Original Article

Prevalence and Molecular Detection of *Cryptosporidium* Species in AL-Anbar Province

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Abstract

Backgrounds: Cryptosporidiosis is a parasitic disease caused by *Cryptosporidium* spp. These parasites belong to the phylum Apicomplexa and are intracellular protozoa that infect mammals, birds, reptiles, and amphibians. They are cosmopolitan, mostly affecting people with immunodeficiency, and in some cases, can be deadly. In immunocompetent individuals. **Objectives:** The current study aimed to investigate the prevalence of *Cryptosporidium* spp. parasite in different regions of Anbar province by different methods, such as the Ziehl–Nelson staining method, as well as the polymerase chain reaction (PCR) method and DNA sequencing, in addition to evaluating the relationship between infection and several demographic factors such as age, sex, geographical area, and seasonal variation. **Materials and Methods:** Investigation on different age groups investigation was carried out on samples of Al-Anbar referral hospital patients with acute diarrhea and other intestinal symptoms. Using modified Ziehl–Neelsen staining, nested PCR, and DNA sequencing, the species of *Cryptosporidium* were identified in samples of stool. **Results:** The prevalence of species of *Cryptosporidium* among patients was 17.5% (36/205) overall. The PCR and DNA semi-nested sequences amplified the *18SrRNA* gene of *Cryptosporidium* spp. in all microscopic-positive samples, revealing the presence of two *Cryptosporidium* species, including *C. hominins* 47.2% (17/36) and *C. parvum* 52.0% (19/36). The correlation between the infection with *Cryptosporidium* spp. and some factors results in nonsignificance differences with all factors under consideration. The results of the phylogenetic tree and sequencing showed close relatedness to Iraqi isolates reference *Cryptosporidium* parasitic isolates *C. parvum* and *C. hominis* isolates with a total percent identity score of 100%. **Conclusions:** The prevalence of *C. parvum* in the current investigation may corroborate the significance of zoonotic cryptosporidiosis transmission in the Al-Anbar province.

Keywords: Al-Anbar province, *Cryptosporidium*, molecular

Introduction

Cryptosporidium is an obligatorily enteric protozoan parasite that is, a widely recognized pathogen of the alimentary tract. *Cryptosporidium* consists of numerous species with a vast host range.[1] Each species can infect the microvilli border of the gastrointestinal tract and respiratory epithelium of various vertebrate hosts, including humans.[2,3] Due to the fact that the pathogenesis of *Cryptosporidium* varies in relation to the parasite species, age, and immune status of the host, infected individuals exhibit a broad spectrum of clinical symptoms. The most prominent symptoms and signs include diarrhea with watery stools, abdominal pains, anorexia, weight loss, nausea, vomiting, lethargy, and a low-grade fever.[4] Cryptosporidiosis affects a variety of human populations, including infants younger than five and immunocompromised individuals, especially human immunodeficiency virus-positive patients.[4] even though molecular and immunological techniques may safely substitute microscopic methods, the microscopic inspection of stool samples remained an essential component of diagnostic testing for these parasites.[5] Microscopy is inexpensive, but a competent parasitologist is necessary, and the diagnostic outcome depends on an appropriate sample of stool.[6] Relevant to humans are

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C. hominis and *C. parvum,* which differ in host range, genotype, and pathogenicity. *C. hominis* infects only humans, whereas *C. parvum* infects other mammals.[7] For molecular epidemiology evaluation of potential infection sources, species differentiation is indispensable. Conventional methods for detecting the oocysts of *Cryptosporidium* in feces include microscopy detection of oocysts utilizing acid-fast stain with widely reactive species of *Cryptosporidium* antibodies or an improved acid-fast staining approach.[8] For precision parasite identification, our research has focused on the hypervariable region of *Cryptosporidium*'s *18S rRNA* gene. Human (male and female) neonates and (male and female) individuals from diverse geographic regions provided the samples that were analyzed. Our research additionally evaluated the degree of variation in sequence between isolates throughout every species and the impact of this variation on the usage of this gene target for the phylogenetic examination of *Cryptosporidium*.

