Investigation of *Cryptosporidium* Oocyst Contamination in Water from Zabol and Zahedan: A Molecular and Microscopic Analysis

Running title: Investigation of *Cryptosporidium* Oocyst Contamination in Water

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Abstract

Background:*Cryptosporidium* is one of the most important protozoan parasites causing waterborne diseases worldwide. The parasite's oocysts are resistant to conventional water treatment, making molecular detection crucial for identifying contamination sources.

Methods: A total of water samples were collected from different sites in Zabol and Zahedan, southeastern Iran. Microscopic screening was performed after concentration and staining with modified Ziehl–Neelsen and Trichrome methods under 1000× oil-immersion magnification. DNA was extracted from positive samples, and the SSU rRNA gene (~800-900 bp) was amplified by PCR. The resulting products were subjected to enzymatic digestion using AluI and RsaI restriction enzymes, and representative amplicons were sequenced.

Results: Microscopic examination confirmed the presence of *Cryptosporidium* oocysts in several water samples. PCR amplification successfully produced fragments of the expected size without nonspecific bands. AluI digestion revealed distinct fragment patterns consistent with *Cryptosporidium* spp., while RsaI showed no cutting sites. Sequence analysis through BLAST showed high identity (≥99%) with *C. parvum* isolates. The phylogenetic tree constructed using the BLAST distance-tree method grouped the sequence closely with *C. parvum*, confirming its identity.

Conclusion: Molecular characterization of *Cryptosporidium* from water samples in southeastern Iran indicated contamination primarily with *C. parvum*. These findings emphasize the necessity of continuous molecular surveillance to ensure the safety of drinking and recreational waters in the region.

Keywords: *Cryptosporidium,* DNA sequencing, Molecular methods, PCR, Water contamination, Zabol, Zahedan.

Introduction

The primary aim of this study is to investigate the prevalence and genotypic diversity of Cryptosporidium in drinking and surface water sources in the Zabol and Zahedan districts. By employing PCR-RFLP techniques for molecular characterization, this research seeks to fill existing knowledge gaps regarding the origins and transmission pathways of *Cryptosporidium.*

Cryptosporidium is a significant enteric parasite responsible for widespread outbreaks of diarrheal illness globally, notably affecting both immunocompromised and immunocompetent individuals. Transmission through contaminated drinking and surface water has become a critical public-health concern, particularly in regions where water-quality management is inadequate. Recent studies have highlighted the increasing prevalence of *Cryptosporidium* infections, underscoring the urgent need for comprehensive research to understand its epidemiology and transmission dynamics. The focus of this study is to investigate the prevalence and genotypic diversity of *Cryptosporidium* in the Zabol and Zahedan districts of Southeast Iran, an area where such outbreaks have not been officially reported. This research is vital for developing targeted intervention strategies that can mitigate the risks associated with waterborne transmission of this pathogen, especially in vulnerable populations (1).

Interest in *Cryptosporidium* research has surged among both scientists and public-health practitioners, driven by growing recognition of the parasite as a leading cause of waterborne disease. This has prompted extensive investigations into its genetic diversity and transmission pathways. Recent advancements in molecular techniques, such as PCR-RFLP, have facilitated more accurate identification of *Cryptosporidium* species and genotypes, enhancing epidemiological tracking (2).

Furthermore, the emergence of new strains and their potential zoonotic implications has heightened awareness regarding the need for continuous surveillance and research. Despite substantial progress, several challenges persist. One major issue is the organism's ability to survive harsh environmental conditions and resist conventional disinfection methods, complicating its control (3). Additional gaps remain concerning the specific sources of contamination and ecological factors contributing to outbreaks. However, recent technological advancements, including high-throughput sequencing and improved culture methods, offer new opportunities to address these challenges by providing deeper insights into Cryptosporidium biology and transmission.Current literature also reveals several areas that require further exploration. While previous studies have contributed valuable data on the epidemiology of *Cryptosporidium* ,(4) many aspects remain understudied, particularly regarding regional variations in prevalence and genetic diversity. Limited data exist on the specific genotypes circulating in Southeast Iran and their potential reservoirs within local wildlife or livestock populations. Addressing these gaps is essential for developing a comprehensive understanding of *Cryptosporidium* dynamics in this region and for improving preventive public-health measures.

Methods

Sample Preparation

Water samples were collected between April 2021 and August 2022 from various locations in Zahedan and Zabol cities, including surface water, tap water, bottled water, well water, and wastewater sources. A total of 180 samples were systematically collected (80 from Zabol and 100 from Zahedan), with each sample labeled for identification during subsequent analyses for *Cryptosporidium* parasites.

