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# Molecular Diagnosis and Typing of *Cryptosporidium* spp. Species in Human Stools with Diarrhea

## *İshalli İnsan Dışkılarında Cryptosporidium spp. Türlerinin Moleküler Tanısı ve Tiplendirilmesi*

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## ABSTRACT

**Objective:** This study was conducted to molecularly identify and classify *Cryptosporidium* spp. in fecal samples (n=150) from patients with diarrhea received at the microbiology laboratory of a private hospital in Denizli.

**Methods:** In this study, the positivity of *Cryptosporidium* spp. in fecal samples was investigated using direct microscopy, Kinyoun's acid-fast staining method, and Nested polymerase chain reaction (PCR) techniques. Positive PCR products were sequenced.

**Results:** In the examined fecal samples of patients with diarrhea, no parasites were detected through direct microscopic examination. Using the Kinyoun acid-fast staining method, *Cryptosporidium* spp. was identified in 2.7% (n=4) of the samples, while Nested PCR detected it in 4.67% (n=7) of the samples. The four positive samples were sequenced using primers that amplify the *18S rRNA* gene region. The sequencing results identified the isolates as *C. parvum*.

**Conclusion:** Cryptosporidiosis is an important public health issue as it is a zoonotic disease caused by the *Cryptosporidium* parasite that can be transmitted from animals to humans. This study focuses on the molecular characterization of *Cryptosporidium* species detected in human fecal samples, which is significant for understanding which specific strains or species are involved in human infections. According to the findings, it is recommended that control measures be implemented to reduce the risk of exposure to *Cryptosporidium* in both humans and animals in Türkiye.

**Keywords:** *18s rRNA*, *Cryptosporidium*, Nested PCR, PCR, sequencing

## ÖZ

**Amaç:** Bu çalışma, Denizli'deki bir özel hastanenin mikrobiyoloji laboratuvarına gelen ishalli hastalardan alınan dışkı örneklerinde (n=150) *Cryptosporidium* spp. türlerinin moleküler olarak tanımlanması ve tiplendirilmesi amacıyla yapılmıştır.

**Yöntemler:** Çalışma kapsamında dışkı örneklerinde *Cryptosporidium* spp. pozitifliği, direkt mikroskobik bakı, Kinyoun'un asit fast boyama yöntemi ve Nested-polimeraz zincir reaksiyon (PZR) yöntemleriyle araştırılmıştır. Pozitif PZR ürünleri sekanslanmıştır.

**Bulgular:** İshalli hastaların incelenen dışkı örneklerinde direkt mikroskobik inceleme ile parazit tespit edilmemiştir. Kinyoun'un asit fast boyama yöntemiyle %2,7 (n=4) oranında, Nested PZR ile %4,67 (n=7) oranında *Cryptosporidium* spp. tespit edilmiştir. Pozitif bulunan 4 örnek *18S rRNA* gen bölgesini amplifiye eden primerlerle sekanslanmıştır. Sekanslama sonucunda izolatlar *C. parvum* olarak tanımlanmıştır.

**Sonuç:** Cryptosporidiosis, *Cryptosporidium* parazitinin neden olduğu ve hayvanlardan insanlara bulaşabilen bir zoonotik hastalık olduğu için önemli bir halk sağlığı sorunudur. Bu çalışma, insan dışkı örneklerinde tespit edilen *Cryptosporidium* türlerinin moleküler karakterizasyonuna odaklanmakta olup, insan enfeksiyonlarında hangi özel suşların veya türlerin yer aldığını anlamak açısından önemlidir. Bulgulara göre, Türkiye'de hem insanlarda hem de hayvanlarda *Cryptosporidium*'a maruz kalma riskini azaltmak için kontrol önlemlerinin uygulanması önerilmektedir.

