Prevalence and Molecular Genotyping of Cryptosporidium Spp. in Diarrheic Patients from Bandar Abbas City, Southern Iran

Majid Najafi-Asl 1, Habibollah Faraji 2, Saeed Hosseini Teshnizi 3 and Khojasteh Sharifi-Sarasiabi 4, 2, *

1Department of Parasitology, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
2Molecular Medicine Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
3Department of Biostatistics, Faculty of Nursing and Midwifery, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
4Infectious and Tropical Diseases Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

*Corresponding author: Infectious and Tropical Diseases Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran. Email: sharifisarasiabi@gmail.com

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Abstract

Background: Cryptosporidium species are recognized as one of the most important gastrointestinal pathogens of humans and livestock.

Objectives: This study aimed to determine the prevalence and sub-genotypes of Cryptosporidium spp. among diarrheic patients in Bandar Abbas City, Iran.

Methods: Diarrheic fecal samples were collected from 170 patients in three hospitals of Bandar Abbas, Iran, from October 2018 to May 2019. Initial parasitological identification of Cryptosporidium spp. was performed by modified Ziehl-Neelsen (ZN) staining. For molecular analysis, the positive specimens and the suspected ones of Cryptosporidium spp. were evaluated by sequence analysis of the 60-kDa glycoprotein gene (gp60). The collected data were analyzed using SPSS software and the relationship between the variables and the presence of Cryptosporidium spp. assessed by the chi-square test. To assess the degree of agreement between PCR and ZN staining, Cohen’s kappa-index was applied.

Results: Of the 170 diarrheic patients, 98 (57.6%) were male, and 72 (42.4%) were female. Prevalence of Cryptosporidium spp. by parasitological examination was 1.8% (3/170). However, using PCR, Cryptosporidium spp. was detected in 12% (6/50) of the positive microscopically samples (3 samples) and 47 suspected specimens. Sequence analysis of the gp60 gene showed that all of the positive isolates were Cryptosporidium parvum in which all subtypes belonged to allele family IId. Two distinct nucleotide sequences obtained from this study were deposited in GenBank under the accession numbers MN820453 and MN820454.

Conclusions: The predominance of C. parvum (subtype family IId) in this study emphasizes the importance of zoonotic Cryptosporidium transmission in Bandar Abbas, Southern Iran.

Keywords: Genotypes, Subtypes, Cryptosporidium, gp60 gene, Diarrhea, Iran

1. Background

Cryptosporidiosis is considered to be one of the most important diarrheal diseases to humans and many vertebrate animals (1, 2). Exposure to low doses of Cryptosporidium oocysts can cause disease, so it has major importance in public health (3). The parasites’ oocyst is highly resistant to chlorination and disinfectants, which can survive for a long time in the environment (3, 4). It causes up to 6% of diarrhea in immunocompetent individuals (5). Cryptosporidiosis is usually a self-limiting disease; on the other hand, it can be life-threatening in people with immune deficiencies or malnutrition (2).

Medical diagnostic laboratories of Bandar Abbas run routine procedures for the detection of intestinal parasites, but they do not use the proper method for detection of this parasite, unless it is requested by the physician. As performed previously in children with diarrhea in Bandar Abbas, the prevalence of Cryptosporidium spp. was reported as 7% by modified Ziehl-Neelsen (ZN) staining (6). The use of molecular tools in epidemiologic investigations has provided new insights into the diversity of the Cryptosporidium spp. as humans and animal infecting factors (7). The 60-kDa glycoprotein gene (gp60) has a high degree of polymorphism among species isolated from Cryptosporidium and several subgroups, and sub-genotypes have been identified, including Cryptosporidium parvum Ila and IId subtype groups, which are capable of transmission by animals.
The C. parvum subtype family Ila, preferably infects cattle, whereas IId sheep and goats.

2. Objectives

The present study was performed in order to find prevalence and genotypes of Cryptosporidium spp. among patients with diarrhea in Bandar Abbas city, Southern Iran.

3. Methods

3.1. Study Area

In this descriptive cross-sectional study, a single fecal specimen was collected from 170 diarrheic patients in 3 hospitals of Bandar Abbas, Iran, from October 2018 to May 2019. This city is located in southern Iran, a tropical region attached to the Persian Gulf with a high humidity and warm climate.

