Kafkas Univ Vet Fak Derg
19 (6): 969-974, 2013
DOI: 10.9775/kvfd.2013.9187

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Prevalence of Cryptosporidiosis and Molecular Characterization of *Cryptosporidium* spp. in Calves in Erzurum [1]

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[1] *This study was supported by Ataturk University Scientific Researches Projects (Project number: BAP-2008-186)*

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Makale Kodu (Article Code): KVFD-2013-9187

Summary

This study was conducted to determine the prevalence of cryptosporidiosis and to identify *Cryptosporidium* species found in preweaned calves, in Erzurum, Turkey. Fecal samples were collected from 307 calves up to one month old from 5 dairy farms. Genomic DNA was obtained by DNA extraction (QIAamp DNA Stool kit). The prevalence of cryptosporidiosis was determined based on identification through a nested PCR protocol to amplify fragments of the *Cryptosporidium* SSU rRNA gene. 3.9% of calves were positive for *Cryptosporidium*. Calves that were subjected to traditional herd management, were female, aged 2 weeks, and had watery feces were affected by the disease at a greater incidence than those were subjected to planned herd surveillance program, were males, were older than 3 weeks, and had firm feces. DNA sequence analysis of the SSU rRNA gene on all of the PCR positive samples ascertained that *C. parvum* was the only species present. Further studies should be performed comprehensive fecal analysis for other causative agents for association *Cryptosporidium* species in calf diarrhea and mortality resulting in economic loss in the region.

Keywords: *Calf, Cryptosporidium, Nested-PCR, SSU rRNA, Erzurum*

Erzurum Yöresinde Buzağılarda Cryptosporidiosisin Prevalansı ve *Cryptosporidium* Türlerinin Moleküler Karekterizasyonu

Özet

Bu çalışma, Erzurum yöresindeki sütten kesim öncesi dönemdeki buzağılarda cryptosporidiosisin prevalansının ve *Cryptosporidium* türlerinin moleküler karekterizasyonunun ortaya konması amacıyla yapılmıştır. Bu amaçla beş süt işletmesinden, bir aydan küçük 307 buzağının dışkı örnekleri toplanmış ve DNA ekstraksiyonu (QIAamp DNA Stool kit) yapılarak genomik DNA elde edilmiştir. Nested PCR protokolü ile *Cryptosporidium* SSU rRNA gen bölgesinin kısmi amplifikasyonu yapılmış ve cryptosporidiosis prevalansı %3.9 olarak belirlenmiştir. Geleneksel yöntemlerle yetiştirilen, dişi, 2 haftalık yaşta ve sulu dışkıya sahip buzağıların modern işletmelerde yetiştirilen, erkek, 3 haftadan büyük ve katı kıvamlı dışkıya sahip olanlara göre hastalıktan daha çok etkilendiği saptanmıştır. PCR pozitif örneklerin SSU rRNA gen bölgesi hedef alınarak yapılan DNA dizi analizleri sonuçlarına göre *C. parvum*'un hayvanlarda bulunan tek tür olduğu anlaşılmıştır. Sonuç olarak, yörede ekonomik kayıplara neden olan buzağı ishalleri ve ölümlerinde *Cryptosporidium* türleri ile diğer hastalık etkenlerinin etkileşimlerinin ortaya konması amacıyla daha kapsamlı çalışmaların yapılmasının gerekliliği sonucuna varılmıştır.

Anahtar sözcükler: *Buzağı, Cryptosporidium, Nested-PCR, SSU rRNA, Erzurum*

INTRODUCTION

Cryptosporidiosis is a zoonotic protozoan disease that has a very broad and versatile geographic distribution including the Antarctic region [1]. *Cryptosporidium* is the causative agent and infects mainly the intestinal tract and rarely the respiratory system of diverse species including human, ruminant, feline, canine, rodent, avian, reptile

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and fish. Transmission usually occurs through the direct fecal-oral route or through ingestion of water or food contaminated with oocysts [2,3].

Bovines are the most common species of mammals, infected with *Cryptosporidium* and considered the major reservoir of *Cryptosporidium* for human infections [3]. Cryptosporidiosis in cattle is mainly caused by *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae* [2,3]. At least 10 other *Cryptosporidium* species or genotypes such as *C. felis*, *C. meleagridis* and *C. suis* can also play role in etiology [4,5].