**Anahtar Kelimeler:** *18s rRNA*, *Cryptosporidium*, Nested PZR, PZR, sekanslama

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Türkiye PARAZİTOLOJİ Dergisi

## INTRODUCTION

Cryptosporidiosis, is the name given to a wide range of diseases caused by *Cryptosporidium* spp. species, which have different genetic characteristics, from non-symptomatic infections to acute enteric diseases in animals and humans (1-3). *Cryptosporidium* spp. is widespread throughout the world, including in underdeveloped countries, rural areas of developed countries or urban centers (4-6). This prevalence, has been reported to be detected at rates ranging from 0.7 to 20% in more than 40 countries in the world; it has been observed that it is higher in developing or underdeveloped countries where sanitation measures are not taken and social and personal cleaning is not fully implemented (7-9).

By amplifying specific gene regions using molecular methods, more information was obtained about the transmission routes, species-specific data and biological characteristics of *Cryptosporidium* species (8).

Of the two different genotypes responsible for human cryptosporidiosis; it has been determined that the human genotype (I) is found only in humans while the bovine genotype (II) can cause infection in humans, sheep, cattle, deer, rarely pigs and mice. Genotype II has been reported to constitute the majority of human infections (3,10). The type I genotype responsible for human infection was determined to be *C. hominis* (previously called type I of *C. parvum* or the human genotype of *C. parvum*) and the type II genotype (previously named type II or bovine genotype) was *C. parvum*. It has been reported that *C. hominis* has very high host specificity and very low genetic variation while *C. parvum* has very low host specificity and very high genetic variation (1,5,8,11,12). The fact that *C. parvum* unlike *C. hominis* can infect not only humans, but also domestic and wild ruminants and rodents, proves that it is a zoonotic species (1). Although *C. parvum* with the zoonotic character and *C. hominis* with the anthroponotic character are the most common causes of cryptosporidiosis in humans (5), cases of *C. muris, C. meleagridis, C. suis, C. canis* and *C. felis* have also been reported to cause infections in humans (4,10,13). *Cryptosporidium parvum* species plays a dominant role especially in zoonotic cryptosporidiosis in humans (14).

The incubation period of cryptosporidiosis generally ranges from 5-28 days (7,15). Diarrhea is the most important clinical manifestation of the disease in both immunologically intact and suppressed individuals. Less frequently, patients also experience abdominal pain, fever, nausea-vomiting and weight loss (15). Resistant oocysts excreted in the feces of infective hosts are the route of transmission. Livestock especially calves and other domestic animals play a major role in human transmission (15). Human-to-human transmission is also possible. Human-to-human contamination can occur via fecal-oral route (15,16). Human-to-human infections were first seen in kindergartens (17). Epidemics, especially seen in child care homes and hospitals, are the biggest indicator that proves the importance of transmission between people. Today, *Cryptosporidium* is also known as the causative agent of tourist disease (15,18). Travels from developed countries to less developed countries also contribute to the spread of *Cryptosporidium*. It is known that cryptosporidiosis is transmitted homosexually among HIV-positive patients (17).

In epidemics, water is also shown as a source. The high rate of *Cryptosporidium* in spring and surface waters, the resistance of oocysts to disinfectants such as chlorine, the ability to pass through the filters of drinking water treatment networks and the very few oocysts that cause infection cause water-borne epidemics (1,14-16).

It is known that cryptosporidiosis, like all parasitic diseases, poses a major public health problem and causes economic losses. Due to the long duration of the treatment and the high cost of treatment, it is necessary to pay attention to the prevention of the disease (7). Since there is no effective treatment against this parasite yet, the infection in patients can become chronic and life-threatening. Since the basic biology of *Cryptosporidium* is not fully understood, an effective treatment method against the disease has not been determined (1,7).

Except for a few studies with small sample sizes, little is known about the molecular characterization of human *Cryptosporidium* species in Türkiye. Therefore, our study, aimed at molecular identification and characterization of the *Cryptosporidium* spp. species in diarrheal human stools.

## METHODS

### Sampling and Identification of *Cryptosporidium* Oocysts

A total of 150 stool samples (69/F, 81/M) collected from individuals aged 0-87 who presented to a private hospital in Denizli were examined for *Cryptosporidium*. In the analysis of the collected samples, only "age and sex" data were considered (Table 1).