Sampling sites were precisely delineated, and random sampling protocols were followed to ensure representativeness. Health and safety protocols were strictly adhered to, including the use of personal protective equipment (PPE), biosafety cabinets, and disinfectants. A minimum of 10 liters of water was collected per site following EPA Method 1623.1.

Upon collection, samples were filtered through membranes with a pore size of 2–3 μm using the Centrifugal Vacuum Filtration method. Pellets trapped in the filter were removed and centrifuged to concentrate the oocysts. The sediment was excised from the filters using a scalpel and transferred into 1.5 ml microtubes. To preserve sample integrity, they were stored at 4°C in a preserving solution of 2.5% potassium dichromate.

DNA Extraction

DNA extraction was performed using the YEKTA-TAJHIZ DNA Extraction Kit according to the manufacturer's instructions. Briefly, a freeze–thaw cycle was applied to lyse the cells, followed by washing with PBS to remove residual contaminants. Glass bead powder was then added to enhance mechanical cell disruption during vortexing. After incubation to ensure complete lysis, DNA was isolated following the kit protocol and finally eluted in elution buffer and stored at −20°C until further analysis.

The quality of the extracted DNA was evaluated by agarose gel electrophoresis. Approximately 5 µl of extracted DNA, mixed with loading dye, was loaded onto a 1% agarose gel containing ethidium bromide and electrophoresed at 75 V for 60 minutes. The gel was visualized using a gel documentation system, and the resolved DNA bands were compared with a standard molecular weight marker.

PCR Method

The PCR reaction mixture consisted of 5 µl extracted DNA, 12.5 µl master mix (Brand/Type), 4.5 µl sterile distilled water, and 1 µl of each primer. PCR amplification was performed in an Eppendorf thermocycler under the following conditions: initial denaturation at 94°C for 5 minutes; followed by denaturation at 94°C for 45 seconds; annealing at 55°C for 45 seconds; elongation at 72°C for 60 seconds; with a final elongation step at 72°C for 7 minutes over a total of 35 cycles.

The primer sequences and PCR product size are shown in Table 1.

Table 1. The SSU-rRNA primer sequences and PCR product

Gene	Primer sequences (5′→3′)	Product size (bp)
SSU rRNA	Forward:GGAAGGGTTGTATTTATTAGATAAAG Reverse: AAGGAGTAAGGAACAACCTCCA	824

RFLP Testing Using PCR Products

Restriction Fragment Length Polymorphism (RFLP) analysis was conducted on PCR products from samples testing positive for *Cryptosporidium*. The enzymes *RsaI* and *AluI* were employed as shown in Table 2.

Table 2. The Restriction Enzymes Used and Their Related Cutting Site

Cutting Site	Enzyme
5′……AG/CT…….3′ 3′…. ……TC/GA…….5′	*Alul*
5′……GT/AC…….3′ 5′……CA/TG…….3′	*Rsa1*

For each enzyme reaction, five microliters of PCR product were combined with two microliters of enzyme buffer and one microliter of either *RsaI* or *AluI* enzyme (Thermo Scientific). The total volume was adjusted to ten microliters with distilled water before incubation at 37°C for one hour. To deactivate the enzymes, microtubes were heated at either 80°C or 65°C for twenty minutes as appropriate.

The digested products were subsequently loaded onto a 1.5% agarose gel for electrophoresis to identify the genotype of *Cryptosporidium.* All procedures adhered to ethical guidelines concerning environmental sampling and laboratory safety protocols.

Results

3.1. Microscopic Examination of Water Samples

A total of 180 water samples were collected from various sources in Zahedan and Zabol. Initial screening was performed using direct microscopic examination, followed by confirmation with modified Ziehl-Neelsen and Trichrome staining methods. Microscopic analysis revealed that 35 samples were positive for *Cryptosporidium* oocysts, 142 were negative, and 3 samples were classified as suspicious (**Figure 1**).

In Zahedan, 9 out of 100 samples (9%) were positive, 2 samples (2%) were suspicious, and 89 samples (89%) were negative. In Zabol, 26 out of 80 samples (32.5%) tested positive, one sample (1.25%) was suspicious, and 53 samples (66.25%) were negative. The highest positivity rates were detected in wastewater (46.9%) and surface water (45.3%), indicating substantial contamination in these sources**.**

3.2. DNA Extraction Results from *Cryptosporidium*

All *Cryptosporidium*-positive samples yielded successful DNA extraction. The incorporation of glass beads together with repeated freeze–thaw cycles effectively enhanced oocyst disruption and improved DNA yield. Following PCR amplification, the products were electrophoresed on a 1% agarose gel and visualized using a gel documentation system. A clear and distinct single band was observed in all positive samples, falling within the expected size range of approximately 800–900 bp, corresponding to the ITS1/SSU rRNA target region (**Figure 2**).