Bovine cryptosporidiosis is considered one of the most common causes of neonatal diarrhea in cattle, leading to economic losses. The severity of infection ranges from mild to severe depending on *Cryptosporidium* species as well as age, previous exposure and immune status of the host. Asymptomatic infection is common in yearling heifers and mature cows [6,7].

Cryptosporidium parvum is a zoonotic species and the predominant in preweaned calves, especially those at age of 1-4 weeks [8]. The agent is responsible for about 85% of cryptosporidiosis in preweaned calves but only 1% of the disease in postweaned calves and 1-2 year old heifers [6,9,10]. Among the other bovine species, *C. bovis* and *C. ryanae* were detected mainly in weaned calves, and *C. andersoni* in yearlings and adult cattle [9,11,12]. While *C. bovis* and *C. ryanae* are considered non-zoonotic, *C. andersoni* has recently been reported in few research involving humans in England [13].

The specific diagnosis of *Cryptosporidium* species is central to the control of the disease and to the understanding of the epidemiology. Lack of distinctive morphologic features of *Cryptosporidium* oocysts makes microscopical examination inconvenient in order to clearly differentiate species and genotypes [14]. Traditionally, *C. parvum* has been diagnosed by microscopy of fecal smears, with or without staining. However, two other species, *C. bovis* and *C. ryanae*, with similar oocyst morphology to *C. parvum*, can only be identified using DNA analysis. That is, microscopy cannot distinguish these three species [6]. Therefore, molecular analyses are required to detect and distinguish *Cryptosporidium* at species/genotype and subtype levels [3,14]. The most frequently used marker for *Cryptosporidium* species and genotype identification is the small subunit of ribosomal RNA (SSU-rRNA) gene [3].

The disease in calves has been studied in many countries, with prevalence ranging from 2.4 to 100% [3,7]. In Turkey, cryptosporidiosis was first diagnosed in calves in 1984 [15]. Since then, other surveys have revealed the prevalence of 7.2-63.9% in calves [16,17]. Most of the studies carried out in Turkey were based on microscopy of stained oocysts in feces [15,16,18-21]. Enzyme-linked immunosorbent assay (ELISA) [17,22] and PCR technique [23-25] have recently become more common to attain the prevalence of cryptosporidiosis. However, few studies have coped with genetic structure of *Cryptosporidium* species in Turkey [26-28]. This study was conducted to determine the prevalence of cryptosporidiosis and to characterize *Cryptosporidium* species based on PCR amplification and sequence analysis of SSU rRNA gene in younger than 1-month-old calves in Erzurum province, Turkey.

MATERIAL and METHODS

Sample Collection

A total of 307 fecal samples were collected from calves less than 1 month old in dairy farms (herd size ranging from 100 to 350 Brown Swiss, and Holstein cows in 3 professional dairy farms and from 40 to 85 Brown Swiss, crossbreed, and Anatolian Red cows in 2 traditional dairy farms) located in Erzurum province between April-2010 and October-2010. Samples were collected directly from the rectum with a gloved hand and transferred into a plastic cup. Fecal consistency was scored as firm, well formed, loose and diarrheic. Samples were kept at 4°C until laboratory analyses.

The study protocol was approved by the Animal Care and Use Committee at Ataturk University (4.4.2008-2008/8 decision number).

DNA Extraction and PCR Amplification

Oocysts were washed and concentrated from feces [29,30] prior to DNA isolation using a QIAamp DNA Stool kit (Qiagen, Maryland, USA). Before eluting, aliquots were added with 100 ml Buffer AE and stored at 20°C.

A nested PCR for the amplification of a fragment of SSU rRNA gene was performed using the protocols and primers as described by Xiao et al.[31] with the following modifications: At the first step of nested PCR, approximately 1.325 bp PCR product was amplified using primers 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAA CAGGA-3′. The PCR contained 1x PCR buffer, 6 mM $MgCl_2$, 0.2 mM (each) dNTP, 200 nM (each) primer, 0.025 U of Taq DNA polymerase, and 1.5 µl of DNA template in a total 25 µl reaction mixture. A total of 35 cycles were carried out at 94°C for 45 s, 55°C for 45 s and 72°C for 1 min. There was also an initial hot start at 94°C for 3 min and a final extension at 72°C for 7 min. A secondary PCR was then performed to amplify 826-864 bp from 1 µl of the primary PCR mixture using primers 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′. The PCR and cycling conditions were identical to the primary PCR. Amplification products were separated by electrophoresis on 1% (w/v) agarose gels, and visualized by ethidium bromide staining.