Materials and Methods

Samples collection

Overall, 205 fecal samples were obtained from different groups of people in Al-Anbar Province admitted to a number of hospitals, including Al-Ramadi Maternity and Children Hospital, Fallujah Teaching Hospital, internally displaced persons (IDP) camps as well as some popular health centres in Al-Anbar province and private laboratories during the period from March, 2022 until February 2023, who suffering acute diarrhea and some intestinal symptoms, every specimen was analyzed to detect *Cryptosporidium* by microscopic examination of direct preparations using normal saline and formalin ether concentration methods, a slide was produced and stained with the modified Ziehl-Neelsen acid-fast technique.[9] Microscopically determined *Cryptosporidium*-positive samples were stored at 4°C in 17.5% (36/205) potassium dichromate prior to DNA extraction.

DNA extraction

Extraction of DNA was made for all positive samples for microscopic examination were collected using a kit of Promega Company (Madison, Wisconsin, USA), which was performed according to the manufacturer's instructions.

Nested PCR

The *18S rRNA* gene was analyzed using a semi-nested two-step PCR procedure. Using NCBI Nucleotide blast and Plus3primer, the outer forward primer 5′-TCTAGAGCTAATACATGCGAAA-3′ and the common reverse primer 3′-CCCATTTCCTTCGA AACAG-5′ were designed for this investigation. Utilizing the following procedure, they amplified a 1325-bp fragment: 5 min at 94°C, followed by 35 cycles of 94°C to stay 45 s, 55°C to stay 45 s, and 72°C to stay 1 min, and a final increase at 72°C to stay 10 min. An 835-bp region was amplified in the second round using the forward primer 5′-GGAAGGGTTGTATTTATTAGAT-3′ and the reverse primer 3′-AAGGAGTAAGGAACAACCTC-5′. The second PCR round was carried out at 94°C to stay 5 min, after which there were 35 cycles at 94°C to stay 45 s per cycle. DNA was amplified in an end volume of 25 L consisting of 12.5 L of 2× PCR master mix (Ampliqon, Denmark), 10 PM of every primer, and 2 L of DNA using a Biorad thermocycler. In each set of PCRS, a DNA-free control sample, and an identified *C. parvum* sample were included as negative and positive controls, respectively. On a 2% agarose gel, the PCR product was electrophoresed for one hour. A red safe was used to stain the gel, and a UV transilluminator (Uvitec, UK) was used to visualize the products.[6]

DNA sequencing

The second PCR products were shipped to Microgene Company (New Delhi, India), and the results were compared with sequences previously deposited in the GenBank database using NCBI-BLAST software.

Statistical analysis

Using statistical software SPSS version 16 (Chicago, Illinois), the Mann–Whitney and chi-square tests with a *P*-value of 0.05 were applied to the outcomes.[10]

Ethical endorsement

The research was conducted in accordance with the Helsinki Declaration's moral criteria. Prior to taking the sample, the patient's verbal consent was obtained as well as analytical consent. On September 26, 2022, a local ethics committee reviewed and approved the research protocol, subject information, and consent form in line with document number 363.

Results

During the period from March 2022 to February 2023, stool samples from 205 patients with acute diarrhea and other intestinal symptoms, such as abdominal pain, vomiting, nausea, etc., who were admitted to hospitals such as Al-Ramadi Maternity and Children Hospital, Fallujah Teaching Hospital, IDP camps, as well as some popular health centers in Al-Anbar province and private laboratories.

Using modified Ziehl–Neelsen staining, nested PCR, and DNA sequencing, species of *Cryptosporidium* were identified in stool samples. Figure 1 displays that the aggregate prevalence of *Cryptosporidium* spp. among patients was 17.5% (36/205). The *18SrRNA* gene of *Cryptosporidium* spp. was amplified successfully by semi-nested PCR as well as sequenced in all microscopic-positive samples, revealing the presence of two species of *Cryptosporidium*, *C. hominis* 47.2% (17/36) and *C. parvum* 52.0% (19/36).

The difference between sexes was not of statistical significance ($P = 0.05$). Patients older than 30 years had a higher infection rate, but there were no statistically significant differences. According to Table 1, there was no relationship between the occurrence percentage of infection with *Cryptosporidium* and any other variables.

Semi-nested PCR amplified the *18SrRNA* gene of *Cryptosporidium* in all 36 microscopy-positive samples [Figures 2 and 3].