The absence of nonspecific bands or smearing indicated a high level of amplification specificity and adequate DNA purity. Furthermore, the position of the band relative to the 500 bp marker confirmed that the amplified fragment was approximately 824 bp in length, which is consistent with previously reported sizes for *Cryptosporidium* ITS1/SSU rRNA gene fragments.

3.3. Results of Enzymatic Digestion with AluI and RsaI Enzymes

RsaI digestion:

No clear restriction fragments were detected following digestion with RsaI. The absence of visible bands is most likely due to the lack of RsaI recognition sites within the amplified ITS1/SSU rRNA region, indicating that this fragment is not cut by RsaI under standard conditions. Therefore, the undigested pattern is considered a normal finding rather than an indication of enzyme failure.

AluI digestion:

Digestion with AluI produced a characteristic multi-band pattern. A prominent fragment of approximately 850 bp was observed, representing partial digestion of the original PCR product. Additional fragments of approximately 720 bp, 480 bp, 456 bp, and 410 bp were detected, confirming that AluI cleaved the target sequence at multiple restriction sites. The coexistence of a large undigested band together with multiple smaller fragments is consistent with expected RFLP profiles for *Cryptosporidium* ITS1/SSU rRNA amplicons. Overall, the observed AluI banding pattern supports the identification of *Cryptosporidium* spp. (**Fig. 3**).

3.4. PCR Sequencing of SSU rRNA in *Cryptosporidium*

Selected PCR-positive samples were subjected to Sanger sequencing using a commercial sequencing service (Bioneer Co.). High-quality chromatograms were obtained, and the forward and reverse reads were assembled to generate a consensus sequence. Raw sequence data were inspected and edited using Chromas Lite version 2.1, and the final consensus sequence was compared with reference sequences available in the NCBI GenBank database using the BLASTn algorithm.

The nucleotide sequence generated in this study is provided in Appendix 1. BLAST analysis demonstrated a strong and significant sequence identity and an E-value of 0.0 (**Fig. 4**). These findings confirm that the mplified SSU rRNA fragment corresponds to *C. parvum*.alignment with multiple isolates of *Cryptosporidium parvum*, showing 98.84%

3.5. Phylogenetic Analysis

Phylogenetic analysis was performed to evaluate the evolutionary placement of the SSU rRNA sequence obtained in this study. A distance-tree was generated using the BLAST pairwise alignment tool, which grouped the sequence (Crypto-F) together with multiple reference isolates of *C. parvum*. No clustering with *C. hominis* or other *Cryptosporidium* species was observed.

The close association of the query sequence with *C. parvum* isolates, combined with the high sequence identity (98.84%) and high query coverage obtained from BLAST analysis, confirms that the sample belongs to the *C. parvum* lineage (**Fig. 5**).

In conclusion, the phylogenetic findings indicate that the SSU-rRNA sequence obtained from the water samples collected in Zabol and Zahed shows clear genetic affinity to established isolates of Cryptosporidium parvum. As illustrated in Fig. 5, the sequence clusters within the C. parvum lineage and shares conserved regions of the 18S rRNA (SSU-rRNA) gene with these reference isolates. This strong genetic similarity provides robust molecular evidence confirming that the detected organism belongs to C. parvum.

Discussion

Cryptosporidium spp. are major waterborne pathogens with substantial public health implications. Human and animal fecal contamination of environmental sources such as soil, water, and food constitutes a key transmission route for this protozoan, which remains one of the leading causes of diarrheal disease worldwide. The World Health Organization (WHO) has recognized Cryptosporidium as a priority pathogen for global water quality monitoring, highlighting its significance in public health surveillance and waterborne disease management (6). Therefore, the detection and molecular characterization of *Cryptosporidium* spp. in environmental samples are essential for understanding transmission dynamics and implementing effective control measures.

Genotyping of *Cryptosporidium* isolates is a critical tool for distinguishing species and genotypes and for elucidating epidemiological patterns. Due to the difficulty of culturing this parasite—an approach that is both time-consuming and susceptible to contamination—PCR-RFLP has become an important molecular method for genotyping (7). This technique differentiates species based on the digestion of PCR-amplified DNA by restriction enzymes that cleave specific nucleotide sequences. The resulting fragment patterns allow

comparison with known profiles and facilitate accurate species identification (8). Variations in banding patterns may reflect genetic polymorphisms, particularly in regions where multiple species or genotypes co-circulate.

The epidemiology of *Cryptosporidium* is further complicated by the coexistence of multiple transmission pathways, including waterborne, foodborne, and zoonotic routes. Genotyping plays an essential role in identifying infection sources and understanding transmission mechanisms by revealing the genetic diversity within *Cryptosporidium* populations. Previous studies indicate that *C. parvum* and *C. hominis* are responsible for the majority of human infections, with *C. parvum* accounting for approximately 90% of cases in many regions. To date, more than 300 human cases have been genotyped, revealing at least nine infective *Cryptosporidium* species, including *C. andersoni*, *C. felis*, and *C. meleagridis*, among others(9).