3.2. Sample Collection and Processing

After obtaining written consent, the researcher administered a comprehensive questionnaire to each patient in the period of time mentioned above. Recipients of antiparasitic drugs and diarrheic patients by Shigella spp. were excluded. The checklist included items on patient demographic aspects. Subsequently, a single fecal specimen was collected from 170 diarrheic patients.

3.3. Microscopic Examination

To identify the oocysts of Cryptosporidium spp., a permanent slide was prepared for each sample after the formalin-ether concentration method. Slides stained with the modified ZN-staining were viewed under a light microscope at a final magnification of 1,000 to observe Cryptosporidium oocysts.

3.4. DNA Extraction and Nested-PCR

The positive specimens were determined by the staining method, and 47 suspected ones of Cryptosporidium spp. were stored in 2.5% potassium dichromate (K2Cr2O7) and stored at 4°C for DNA extraction. Approximately 200 µL of fecal suspension was washed three times in distilled water before extraction. Genomic DNA was then extracted using the FavorPrep Stool DNA Isolation Mini Kit (FAVORGEN, Taiwan) according to the manufacturer’s instructions. Subtype analysis of Cryptosporidium targeted a gp60 gene fragment (400 bp) using nested PCR as previously described.

Briefly, in both reactions, the total volume was 20 µL containing 3 µL of MgCl2 solution (25 mM), 2 µL of 10× reaction buffer, 1.5 µL of 10 mM dNTPs mix, 2 µL of primer mix (10 pm/µL), ~4 ng of DNA template and 0.25 µL of Taq DNA polymerase (5 U/µL) (all from Parstous, Mashhad, Iran). Two PCR cycles were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 55/58°C for 1 min/45 s, and 72°C for 60 s, then final extension at 72°C for 7 min. For the first reaction, outer primers GP60 forward 1 (5’-ATAGTCTCCGGTGTAATT-3’) and GP60 reverse 1 (5’-GCAGAGGAACACGGC-3’) were used at annealing temperature 55°C, with a product size of 980-1,000 bp. For the second reaction, inner primers GP60 forward 2 (5’-TCCGGTGTAATCTCCAGGC-3’) and GP60 reverse 2 (5’-GAGATATCTTTGTTGCG-3’) were used at annealing temperature 58°C with a product size of nearly 400 bp.

3.5. Sequence Analysis

For final confirmation, PCR products of the gp60 gene (approximately 400 bp) were sequenced on an automated sequencer using primers 5’-TCCGGTGTAATCTCCAGGC-3’ and 5’-GAGATATCTTTGTTGCG-3’ (Bioneer Corp). After trimming low-quality sequencing reads at the 5’ and 3’ ends, nucleotide BLAST (Basic Local Alignment Search Tool) similarity searching (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed between the PCR-related sequences and sequence databases and the statistical significance was calculated (percent identity). In addition, by using Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo), multiple alignment of the trimmed nucleotide sequences (370 bp) was carried out. For enabling classification of C. hominis and C. parvum, as described by Chalmers and colleagues, firstly, an allelic family is identified from a conserved sequence of a 3’primer region of the gp60 gene.
Table 1. The Demographic Characteristics of the 6 Cryptosporidium spp. Positive Patients, Bandar Abbas, Iran.

| Variable                  | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 |
|---------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Age                       | 1         | 3         | 8         | 31        | 32        | 33        |
| Gender*                   | male      | female    | female    | female    | female    | female    |
| Occupation                | -         | -         | housewife | housewife | housewife |
| Educational level         | -         | -         | > high school | < high school | > high school |
| Residency                 | rural     | urban     | urban     | urban     | urban     | urban     |
| Type of reception         | inpatient | outpatient | outpatient | inpatient | inpatient | outpatient |
| Contact with animals      | yes/sheep | no        | no        | no        | no        | no        |
| Underlying disease        | no        | no        | no        | yes/organ transplant | no |
| Travel history in recent 3 months | Autumn | Spring | Spring | Winter | Winter | Spring |
| season                    |           |           |           |           |           |           |
| Addict                    | no        | no        | no        | no        | no        | no        |
| Genotyping                | IIdA14G1  | IIdA14G1  | IIdA14G1  | IIdA15    | IIdA14G1  | IIdA14G1  |