All samples were stored at 4 °C until microscopic examination and DNA extraction. The samples were examined parasitologically by the native-Lugol direct examination method. Then, some feces were taken from the samples and subjected to formol-ethyl acetate precipitation and preparation processes. All samples were stained with Kinyoun's acid-fast staining method and examined microscopically.

The study was approved by the Ethical Committee of Kütahya Health Sciences University (09.02.2021/2021/02-16).

### Genomic DNA Isolation

At this stage, 200 µL of each of the thoroughly homogenized stool samples was taken and DNA extraction was performed in accordance with the "Thermo Scientific GeneJET™" DNA Purification Kit procedure. The obtained DNAs were stored at -20 °C for use in the polymerase chain reaction (PCR) process.

### SSU rRNA Nested PCR

Primer sets (Crypto F1 and Crypto R1) targeting a 1325bp region of the SSU-rRNA region and used in previous studies were preferred in PCR applications (19-21). In the first step of PCR, primers Crypto F1 (PCR Forward Primer) and Crypto R1 (PCR Reverse Primer) were used, which amplify a 1325bp DNA fragment from the SSU rRNA encoding DNA region of *Cryptosporidium*

**Table 1.** Gender-age distribution of sample owners

| | Number of individuals | Age | (Range/average) |
|---|---|---|---|
| Male | 81 | 0-86 | 24.3 |
| Female | 69 | 0-87 | 24.3 |

species. With the help of the methods used in previous studies, the Nested-PCR reaction was performed using Crypto F2 (Nested PCR Forward Primer) and Crypto R2 (Nested PCR Reverse Primer) primers, which amplify a region of 826-864bp (Table 2) (19-21). In order to detect amplicons obtained from genomic DNAs that underwent PCR and Nested PCR procedures, they were subjected to electrophoresis on a 1% agarose gel and visualized.

### Sequence Analysis of SSU rRNA

Agarose gel electrophoresis was performed to detect the sequences obtained from genomic DNAs that underwent PCR and Nested PCR procedures. The specific bands obtained after the imaging process were cut from the gel and purified. During the purification process, the Gene Jet Purification Kit procedure was followed.

Sequence analysis was performed by a commercial firm. The data obtained as a result of the sequence analysis were compared with the data in GenBank (https://www.ncbi.nlm.nih.gov). Phylogenetic analyzes of the obtained sequences were performed using the MEGAX software (22,23).

### Statistical Analysis

Relationships such as whether the positive results were related to age and gender, and whether there was a statistically significant difference, were examined using the chi-square test. The threshold level of statistical significance was taken as $p<0.05$ [SPSS version 20.0 (Statistical Package for the Social Sciences-SPSS, IBM, Chicago, USA) package software" chi-square test ($X^2$) (Two-Way Table in Worksheet)" test].

## RESULTS

No oocysts could be detected in any of the samples examined by the native-lugol method to search for *Cryptosporidium* spp. oocysts in stool samples sent to the laboratory for different examinations. After the condensation process with formol-ethyl acetate, the preparations were prepared and examined by Kinyoun's acid-fast dying method. After the examination, positivity was detected in 4 samples (2.7%) (Figure 1).

As a result of molecular examinations, positivity (Table 3) was detected in 7 samples (4.67%). Four of them are samples that were found positive by microscopic examination (Figure 2). The other 3 had negative results in microscopic examination.

It was determined that 54% of all samples studied were men and 46% were women, and 4 of the positive samples were found in the feces of females and 3 of them were found in male feces. According to the data obtained, no significant relationship was found between gender and positivity (p=3.24).

In this study, 71.43% of the positives were isolated from children in the 0-10 age group (57% especially in the 0-5 age group) and 28.57% from adults aged 24-35, which we can define as the young age group. *Cryptosporidium* has not been found in any of the individuals over the age of 35. This situation was statistically significant ($p<0.05$). According to the data obtained, it was understood that the disease was associated with age (Figure 3).