For species identification, the *18s rRNA* gene PCR-positive products of 36/205 specimens from diarrhea patients were sequenced. Nineteen of these human specimens were similar to *C. parvum* (GenBank accession number MK426796.1 [Iraq]), while 17 were similar to *C. hominis* (GenBank accession number MK982514.1 [Iraq]). Figure 4 depicts the isolated *Cryptosporidium* parasite sequences from the patients studied.

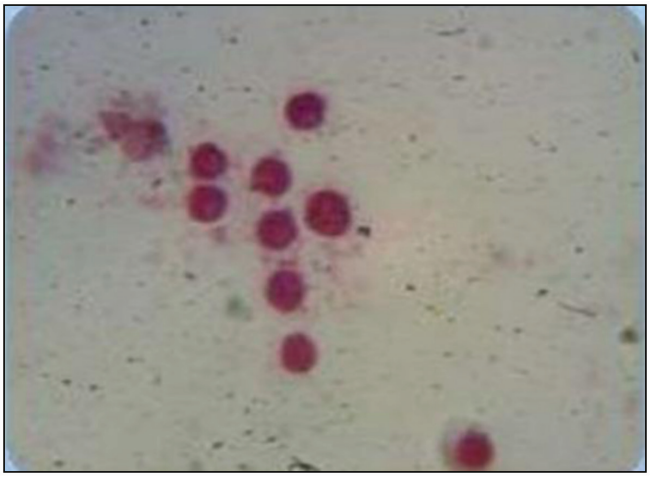

Figure 1: *Cryptosporidium* oocysts were detected in the stool sample (100×)

Discussion

The parasite of protozoan *Cryptosporidium* is one of the largest and most important intestine organisms and the main cause of digestive disorders in human beings, among parasites that impact the gastrointestinal tract. It is fetal only in immunocompromised patients; consequently,

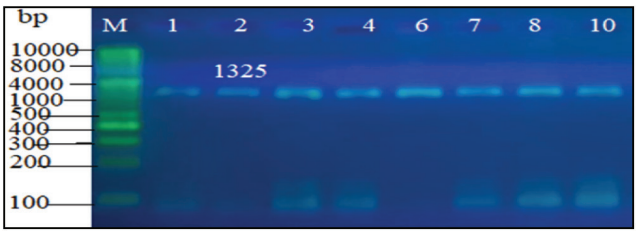

Figure 2: Illustrates the gel electrophoresis profile of 1325 bp of the 18S small subunit ribosomal RNA gene utilizing 2% agarose stained alongside a safe red DNA dye as well as electrophoresed at 5 vol/cm in TBA solution. Lane M.DNA Identifier 100–10,000 bp

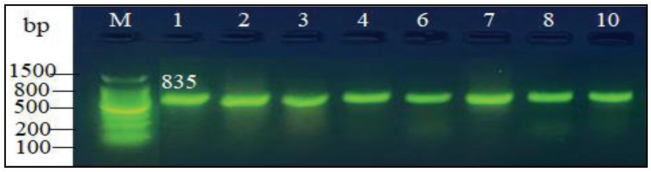

Figure 3: The gel electrophoresis profile of 835 bp of the 18S small subunit ribosomal RNA gene using 2% agarose stained with a safe red DNA dye and electrophoresed at 5 vol/cm in TBA buffer. Lane M.DNA Marker 100–1500 bp

Table 1: Al-Anbar province, sociodemographic features of the patients based on species of *Cryptosporidium* presence or absence

Variable		Infected (%)	Noninfected (%)	Total	*P*-value
Gender	Male	18 (8.7%)	69 (33.6%)	87 (42.4%)	1.022
	Female	18 (8.7%)	100 (48.7%)	118 (57.5%)	
Age groups	(0–30)	22 (10.7%)	94 (45.8%)	116 (56.5%)	61.381
	(30–60)	10 (4.8%)	58 (28.2%)	68 (33.1%)	0.446
	(60–90)	4 (1.9%)	17 (8.2%)	21 (10.2%)	7.119
Residency	Rural	22 (10.7%)	93 (45.3%)	115 (56.09%)	
	Urban	14 (6.8%)	76 (37.07%)	90 (43.9%)	
Seasonal variation	March	1 (0.48%)	8 (3.9%)	9 (4.3%)	
	April	4 (1.9%)	6 (2.9%)	10 (4.8%)	
	May	1 (0.48%)	4 (1.9%)	5 (2.43%)	
	June	3 (1.4%)	8 (3.9%)	11 (5.3%)	
	July	2 (0.97%)	7 (3.4%)	9 (4.3%)	
	August	6 (2.9%)	20 (9.7%)	26 (12.6%)	
	September	2 (0.97%)	11 (5.3%)	13 (6.3%)	
	October	2 (0.97%)	11 (5.3%)	13 (6.3%)	
	November	6 (2.9%)	29 (14.1%)	35 (17.07%)	
	December	4 (1.9%)	21 (10.2%)	25 (12.1%)	
	January	5 (2.43%)	44 (21.4%)	49 (23.9%)	