*P value = 0.039

Variation in a 5’ trinucleotide repeat region of the gene then identifies subtypes within each family (e.g., A15G1). Finally, in some gp60 families, the number of contiguous copies of a short repeat sequence, Rn, located in a region between two primer regions of the gene, can also contribute to subtype identification (e.g., R1). In this study, in order to recognize isolated subtypes, trinucleotide repeats TCA (red ink), and TCG (green ink) in variable regions located in the 5’primer region of gp60 gene were enumerated (Figure 2).

3.6. Statistical Analysis

The collected data were analyzed using SPSS software (version 20, Chicago, IL, USA), while the relationship between the variables and the presence of Cryptosporidium spp. was assessed by the chi-square test. Frequency (n) and percentage (%) was used to describe qualitative variables. To assess the degree of agreement between PCR and ZN staining for detecting Cryptosporidium spp. Cohen’s kappa-index: poor agreement (k < 0.20), fair agreement (k = 0.21 - 0.40), moderate agreement (k = 0.41 - 0.60), substantial agreement (k = 0.61 - 0.80) and perfect agreement (k = 0.81 - 1.00) was applied (14). For all statistical analyses P < 0.05 was considerate as statistically significant.

4. Results

One hundred and seventy individuals with diarrheic stool were recruited, of which 98 (57.6%) were males and 72 (42.4%) were female. The median age of the study participants was 28.5 yr. (range: 1 d to 91 yr.). Prevalence of Cryptosporidium spp. by modified ZN staining was 1.8% (3/170). However, PCR in positive patients (3 individuals) and 47 suspected specimens revealed infection to Cryptosporidium spp. in 12% (6/50). The youngest infected patient was a one-year-old and the oldest was 33. Evaluating the positive cases of infection, we found that 3 of the cases were housewives.

The results of the chi-square test with the variables showed that the frequency of Cryptosporidium spp. was significantly related only to gender (Table 1). One of the patients had already endured a kidney transplant. Also, 3 of the patients were children and one of them came from the rural area whose parents bred sheep at home. There was no significant difference between age, occupation, education level, residency, type of reception, contact with animals, underlying disease, travel history within the last 3 months, season, and addiction. The other demographic characteristics of the 6 positive patients are presented in Table 1. With regard to Cohen’s kappa-index definition, the agreement level between the two methods, PCR and ZN staining to detect Cryptosporidium spp. was above average (Kappa = 64%). In other words, there is a substantial agreement between the two methods (14). However, PCR detection power was significantly higher than that of ZN staining (P < 0.001) (Table 2).

4.1. Nucleotide Sequence Accession Number

Nucleotide BLAST similarity results showed that all representative isolates belonged to the C. parvum IId family (13). Isolates 1, 2, 3, 5, 6 had percent identity up to 99.73% with C. parvum IIdA14G1 subtype family under the accession number KT716847.1. Further, isolate 4 exhibited percent identity 97.57% with C. parvum IIdA15G1 subtype family under the accession number HQ241928.1. Multiple sequence
Figure 2. Multiple sequence alignment of Cryptosporidium parvum isolates obtained from PCR products of gp60 gene. Red and green boxes indicate trinucleotide repeats TCA and TCG in the variable region. Isolates 1, 2, 3, 5, 6 were identical in nucleotides with 14TCA and 1TCG repeats (subtype family IIdA14G1), whereas isolate 4 contained 15TCA repeats without TCG trinucleotide (subtype family IIdA15). The asterisks indicate identical nucleotides.

| Isolate | Sequence                                      |
|---------|-----------------------------------------------|
| 1       | GTTTCTGGTGAAGGTTCATCAGCATCATCATCATCATCATCATC |
| 2       | GTTTCTGGTGAAGGTTCATCAGCATCATCATCATCATCATCATC |
| 3       | GTTTCTGGTGAAGGTTCATCAGCATCATCATCATCATCATCATC |
| 5       | GTTTCTGGTGAAGGTTCATCAGCATCATCATCATCATCATCATC |
| 6       | GTTTCTGGTGAAGGTTCATCAGCATCATCATCATCATCATCATC |
| 4       | ATGTCATACATTAAAGGAGATGCGGTACTTCACTTGTATTGTCGAGAGGTTGTTGG |