Four of the positive samples were sequenced. As a result of sequencing, 685bp, 746bp, 832bp and 748bp sequences were obtained in the *18S rRNA* gene region. Table 4 was obtained as a result of comparing the sequences obtained in this study with other sequences known to be deposited in GenBank. As a result of the Blast and phylogenetic analyzes, the phylogenetic tree given below was formed (Figure 4). According to the data obtained, it was determined that 99.997-100% of the isolates were identical with *Cryptosporidium parvum*.

While examining the evolutionary history of the sequences we obtained, the "Neighbor-Joining" method was used (24). The optimal tree is shown with length=0.59990219. The percentage of replicated (1000 copies) trees associated with the clustered taxon is given together with tree branches in the bootstrap test (25). Evolutionary distance was calculated using the "Maximum Composite Likelihood" method (26) and units of base numbers per region. This analysis includes 28 nucleotide sequences. All ambiguous positions (with double delete option) have been removed for each row pair. A total of 1909 positions were found in the latest data set. Evolutionary analyzes were performed using MEGA X (22,23).

Sequence data of *Cryptosporidium* isolates obtained in the study are deposited in GenBank with accession numbers OL621907, OL689399-401.

## DISCUSSION

*Cryptosporidium* species are coccidian protozoa (15) and their sizes vary according to the species and reproduction stages (21). *Cryptosporidium* species are monoxen parasites and transmission occurs by oral ingestion of fecal oocysts (2,12). People who live in the same house with infected people, people they have sexual contact with, health workers, veterinarians, people dealing with agriculture and animal husbandry, people traveling to endemic areas and nursery children are at risk. It can also be transmitted to humans from pets, laboratory animals and farm animals. Oocysts are seen in untreated wastewater, surface, and underground

**Table 3.** Investigation methods and positivity status

| | Native-Lugol | Kinyoun's acid-fast | PCR/Nested PCR |
|---|---|---|---|
| Positive | 0 | 4 (1F/3M) | 7 (4F/3M) |
| Negative | 0 | 146 (68F/78M) | 143 (65F/78M) |

F: Female, M: Male, PCR: Polymerase chain reaction

**Table 2.** Used primer sequences

| PCR/Nested PCR primers | Sequences | Long (bp) |
|---|---|---|
| Crypto F1 (PCR forward primer) | 5'-TTCTAGAGCTAATACATGCG-3' | 1325 |
| Crypto R1 (PCR reverse primer) | 5'-CCCATTTCCTTCGAAACAGGA-3' | |
| Crypto F2 (Nested PCR forward primer) | 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' | 826-864 |
| Crypto R2 (Nested PCR reverse primer) | 5'-AAGGAGTAAGGAACAACCTCCA-3' | |

PCR: Polymerase chain reaction

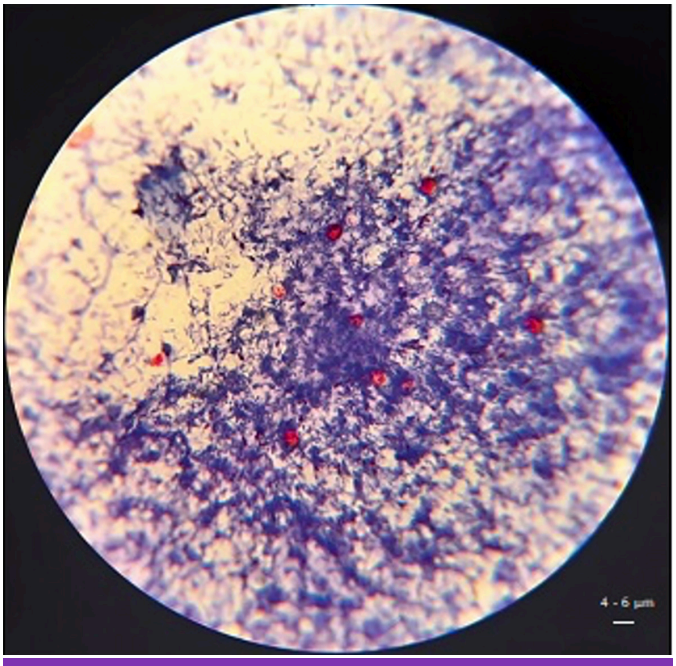
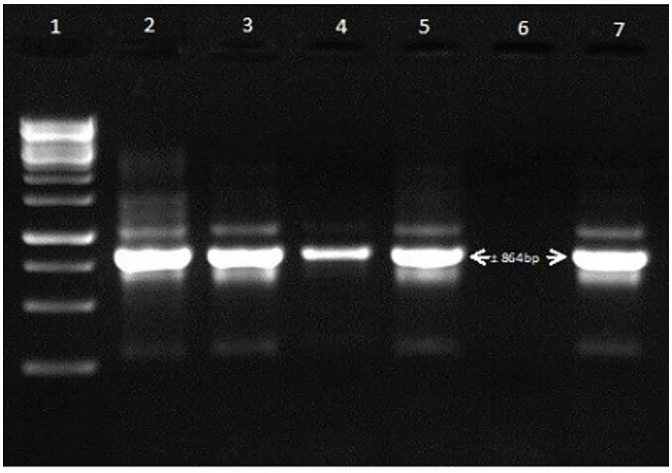