DNA Sequence Analysis and Phylogenetic Analysis

Successfully amplified samples were subjected to DNA sequence analysis for species determination. Sequencing

was performed using the ABI PRISM® BigDye terminator cycle sequencing kit in ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). Sequence data were then subjected to BLASTN (RefSeq) searches of the *Cryptosporidium* genome database at the National Center for Biotechnology Information *(http://www.ncbi nlm.nih.gov/)*. All sequence data were edited using BioEdit 7.0 *(http://www.mbio.ncsu.edu/BioEdit/bioedit.html)* and FinchTV Version 1.4.0 *(http://www.geospiza.com/finchtv)* following naked eye checking. Multiple sequence alignments were made with the Clustal W method with BioEdit 7.0 software [32]. The neighbor-joining (NJ) method as implemented in the MEGA5.1 program [33] was used for the phylogenetic analysis based on SSU rRNA, utilizing *Eimeria tenella* sequence (HQ680474) as out-group. The branch reliability was assessed by the bootstrap method with 1000 replications.

Data Analysis

The PROC MEANS and FREQ were computed to obtain descriptive statistics [34]. Animals were categorized by breed (culture, crossbreed and local), age (1-7, 8-15 and 16-30 days) and fecal consistency (firm, well formed, loose and diarrheic) before establishing cross-tables to reveal association of risk factors with cryptosporidiosis using Chi-square. The associations were considered significant at P<0.05.

RESULTS

The nested PCR showed a total of 12 (3.9%) positive amplfications in 307 fecal samples *(Table 1)*. The cryptosporidiosis prevalence among calves raised in modern farm was lower than those raised in farms with poor infrastructure (1.5 vs. 8.5%, P<0.003). The infection rate in culture breed was insignificantly higher (4.5%) than the other breeds. As the age advanced, the frequency of animals infected by *Cryptosporidium* increased quadratically, being the highest among calves aged 8-15 days (P<0.0001). Cryptosporidiosis was more common in females than males (6.8 vs. 1.3%, P<0.01). The cryptosporidiosis prevalence increased with the fecal water content (P<0.0001), 13.6% in calves with the diarrheic feces and 7.1% in calves with the loose feces *(Table 1)*.

All PCR-positive isolates in Erzurum were confirmed to be *C. parvum (Fig. 1)*. The sequences were deposited into GenBank under accession numbers KC437395 to KC437406, respectively. *C. parvum* [GenBank: JN245618, JQ250804, JX886767, DQ656355, AB513881, JX948126, JX416362, JQ413434, JQ182993], *C. andersoni* [GenBank: JX948125, JX437080], *C. bovis* [GenBank: JX515546, JX886773, JN245624] and *C. ryanae* [GenBank: JX886771, JN245623] were reference species, whereas *E. tenella* (HQ680474) was an out-group reference in comparisons. *Fig. 1* depicts phylogenetic relationship among *C. parvum* in Erzurum isolates and the other *Cryptosporidium* isolates as inferred by the NJ analysis of the partial SSU rRNA gene sequences. *C. parvum* in Erzurum isolates were grouped into the same clade with respective reference *C. parvum* sequences. In the present experiment, the percent identities were 99.3-100% among *C. parvum* in Erzurum isolates, 98.7-100% with other *C. parvum* isolates and 87.8-94% with other *Cryptosporidium* species from GenBank.

Table 1. *Factors affecting cryptosporidiosis in calves younger than one month in Erzurum (n=307)* *

Tablo 1. *Erzurum yöresinde bir aydan küçük buzağılarda cryptosporidiosise etki eden faktörler (n=307)* *