**Alignment of PCR sequences for the gp60 gene illustrated that isolates 1, 2, 3, 5, 6 were identical in nucleotides, whereas isolate 4 revealed a percent identity of 97.84% with the rest. In order to recognize isolate subtypes, trinucleotide repeats TCA (red ink), and TCG (green ink) in variable regions located in the 5' region of the gp60 gene were**
enumerate. As a result, Isolates “1, 2, 3, 5, 6” and 4 were assigned as IIdA14G1 and IIdA15 subtype families, respectively (Figure 2). Finally, two distinct nucleotide sequences obtained from this study were deposited in GenBank under the accession numbers MN820454 and MN820453.

5. Discussion

In our study, the infection rate of Cryptosporidium spp. was 1.8%. Prevalence of Cryptosporidium spp. in patients with gastroenteritis in other regions of Iran varies, in Mazandaran province, northern Iran 0.1% (15), Nahavand county in western Iran 1.3% (12), Iranian children of Tehran 2.4% (16), Gonbad Kavoos city, northern Iran 4.94% (17) and in Shiraz, Fars province 25.6% (18). There are differences between our study and a previous one performed in Bandar Abbas (6). This study had less prevalence since our subjects included all individuals with diarrhea, whereas the previous study (6) was performed among children with diarrhea. As a result, the latter had a higher prevalence (7%). Unfortunately, there was no animal study of the prevalence and genotype of Cryptosporidium in Hormozgan to be associated with the results of our study. The prevalence of Cryptosporidium spp. in the other countries of the world also varies. In the rural population of the Buner district, Pakistan, the prevalence of this parasite was found to be 29.88% (19), in Lebanon 11% (20), and in New Zealand 10% (21). This discrepancy may be due to the study population, exposure to animals, residency, geographical climates, nutritional habits, and especially, the type of detection methods (22).

In a systematic review and meta-analysis study in Iran (22), and Lebanon (20), the prevalence of this parasite in children was significantly higher than the other groups, in contrast to our study where there was no significant difference between age and parasitic infection. There found to be a significant difference between the occurrence of infection and gender, consistent with the study of Keshavarz et al. (23) and Khalili and Mardani (24) and inconsistent with the study of Saneian et al. (25). All Cryptosporidium isolates from patients with diarrheal complaints were C. parvum, and none belonged to C. hominis; it indicates transmission of infection from animal to human similar to the study of Sharbatkhori et al. in the northern Iran (17). It is noteworthy that only one patient had direct contact with sheep. Other contamination may have been due to indirect exposure to animal feces, such as polluted vegetables or fruits.

As we can see, all three women are housewives and likely to be infected with dirty vegetables. Most of the infections in Iran are C. parvum (26-28). Molecular studies in the Middle East countries showed C. parvum, as the most dominant species in human infections (22) this is contrary to study of Squire and Ryan, which shows that C. hominis is the most cases of infection in Africa (29) as well as the study of Osman et al. in Lebanon (20) and Sannella et al. in Thailand (30). A few numbers of isolates in the study of Keshavarz et al. (23) in Tehran and Qazvin, Ranjbar et al. and Taghipour et al. in Tehran (16, 28) Rafeie et al. in Ahvaz (10) as well as Mohammadian et al. in Zabol, eastern Iran (27), detected C. hominis while none of the isolates in the present study were C. hominis. In contrary to our study, the other species of Cryptosporidium, C. meleagridis is one of the major human parasitic pathogens in African countries (29).