**Figure 1.** *Cryptosporidium* spp. oocysts




1: 1 kb ladder; 2-5: Nested 1-Nested 4 isolates; well 6. Nested negative control; well 7. Nested positive control.

**Figure 2.** Band images of isolates obtained in the Nested PCR
PCR: Polymerase chain reaction

waters, as well as in pool waters, especially as a result of fecal contamination of small children's pools. It has also been reported that contamination can be seen with certain foods consumed, such as sausages, offal, and raw milk, which are prepared by not paying attention to their cleanliness. Respiratory transmission occurs in immunocompromised patients and AIDS patients. In addition, it can be transmitted by carrier hosts such as soil, arthropods and birds (16,27). As with malaria, there are indications that the spread of cryptosporidiosis across geographic regions is likely related to modes of transmission. Reasons such as suppression of the host's immune system, low number of oocysts causing infection, the ability of oocysts to remain in the latent phase in the external environment for a long time (for months at 20 °C) and to be resistant to many disinfectants, the infectivity of oocysts when excreted from the host, animals as reservoirs for some genotypes are factors that determine the epidemiology of *Cryptosporidium*

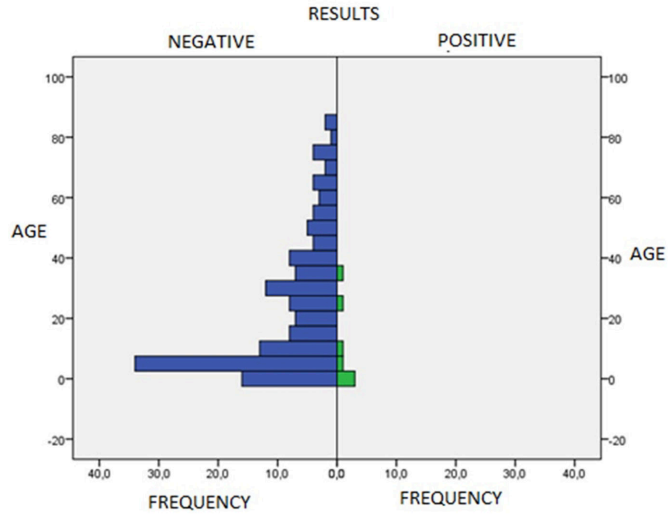



**Figure 3.** Frequency of positive and negative samples by age

(2,7,27). Currently, regional studies are carried out to detect cryptosporidiosis in healthy and immunocompromised individuals both in Türkiye and around the world.

