region confirmed the results of the qPCR assay. Development of accurate qPCR assays to determine and discriminate the bovine *Cryptosporidium* species might be useful for epidemiological investigations.

We determined high *C. parvum* infection in pre-weaned calves with diarrhoea with an overall prevalence of 53.6%. Neonatal calf diarrhoea has a multifactorial etiology and *C. parvum* is recognized as frequently associated with neonatal diarrhoea (8,40). The dominancy of *C. parvum* as the causative pathogen of diarrhoea in pre-waned calves has been also reported in the herds of several countries (29,41–43) as well as Turkey (24,25,44). On the other hand, the other bovine *Cryptosporidium* species such as *C. bovis* and *C. ryanae* are responsible for the majority of cryptosporidiosis in post-weaned calves and heifers (25,45–49). The other bovine *Cryptosporidium* species,

C. andersoni is commonly found in adult cattle (9,45,50). The absence of all the above species in diarrheic calves in our study further confirmed the age-related future of the bovine *Cryptosporidium* species. The predominance of *C. parvum* in our study is somewhat expected and consistent with the age of the studied calves, as this is the most frequent species in neonate animals. This approach allowed us to identify a unique subtype IIaA13G2R1 of *C. parvum*, within the family IIa, which is widely recognized for its zoonotic potential. This subtype has been previously characterized as a common subtype in calves. On the other hand, some researchers also reported the presence of *C. bovis* and *C. ryanae* in pre-weaned calves in the absence of *C. parvum* (49,51). Conversely, *C. bovis* was determined to be the most prevalent species in pre-weaned dairy calves in some countries such as China (52,53), Sweden (54), Ethiopia (49) and Western Australia and New South Wales (55), and some authors highlighted that this status may be associated with different farming practices and seasonal differences (56). We also determined that *C. parvum* was more prevalent in the early pre-weaned calves (≤15 days old) than the pre-weaned calves of 15 to 40 days old. However, this difference was not statistically significant and *C. parvum* could affect pre-weaned calves during the growing period as indicated by several researchers (50,57–59).

Sequence analysis of the *gp60* gene revealed the presence and wideness of unique subtype IIaA13G2R1 of *C. parvum* in the zoonotic IIa family in the study area. In addition to *C. parvum* speciation, we decided to pursue subtyping using a nested PCR assay targeting the *gp60* gene followed by sequencing. IIaA13G2R1 has been previously characterized as a common subtype in calves from the different regions of Central Anatolia and Mediterranean regions in Turkey (25). This subtype has also been reported in calves in Canada (60), Belgium (61), Algeria (62), the Netherlands (63), in people with HIV/AIDS in Malaysia (64), in goat kids and lambs in Algeria (65), in ponies in the United States (66). The occurrence and wideness of IIaA13G2R1 in several ruminants, equids, and also humans indicate the risk of zoonotic transmission of cryptosporidiosis in the research area.

Conclusion

In conclusion, our study confirms the usefulness of nested PCR-RFLP and qPCR assays in the accurate diagnosis of *Cryptosporidium* in calves. Regarding the high prevalence of zoonotic subtype IIaA13G2R1, our results highlight the potential risk of pre-weaned calves for human infections with *C. parvum* in the research area. Thus, control measures should be considered for reducing the risk of the zoonotic transmission of *C. parvum*. Comparative molecular surveys on humans from different geographic regions, especially in farmworkers that are in close contact with *C. parvum*-infected animals are needed to improve our understanding of cryptosporidiosis epidemiology and *C. parvum* subtype diversity in Turkey.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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