>Seq1: *C. parvum*
TTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTACGGATCACATTAAATGTG
ACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCTACCGTGGCAATGACGGG
TAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATCCTAATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGT
TTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGC
AGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTT
CTGTTAATAATTTATATAAAATATTTTGATGAATATTTATATAATATTAACATAATTCATATTACTATAT
ATTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTTGAATACTCC
AGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAAGAATAATGATTAATAGGGA
CAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCGA
AAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGT
CGTAGTCTTAACCATAAACTATGCCAACTAGAGATTGGAGGTTGTTCCT

>Seq6 : *C. hominis*
TTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTACGGATCACAATTAATGTG
ACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCTACCGTGGCAATGACGGG
TAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATCCTAATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGT
TTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGC
AGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTT
CTGTTAATAATTTATATAATATATTTTGATGAATATTTATATAATATTAACATAATTCATATTACTATTT
TTTTTTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTTGAATACTC
CAGCATGGAATAATATTAAAGATTTTTATCTTTTTTATTGGTTCTAAGATAAGAATAATGATTAATAGGG
ACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAATGCG
AAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG
TCGTAGTCTTAACCATAAACTATGCCAACTAGAGATTGGAGGTTGTTCCTTACTCCTT

Figure 4: DNA sequences found in *C. hominis* and *C. parvum*

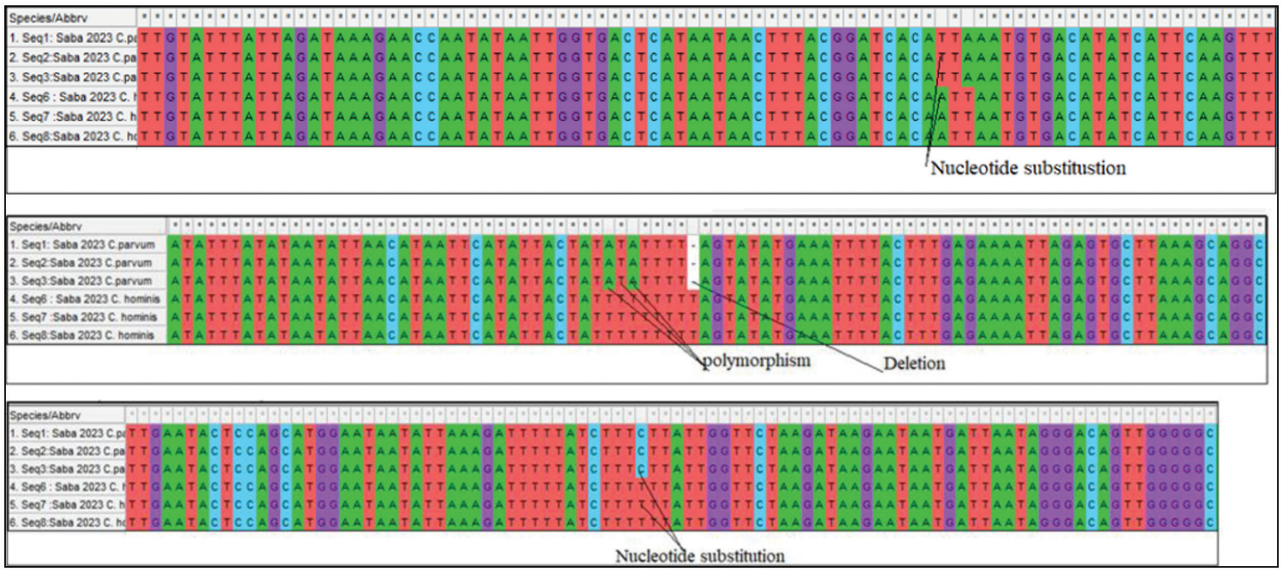

Figure 5: Variation in the 18srRNA gene nucleotide sequences in the primer regions between *C. parvum* and *C. hominis*