Variable	Infection Status	
	PCR – (n = 295, 96.09%)	PCR + (n = 12, 3.91%)
Enterprise		
Traditional (n=106, 34.53%)	97 (91.5)	9 (8.5)
Professional (n=201, 65.47%)	198 (98.5)	3 (1.5)
	X^2 = 9.05, P<0.003	
Breed		
Culture (n=264, 85.99%)	252 (95.4)	12 (4.5)
Crossbreed (n=8, 2.61%)	8 (100)	0
Local (n=35, 11.40%)	35 (100)	0
	X^2 = 2.03, P<0.36	
Age (d)		
1-7 (n=30, 9.77%)	28 (93.3)	2 (6.7)
8-15 (n=71, 23.13%)	62 (87.3)	9 (12.7)
16-30 (n=206, 67.10%)	205 (99.5)	1 (0.5)
	X^2 = 21.56, P<0.0001	
Sex		
Female (n=148, 48.21%)	138 (93.2)	10 (6.8)
Male (n=159, 51.79%)	157 (98.7)	2 (1.3)
	X^2 = 6.17, P<0.01	
Fecal consistency		
Firm (n=11, 3.58%)	11 (100)	0
Well formed (181, 58.96%)	181 (100)	0
Loose (n=56, 18.24%)	52 (92.9)	4 (7.1)
Diarrheic (n=59, 19.22%)	51 (86.4)	8 (13.6)
	X^2 = 24.00, P<0.0001	

* *Data are n (%)*

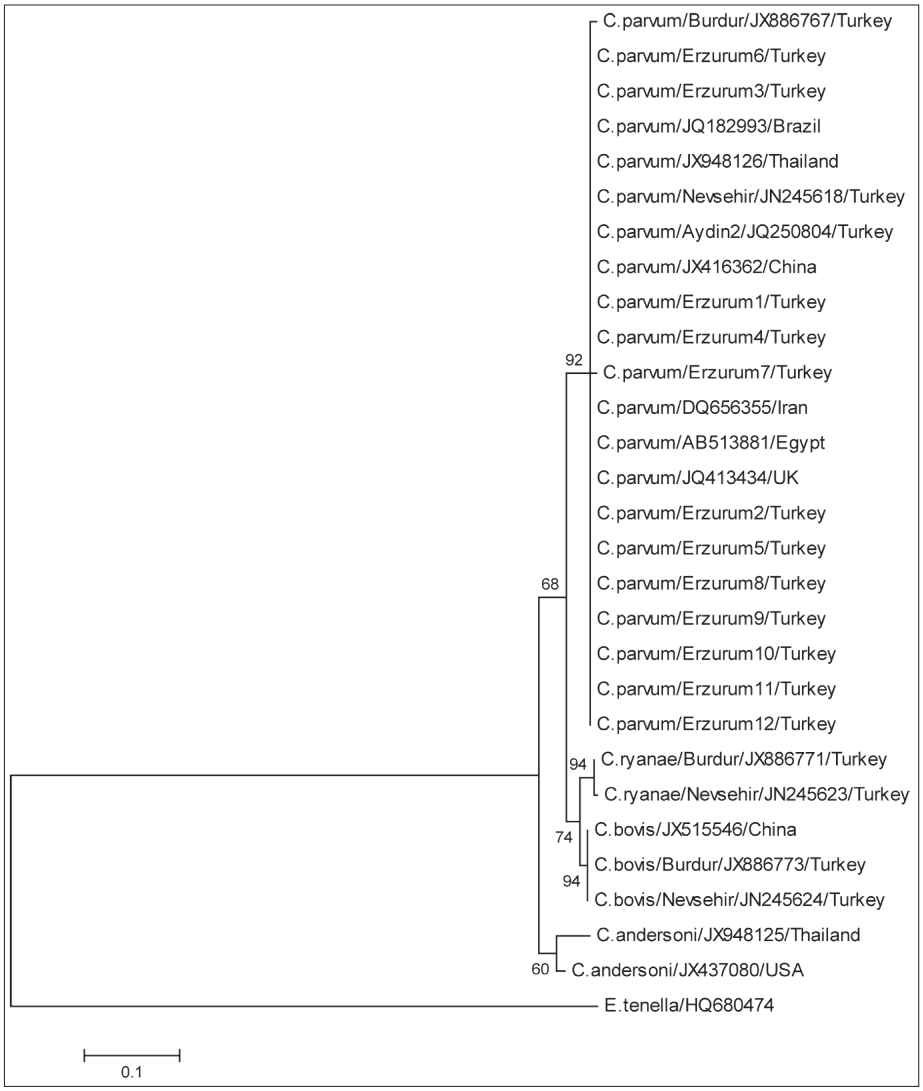

Fig 1. Phylogenetic relationship among *Cryptosporidium* isolates as inferred by neighbor-joining analysis of SSU rRNA nucleotide sequences. The sequence for *E. tenella* (HQ680474) was used as an out-group. Numbers on branches indicate percent bootstrap values from 1000 replicates. All of Erzurum isolates were identified as *C. parvum*