When we look at the studies done in the world, it has been reported that cryptosporidiosis is a zoonotic disease seen in all regions with a hot climate except Antarctica (4,7,15). Recent studies have focused more on the molecular typing, genotypic properties, methods used for the detection of the parasite and the comparison of these methods, isolation from water resources and ecological environment, its effect on epidemics, sanitation or ways of protection etc. rather than the incidence of *Cryptosporidium*. In order to examine the zoonotic potential of *Cryptosporidium* in the Netherlands, 70% of *C. hominis*, 19% of *C. parvum*, 10% of both combinations and 1% of *C. felis* isolates were detected from humans and 100% *C. parvum* isolates from cattle by genotyping from infected human and bovine feces (28). In a study conducted in environmental water samples in Germany, *Cryptosporidium* was detected at a rate of 30.4% by IFA method, 41.9% by Nested PCR, and 43.6% by LAMP method (27).

There are many studies conducted in Türkiye on human feces with diarrhea. In the examinations made in the Mediterranean Region, *Cryptosporidium* positivity was reported at a rate of 5.3% by staining method, 10.6% with ELISA method in Adana (7), in another study, it was 1.3% by direct examination method, 5.2% by staining method and 24% by ELISA method (16).

In studies conducted in the Eastern Anatolia Region, one study reported a *Cryptosporidium* positivity rate of 2.2% (11), while another study determined it to be 20% (13). In patients with chronic renal failure, the positivity rate was 32%, and in healthy individuals, it was 3.3% (10). In a study conducted in Ağrı, *Cryptosporidium* spp. was positive in 7 cases (3.80%) and identified as *C. parvum*, belonging to the IId subtype family (9). In the researches carried out in the Aegean Region, *Cryptosporidium* positivity was reported rate of 3.6% with the direct view method, 4.4% with the painting method in Afyonkarahisar (18), 2.7% by staining method and 3.4% by ELISA method in Kütahya (12). In the studies carried out in the South East Anatolian Region, the rate was 3.2% with the modified acid-fast staining method and 5.8% with the ELISA method in Diyarbakır (15), in another study conducted in the same city, it was 3% with the

**Table 4. *Cryptosporidium* isolates with phylogenetic comparisons around the world**

| Accession number | Isolation source | Species | Isolate | Origin |
|---|---|---|---|---|
| MN803326 | Calf | *Cryptosporidium ryanae* | ERU-KyCrya1 | Türkiye-Kayseri |
| MN803325 | Calf | *Cryptosporidium parvum* | ERU-KyCpar1 | Türkiye-Kayseri |
| MN803324 | Heifer | *Cryptosporidium bovis* | ERU-KyCbov1 | Türkiye-Kayseri |
| JX644908 | Human | *Cryptosporidium viatorum* | 31332 | Chinese |
| KP730318 | Brushtail rock kangaroo | *Cryptosporidium fayeri* | BW993 | Australia |
| JQ029723 | Human | *Cryptosporidium hominis* | 27156 | Chinese |
| KC305650 | Horse | *Cryptosporidium* sp. hedgehog gnt. | M1047 | Algeria |
| GQ983349 | Human | *Cryptosporidium parvum* | W14595 | United Kingdom |
| AF329187 | Human | *Cryptosporidium meleagridis* | 5095 | Peru |
| AF108862 | Cat | *Cryptosporidium felis* | C Horse 1 (131) | Australia |
| KP899827 | Human | *Cryptosporidium ubiquitum* | VE20 | Venezuelan |
| KJ790244 | Pig | *Cryptosporidium suis* | CTC2 | Chinese |
| MH807493 | Human | *Cryptosporidium occultus* | GX996 | Chinese |
| AB210854 | Dog | *Cryptosporidium canis* | - | Japan |
| AF093495 | Chicken | *Cryptosporidium baileyi* | CBA01 | USA |
| FJ463193 | Dairy cow | *Cryptosporidium ryanae* | 23 | Chinese |
| FJ896053 | Sheep | *Cryptosporidium xiaoi* | 191.1 | USA |
| AY741305 | Cattle | *Cryptosporidium bovis* | - | USA |
| AF151376 | Snake | *Cryptosporidium serpentis* | - | US |
| HM116388 | Eurasian silktail (bird) | *Cryptosporidium galli* | 14 | Chinese |
| EU162751 | Javanese frog | *Cryptosporidium fragile* | Clone A | Malaysia |
| AY642591 | Large Japanese dormouse | *Cryptosporidium muris* | Kaw Horseabi | Japan |
| FJ463171 | Dairy cow | *Cryptosporidium andersoni* | 1 | Chinese |