cryptosporidiosis is a significant issue of global public health with a wide range of prevalence. Similar to recent studies, our findings revealed no statistically significant deviations between infection with *cryptosporidium* spp. and studied variables such as gender, age, seasonal variation, and residence ($P = 0.05$).[11,12] Although the sample size is insufficient to draw a conclusion, it is consistent with a study conducted in the Philippines, which found that there was no correlation between the gender of youngsters and the prevalence of being infected by this parasite.[13,14]

In addition, the findings were consistent with those of Natividad *et al*.[15] and Ke-Xia *et al*.[16] In impoverished societies, intestinal parasites are prevalent, and among parasitic diseases, *Cryptosporidium* poses a significant hazard to public health, particularly in developing nations.[17,18]

The authors analyzed the progression of an infection within 128 patients. Patients with less severe immunosuppression were more likely to contract transient infections, which were detected in 28.7% of immunocompromised patients.[19] 7.8% of patients passed over 2 L of stool/day, but just those alongside a CD4 count below 50/mm^3.[20] Among AIDS patients, cryptosporidiosis remains one of the most prevalent causes of diarrhea.[21] Currently,

cryptosporidiosis is the most prevalent waterborne disease in the world.[22]

The *18SrRNA* gene of *Cryptosporidium* spp. was efficiently amplified by nested PCR and sequenced in all microscopic-positive samples, revealing the presence of a pair of *Cryptosporidium* species, including *C. hominins* 47.2% (17/36) and *C. parvum* 52.0% (19/36). The substantial number of *C. parvum* may be attributable to the high incidence of zoonotic transmission cryptosporidiosis in this region, as well as anthroponotic transmission associated with *C.hominis* and the lack of host specificity, which may give it a real chance to spread widely among humans as well as animals,[23] as reported by Al-Braiken *et al*.[24] *C. parvum* and *C. hominis* were identified in 37% and 42.9% of cases, respectively, in Saudi Arabia. Mixed infections with *C. hominis* and *C. parvum* were detected in 2.9% of infected children, and 2.9% were infected with other species. Rafiei *et al.*[25] recorded infection rates of 68%, 25%, and 6.2% among juveniles infected with *C. parvum*, *C. hominis*, and other species in Iran. In Egypt, *C. hominis* is predominant among humans (60.5%), followed by *C. parvum* (38.35%), as determined by Helmy *et al*.[26]

In this regard, other researchers used conventional techniques such as formalin-ether concentration and modified Ziehl–Neelsen staining, whereas we used conventional techniques (Zeihl–Neelsen stain) followed by molecular techniques.