The gp60 is the most commonly used genetic locus for subtyping Cryptosporidium spp. (8). Nearly 20 C. parvum subtype families have been described at this locus, IIC appears to be adapted to humans, IIA adapted to humans and a broad range of animals, and IId adapted to animals (sheep, goats, and cattle) (31). In this study, sequence analysis of the gp60 locus identified only one C. parvum subtype family, IId, and two subtypes (IIdA4G1 and IIdA15). According to the subtypes found in this study, it appears that the infected individuals are either directly or indirectly in contact with the animal, and the main mode of transmission in Bandar Abbas is zoonotic. One of the animals bred in the rural areas is sheep and goats, which is probably the reason for the high prevalence of this subtype in these areas. Unlike the study of Ranjbar et al. (28) and Sharbatkhori et al. (17) which identified two subtypes (IIa and IId) and Garcia et al. (21) which identified more subtypes (IIa, IIC, IId, and Ile) among the Cryptosporidium isolates, all of the subtypes in the present study were of the IId subtype.

One limitation of the present study was the low number of samples as well as the number of positive samples, but this was the first study to determine the species and genotypes of the parasite in Bandar Abbas. Of course, more

| PCR          | Modified Ziehl Neelsen Stain | Kappa | P Value  |
|--------------|------------------------------|-------|----------|
|              | Negative                     | Positive | Total   |          |
| Negative     | 44 (88)                      | 0 (0)   | 44 (88)  |          |
| Positive     | 3 (6)                        | 3 (6)   | 6 (12)   | 0.64     |
| Total        | 47 (94)                      | 3 (6)   | 50       | < 0.001  |
molecular studies are suggested to determine the pathways of transmission of this parasite as well as its epidemiology in the wide range of specimens in humans as well as the cattle of Bandar Abbas, Hormozgan province.

5.1. Conclusions

The study confirmed that the transmission of the parasite in Bandar Abbas is more zoonotic than anthroponic. Therefore, these results are useful for researchers to determine the appropriate preventive and therapeutic measures. In addition, there was a significant difference in parasite detection by microscopic methods compared to molecular methods, so molecular methods are suggested as a more accurate and sensitive methods in cases where we suspect this parasite.

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Footnotes

Authors’ Contribution: Study concept and design: Khojasteh Sharifi-Sarasiabi, Majid Najafi-Asl, and Habibollah Faraji; analysis and interpretation of data: Khojasteh Sharifi-Sarasiabi, Habibollah Faraji, Majid Najafi-Asl and Saeed Hosseini Teshnizi; drafting of the manuscript: Khojasteh Sharifi-Sarasiabi and Habibollah Faraji; statistical analysis: Saeed Hosseini Teshnizi; acquisition of data: Majid Najafi-Asl, Habibollah Faraji, Saeed Hosseini Teshnizi, Khojasteh Sharifi-Sarasiabi; critical revision of the manuscript for important intellectual content: Majid Najafi-Asl, Habibollah Faraji, Saeed Hosseini Teshnizi and Khojasteh Sharifi-Sarasiabi

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Ethical Approval: The Ethics Committee of Hormozgan University of Medical Sciences (HUMS) approved the study protocol (IR.HUMS.REC.1397.164).

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Informed Consent: The aim of the study was described to patients or their parents and informed consent was obtained from all the enrolled cases.

References

1. Pumipuntu N, Pirataa S. Cryptosporidiosis: A zoonotic disease concern. Vet World. 2018;11(5):681–6. doi: 10.14202/vetworld.2018.681686. [PubMed: 29995508]. [PubMed Central: PMC593756].

2. Insulander M, Silverlas C, Lebad M, Karlsson L, Mattsson JG, Svenungsson B. Molecular epidemiology and clinical manifestations of human cryptosporidiosis in Sweden. Epidemiol Infect. 2013;141(5):1009–20. doi: 10.1017/S0950268812000665. [PubMed: 22877562].

3. Gatei W, Wamae CN, Mbue C, Waruru A, Mulinge E, Wathera T, et al. Cryptosporidiosis: prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. Am J Trop Med Hyg. 2006;75(1):78–82. [PubMed: 16837712].

4. Plutzer J, Karanis P. Genetic polymorphism in Cryptosporidium species: an update. Vet Parasitol. 2009;165(3-4):187–99. doi: 10.1016/j.vetpar.2009.07.003. [PubMed: 19660869].

5. Chen XM, Keithly JS, Paya CV, LaRussio NF. Cryptosporidiosis. N Engl J Med. 2002;346(22):1723–31. doi: 10.1056/NEJMra013170. [PubMed: 12037153].