dyeing method and 4% with the ELISA method (2). The Central Anatolia, *Cryptosporidium* positivity was reported 5.6% in a study conducted in Ankara (20), in another study at the rate of 0% with the direct view method, 1% with the painting method and 4% with the DFA method in Sivas (3). Studies in the Marmara Region, *Cryptosporidium* positivity was reported 2.1% of dialysis patients with end stage renal disease (17).

In this study, diarrheal stool samples of 150 people (69F/81M) from different age groups (0-87) who applied to a private hospital in Denizli city center were examined. In the examined fecal samples of patients with diarrhea, no parasites were detected through direct microscopic examination. Using the Kinyoun acid-fast staining method, *Cryptosporidium* spp. was identified in 2.7% (n=4) of the samples, while Nested PCR detected it in 4.67% (n=7) of the samples. The four positive samples were sequenced using primers that amplify the 18*S rRNA* gene region. The sequencing results identified the isolates as *C. parvum*. These results show compatibility with previous studies in terms of prevalence values and differences in results between the applied methods and the detected species.

In a study, *Cryptosporidium* was detected in adults older than 20 years of age with an incidence of 1.25% and in those younger than 20 years of age with an incidence of 17.8%. Within these data, cryptosporidiosis was largely recorded as a pediatric disease (9,29). In another study, it was reported that the vast majority (80%) of human cases occurred in children aged 0-9 years (28).

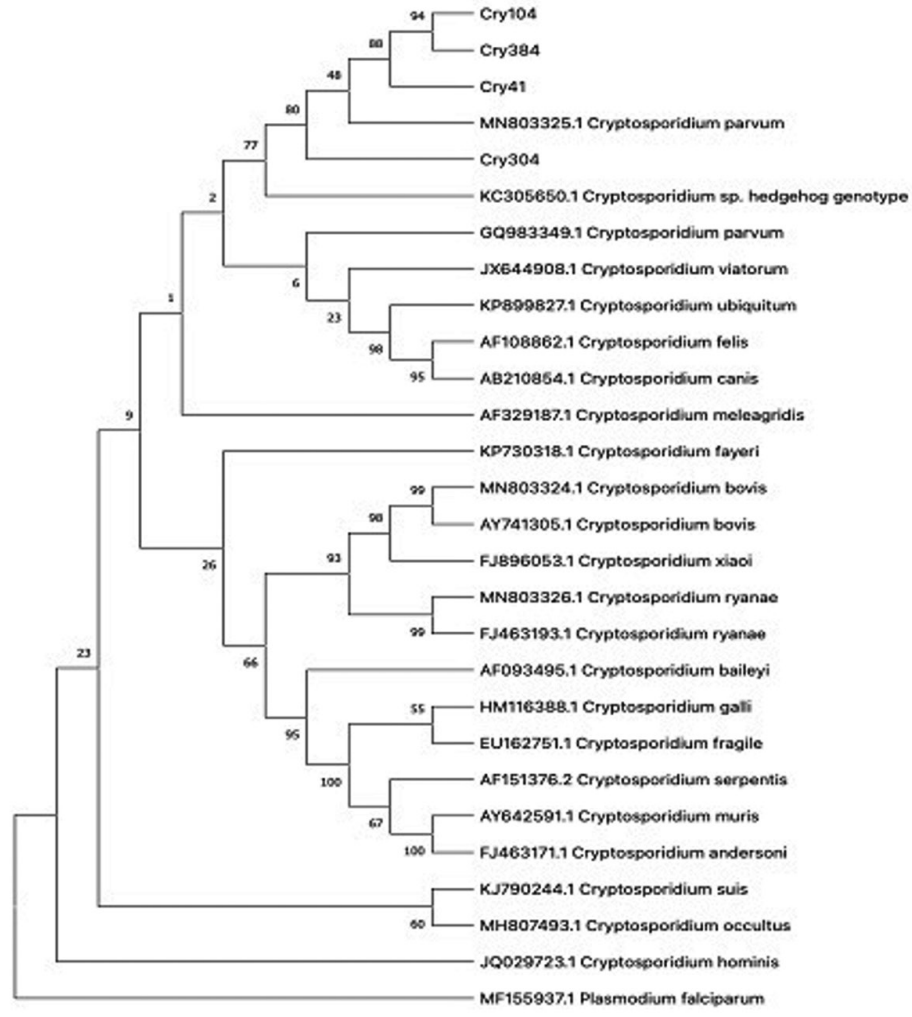
In this study, the majority of the samples detected with cryptosporidiosis belong to children in the 0-10 age group (57% especially in the 0-5 age group). This rate has an incidence of 71.43% among positives. Other positive samples belong to adults aged 24-35 with a rate of 28.57%. No oocyst was detected in any of the individuals over the age of 35. Of the studied samples, 54% belonged to male individuals and 46% to female individuals. These results are in agreement with other studies.