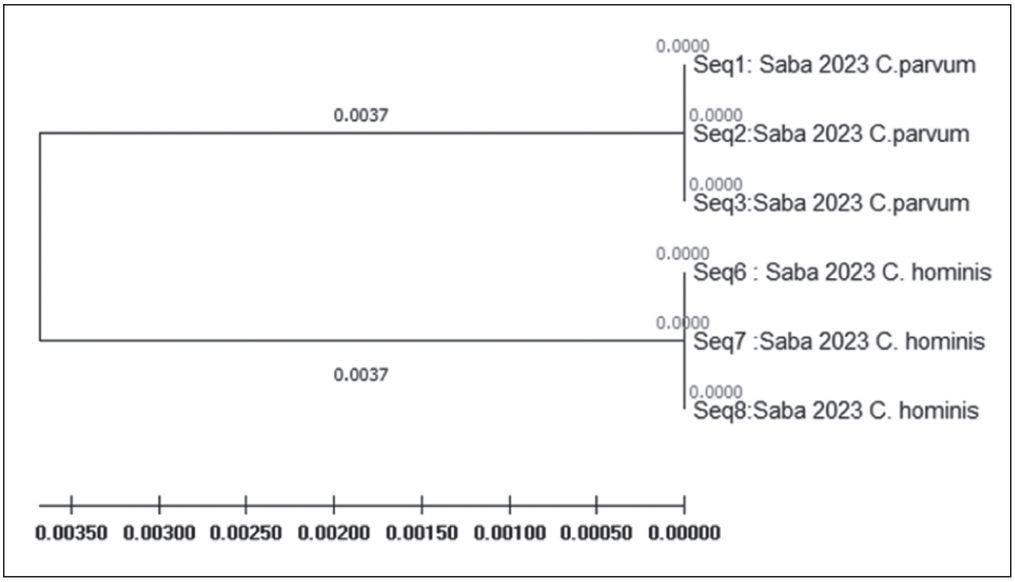

Figure 6: Neighbor-joining tree is used to construct local phylogenetic trees.MEGAX was responsible for the phylogenetic distance analyses

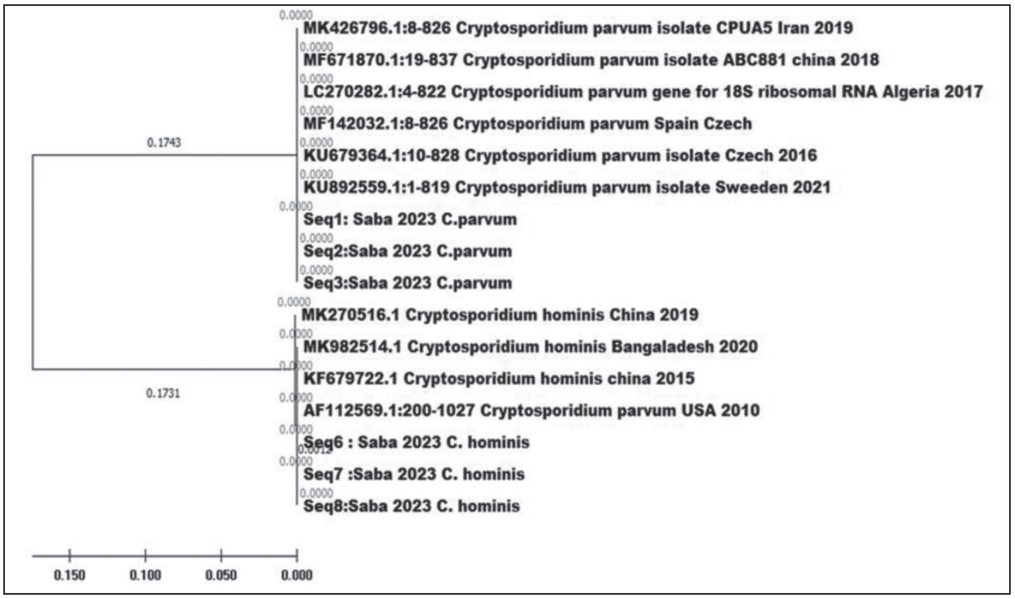

Figure 7: Neighbor-joining tree is used to construct global phylogenetic trees.MEGAX was responsible for the phylogenetic distance analyses

To investigate the degree of total identity and overall similarity degree for the *18s rRNA* gene of the *Cryptosporidium* parasite, which generally affects humans, compared to our Iraqi isolates *C. parvum* and *C. hominis* isolated from humans, the results showed closed relatedness to reference *Cryptosporidium* parasitic isolates *C. parvum* and *C. hominis* isolates with a total percent identity score of 100%. Using the MCL method and Tamura-nei model,[27] we can infer the evolutionary history as Figures 5 and 6.

For detection of the degree of total identity and overall similarity degree for the *18 sr RNA* gene of the *Cryptosporidium* parasite compared with other international isolates that isolate from humans, the results showed closed relatedness to reference *Cryptosporidium* parasitic isolates *C. parvum* and *C. hominis* isolates with a total percent identity score of 100% as Figure 7.

Conclusion

Our findings provide valuable information about the distribution of *Cryptosporidium* species in Al-Anbar province. This study revealed that *Cryptosporidium* spp. is common among children and adults under 30 years old in the study location. *Cryptosporidium parvum* (a zoonotic species) is a particularly common cause of cryptosporidiosis in the region. There is no significant difference between male and female infection rates. The rate of infection in rural areas was considerably higher than in urban areas, but the difference was not statistically noteworthy. The monthly occurrence rate is unaffected by the months of the year.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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