6. Hamedi Y, Safa O, Haidari M. Cryptosporidium infection in diarrheic children in southeastern Iran. Pediatr Infect Dis J. 2005;24(1):86–8. doi: 10.1097/00002263-200501000-00020. [PubMed: 15657180].

7. Cama VA, Bern C, Roberts J, Cabrera L, Sterling CR, Ortega Y, et al. Cryptosporidium species and subtypes and clinical manifestations in children. Peru. Emerg Infect Dis. 2008;14(10):1567–74. doi: 10.3201/eid1410.071273. [PubMed: 18826821]. [PubMed Central: PMC2609889].

8. Feng Y, Ryan UM, Xiao L. Genetic Diversity and Population Structure of Cryptosporidium. Trends Parasitol. 2008;24(11):997–1001. doi: 10.1016/j.pt.2008.07.009. [PubMed: 19010820].

9. Heydari-Hengami M, Hamedi Y, Najafi-Asl M, Sharifi-Sarasiabi K. Prevalence of Intestinal Parasites in Food Handlers of Bandar Abbas, Southern Iran. Iran J Public Health. 2018;47(1):111–8. [PubMed: 29318125]. [PubMed Central: PMC5756585].

10. Rafiei A, Rashno Z, Samarbaftadzeh A, Khademvatan S. Molecular Characterization of Cryptosporidium spp. Isolated From Immunocompromised Patients and Children. Jundishapur J Microbiol. 2004;7(4). doi: 10.5481/jjm.983.

11. Abe N, Matsubayashi M, Kimata I, Iseki M. Subgenotype analysis of Cryptosporidium parvum varm isolates from humans and animals in Japan using the 60-kDa glycoprotein gene sequences. Parasitol Res. 2006;99(3):303–5. doi: 10.1007/s00436-004-0140-6. [PubMed: 16565816].

12. Kiani H, Haghbighi A, Seyyedtabaei SJ, Azargashb E, Zebarast N, Taghipour N, et al. Prevalence, Clinical Manifestations and Genotyping of Cryptosporidium Spp. in Patients with Gastrointestinal Illnesses in Western Iran. Iran J Parasitol. 2017;12(2):309–76. [PubMed: 28764476]. [PubMed Central: PMC5527026].

13. Chalmers RM, Robinson G, Elwin K, Elson R. Analysis of the Cryptosporidium spp. and gp60 subtypes linked to human outbreaks of cryptosporidiosis in England and Wales, 2009 to 2017. Parasit Vectors. 2019;12(1):95. doi: 10.1186/s13071-019-3354-6. [PubMed: 30867023]. [PubMed Central: PMC4674102].

14. McHugh ML. Interrater reliability: the kappa statistic. Biochem Med (Zagreb). 2002;12(3):276–82. doi: 10.3201/eid1410.071273. [PubMed: 29318125]. [PubMed Central: PMC3900052].

15. Vaheedi M, Gohardelhi S, Sharif M, Daryani S. Prevalence of parasites in patients with gastrooenteritis at East of Mazandaran Province, Northwestern Iran. Trop Biomed. 2012;29(4):568–74. [PubMed: 23202601].

16. Taghipour N, Nazemalhosseini- Mohajar E, Haghbighi A, Rostami-Nejad M, Romani S, Keshavarz A, et al. Molecular epidemiology of cryptosporidiosis in Iranian children, Tehran, Iran. Iran J Parasitol. 2011;6(4):41–5. [PubMed: 2234732]. [PubMed Central: PMC3279905].

17. Sharbatkhori M, Nazemalhosseini Mozarak E, Taghipour N, Paghhe AS, Meysarian F. Prevalence and Genetic Characterization of Cryptosporidium Spp. In Diarrheic Children from Gonbad Kavoos City, Iran. Iran J Parasitol. 2015;10(3):441–7. [PubMed: 26622399]. [PubMed Central: PMC4662744].
18. Mirzaei M. Prevalence of Cryptosporidium sp. infection in diarrheic and non-diarrheic humans in Iran. *Korean J Parasitol*. 2007;45(2):133-7. doi: 10.3347/kjp.2007.45.2.133. [PubMed: 17570977]. [PubMed Central: PMC2526311].