Şekil 1. *Cryptosporidium* izolatlarının SSU rRNA gen bölgelerinin nükleotid dizileri baz alınarak neighbor-joining metodu ile yapılan filogenetik analizi. *E. tenella* (HQ680474) sekansı grup dışı olarak kullanılmıştır. Filogenetik ağaçtaki numaralar 1000 tekrar sonucu elde edilen bootstrap değerlerini göstermektedir. Erzurum izolatlarının hepsi *C. parvum* olarak tanımlanmıştır

DISCUSSION

The prevalence of cryptosporidiosis in Turkey varies between 7.2-63.9% in calves [16,17]. To our knowledge, this study delivered the lowest prevalence rate (3.9%) among other reports from different locations of Turkey [17,19-22,24,26-28]. The difference could be due to a vast number of factors such as breed, age, management, environment, and season as well as diagnostic method [5,7,13]. The low prevalence could also be caused by spot fecal sampling instead of serial sampling, which may result in underestimation because of intermittent oocyst excretion [9,11].

The majority of *C. parvum* infections appear to be limited to dairy calves under eight weeks of age [10,35], being highest in calves up to 1-month-old [7,8,36]. In calves, the highest infection rates are reported in calves 7-14 days old [7,37], 8-14 days old [4,38] and 8-21 days old [39]. In accordance with the literature, in the present study, the infection prevalence was highest in calves aged between 8-15 days (12.7%), followed by those aged 1-7 days (6.7%) and 16-30 days (0.5%).

As previously reported by Trotz-Williams et al.[40] in Ontario, Canada, by Aysul et al.[26] in Aydın, Turkey and by Coklin et al.[13] in Prince Edward Island, Canada, *C. parvum* was the only species identified in calves less than 1 month old. On the other hand, the absence of *C. bovis*, *C. andersoni* and *C. bovis* in our study could be a result of the age group (≤ 1 months) because since *C. bovis* and *C. ryanae* are known to be more prevalent in weaned calves and *C. andersoni* in yearlings and adult cattle [6,9,11,12].

Calf diarrhea has a multifactorial etiology, and *C. parvum* is frequently associated with the disease [7,38,39,41]. Besides, viruses and bacteria are other causative agents that can cause this symptom simultaneously or individually. Of 12 *C. parvum* positive fecal samples, 8 were from diarrheic calves and 4 from calves with loose feces *(Table 1)*. In disagreement with some previous studies [35,39,41,42], our results proved an association of fecal consistency with the infection. Studies reporting relationship between fecal consistency and cryptosporidiosis are available [7,12,36]. Because other possible agents were not searched in the present study, it requires caution to make inference that

calves with watery feces are prone to cryptosporidiosis. Another factor to contribute fecal dry matter is feeding scheme because looser feces can be consequence of milk feeding [39]. These suggest that extensive sample analysis is required to confirm the relationship between fecal consistency and cryptosporidiosis.

The molecular characterization of *Cryptosporidium* species in Turkey has been published in three reports, in which *C. parvum* [26-28], *C. bovis* [27] and *C. ryanae* [27] were identified. In our study, homology search proved that all isolates in Erzurum were *C. parvum*. The partial SSU rRNA gene sequences had 100% similarity to reference sequences downloaded from the GenBank (DQ656355, AB513881, JX948126, JX416362, JQ413434, JQ182993 and JN245618). The NJ phylogenetic analysis based on the SSU rRNA *(Fig. 1)* showed that all sequences of *C. parvum* in Erzurum isolates clustered in the intestinal clade with reference *C. parvum* sequences (bootstrap value 92).

In conclusion, the current study elucidated the prevalence of cryptosporidiosis and the molecular characterization of *Cryptosporidium* species found in calves in Erzurum, Turkey. The prevalence of *Cryptosporidium* infection in dairy calves determined by nested PCR was at 3.9%. *C. parvum* was the only causative *Cryptosporidium* species in calves younger than 1 month in Erzurum province as ascertained by sequencing the amplified SSU rRNA regions.

Acknowledgements

The authors thank Mehmet Özkan Timurkan for technical assistance.

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