It has been observed that *Cryptosporidium* species have a very high incidence among parasite species isolated from large epidemics (29), environmental waters (14,27), and rural areas where well water is used as drinking water (5,6,9,13). It has been determined that *Cryptosporidium* species can easily pass through the filters of the water processed in the treatment plants before being used in the city network (14,27). It is stated that *Cryptosporidium* species are resistant to many disinfectants. In addition, the presence of heavy metals pumping carriers of the species supports its ability to survive (30).

The fact that the aforementioned protozoan, which threatens both human and environmental life, was identified as an infectious agent in our study, the inadequacy of drugs used in the treatment of other coccidian protozoa in the treatment of cryptosporidiosis, effective treatment protocols against *Cryptosporidium* infections have not yet been established (30) once again reveals the seriousness of the situation we are facing.

## CONCLUSION

For this reason, *Cryptosporidium* species should be added to the fecal indicator organisms, which are among the water quality




While examining the evolutionary history of the sequences we obtained, the "Neighbor-Joining" method was used (Saitou et al., 1987). The optimal tree is shown with length=0.59990219. The percentage of replicated (1000 copies) trees associated with the clustered taxon is given together with tree branches in the bootstrap test (Felsenstein et al., 1985). Evolutionary distance was calculated using the "Maximum Composite Likelihood" method (Tamura et al., 2004) and units of base numbers per region. This analysis includes 28 nucleotide sequences. All ambiguous positions (with double delete option) have been removed for each row pair. A total of 1909 positions were found in the latest data set. Evolutionary analyzes were performed using MEGA X (Kumar et al., 2018; Stecher et al., 2020).

**Figure 4.** Phylogenetic tree

parameters applied in rural-urban basins. Considering the fecal-oral contamination, care should be taken not to irrigate agricultural products with unfiltered water and sewage mixing water. It is necessary to inform the treatment water facilities and municipal authorities on the subject and to support the studies on the eradication process. Sanitation should be given importance in order to contribute to the economy of both the country and the world, minimize health expenses, and most importantly, to protect public health. In order to ensure personal hygiene and protect against infections, individuals should be made aware, and health policies that improve environmental factors should be developed and implemented.

## *Acknowledgments

This study was summarized from the same titled PhD thesis.

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## *Ethics

**Ethics Committee Approval:** The study was approved by the Ethical Committee of Kütahya Health Sciences University (09.02.2021/2021/02-16).

**Informed Consent:** No personal data was used in the study because no samples were specifically collected from patients and the samples brought to the hospital for routine tests were analyzed a second time.

## *Authorship Contributions

Design: F.Ö., A.İ., Data Collection or Processing: F.Ö., Analysis or Interpretation: F.Ö., A.İ., Literature Search: F.Ö., Writing: F.Ö., A.İ.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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