19. Khan A, Shams S, Khan S, Khan M, Khan S, Ali A. Evaluation of prevalence and risk factors associated with Cryptosporidium infection in rural population of district Buner, Pakistan. *PloS One*. 2019;14(1). e0209188. doi: 10.1371/journal.pone.0209188. [PubMed: 30608576]. [PubMed Central: PMC6314602].

20. Osman M, El Safadi D, Benamrouz S, Guoyt K, Del-Cas E, Allouat el M, et al. Initial data on the molecular epidemiology of cryptosporidiosis in Lebanon. *PloS One*. 2015;10(5). e0125129. doi: 10.1371/journal.pone.0125129. [PubMed: 25950832]. [PubMed Central: PMC4423932].

21. Garcia R, French N, Pita A, Velathanthiri N, Shrestha R, Hayman D. Local and global genetic diversity of protozoan parasites: Spatial distribution of Cryptosporidium and Giardia genotypes. *PloS Negl Trop Dis*. 2017;11(7). e0005736. doi: 10.1371/journal.pntd.0005736. [PubMed: 28704162]. [PubMed Central: PMC5526614].

22. Kalantari N, Ghaffari S, Bayani M. Cryptosporidium spp. infection in Iranian children and immunosuppressive patients: A systematic review and meta-analysis. *Caspian J Intern Med*. 2018;9(2):106-15. doi: 10.22088/cijim.9.2.106. [PubMed: 29732026]. [PubMed Central: PMC592216].

23. Keshavarz A, Athari A, Haghighi A, Kazemi B, Abadi A, Nazem AME, et al. Genetic characterization of Cryptosporidium spp. among children with diarrhea in Tehran and Qazvin provinces, Iran. *Iran J Parasitol*. 2008;30-6.

24. Khalili B, Mardani M. Frequency of Cryptosporidium and risk factors related to cryptosporidiosis in under 5-year old hospitalized children due to diarrhea. *Iran J Clin Infect Dis*. 2009;4(3):51-5.

25. Saneian H, Yaghini O, Yaghini A, Modarresi MR, Soroshnia M. Infection Rate of Cryptosporidium parvum among Diarrheic Children in Isfahan. *Iran J Pediatr*. 2010;20(3):343-7. [PubMed: 23056727]. [PubMed Central: PMC3446045].

26. Meenar AR, Rezaian M, Rezaie S, Mohraz M, Mohebali M, Mohammad K, et al. SSU-rRNA gene analysis of Cryptosporidium spp. in HIV positive and negative patients. *Iran J Public Health*. 2006;2-7.

27. Mohammadian H, Azizi H, Dabirzadeh M. Genetic Study of Cryptosporidium with SSU-rRNA in Children Younger Than Ten Referring to Hospitals of Zabol, Southeast of Iran. *Shiraz E-Med J*. 2019;[In Press](In Press). doi: 10.5812/seMJ.8106.

28. Ranjbar R, Baghaei K, Nazemalhosseini Mojarrad E. Genetic characterization of Cryptosporidium spp. among patients with gastrointestinal complaints. *Gastroenterol Hepatol Bed Bench*. 2016;9(4):301-7. [PubMed: 27995856]. [PubMed Central: PMC5218855].

29. Squire SA, Ryan U. Cryptosporidium and Giardia in Africa: current and future challenges. *Parasit Vectors*. 2017;10(1):195. doi: 10.1186/s13071-017-2111-y. [PubMed: 28427454]. [PubMed Central: PMC5397716].

30. Sannella AR, Suputtamongkol Y, Wongsawat E, Caccio SM. A retrospective molecular study of Cryptosporidium species and genotypes in HIV-infected patients from Thailand. *Parasit Vectors*. 2019;12(1):91. doi: 10.1186/s13071-019-3348-4. [PubMed: 30867022]. [PubMed Central: PMC6417249].

31. Zhang S, Chen L, Li F, Li N, Feng Y, Xiao L. Divergent Copies of a Cryptosporidium parvum-Specific Subtelomeric Gene. *Microorganisms*. 2019;7(9). doi: 10.3390/microorganisms7090366. [PubMed: 31540508]. [PubMed Central: PMC6780254].