This diversity underscores the need for continuous monitoring and molecular surveillance.

Globally, water contamination remains a major public-health concern due to the exceptional resistance of *Cryptosporidium* oocysts to routine water-treatment processes. Oocysts are highly resistant to chlorine and several commonly used disinfectants, complicating their removal from water supplies and reinforcing the need for sensitive detection methods (10). Given the increasing threat of waterborne diseases worldwide, the development and application of rapid molecular diagnostic techniques have become a priority in water-quality monitoring programs (11).

In the present study, 180 water samples were examined, of which 35 tested positive for *Cryptosporidium* oocysts. The primers used successfully amplified an approximately 800–900 bp fragment of the SSU rRNA gene, which is consistent with previous studies targeting this region. Microscopic examination and staining techniques initially confirmed the presence of oocysts. Subsequently, subtyping was performed using AluI and RsaI restriction enzymes, followed by DNA sequencing to further characterize the isolates. Consistent with expectations for the SSU rRNA gene, digestion with AluI produced clear fragment patterns suitable for genotyping, whereas digestion with RsaI did not yield visible fragments—likely due to the absence of an appropriate recognition site within the amplified region rather than a technical error.

The combined application of microscopy, staining, RFLP analysis with AluI, and sequencing revealed notable genetic polymorphism among *Cryptosporidium* isolates from the Zabol and Zahedan districts. This observation indicates substantial genetic diversity among circulating isolates in these geographic regions. The integration of molecular and conventional detection techniques allowed for robust subtyping and comparison of isolates, thereby providing a comprehensive picture of the genotypic profiles present in the study area.

The phylogenetic tree (Fig. 5), constructed using SSU rRNA sequences, demonstrates a distinct clustering of *C. parvum* isolates according to geographic origin and host specificity. Similar findings have been reported previously, showing that *C. parvum* isolates from different regions often form independent clades, suggesting geographic structuring and restricted gene flow (12, 13). These genetic markers are essential for tracking outbreaks and distinguishing between zoonotic and anthroponotic transmission routes. Certain *C. parvum* genotypes may exhibit host adaptation, emphasizing the importance of recognizing host-associated variants for public-health surveillance.

Genetic analyses of *C. parvum* also provide insights into potential environmental and animal reservoirs, helping to identify sources of contamination in water systems. Such information is vital for designing targeted interventions, enhancing disease surveillance, and improving our understanding of transmission routes. Molecular epidemiology using SSU rRNA markers therefore plays a crucial role in monitoring and controlling cryptosporidiosis.

Worldwide, the prevalence of *C. parvum* in diverse water sources continues to present major challenges for water safety. Studies have reported prevalence rates of 46.9% in wastewater, 45.3% in surface water, and 31.6% in untreated raw water (14). These findings reflect the persistence of *Cryptosporidium* oocysts in the environment and their resistance to conventional treatment methods, particularly chlorine-based disinfection (10). In Iran, *C. parvum* is the dominant species, accounting for 83.3% of human cases followed by *C. hominis* with 8.3% (6). In livestock, particularly cattle, *C. parvum* is also the predominant species, representing approximately 36% of infections,with genotypes similar to those identified in countries such as France(15)(16), These data further highlight the importance of zoonotic transmission in regional epidemiology. Globally, *C. parvum* remains one of the most frequently reported pathogens in waterborne outbreaks, with contaminated water serving as a major transmission route (10).

In conclusion, the persistence of *C. parvum* in water sources in Iran remains a considerable public-health challenge. The detection and molecular characterization of *C. parvum* from environmental water samples provide essential information for assessing the risk of waterborne cryptosporidiosis. Addressing

contamination requires improvements in water-treatment infrastructure, enhanced monitoring systems, and effective intervention strategies. The findings of this study—particularly the genotypic and phylogenetic characterization of isolates—contribute valuable insights into the transmission dynamics of *Cryptosporidium* and underscore the importance of molecular tools in surveillance and outbreak prevention.

Author Contributions

1. R S: Conducted extensive field research in Zabol City and its surroundings to gather crucial data. Drafted the introduction and results sections and thoroughly reviewed the manuscript content.
2. M B : Coordinated field study operations and played a key role in formulating the research design, specifically focusing on molecular methodologies. Assisted in interpreting data and participated actively in discussions regarding regional health policies. Approved the final manuscript for publication.
3. M K S: Conducted extensive field research in Zabol City and its environs, collecting critical data pivotal to the study. Analyzed the gathered data, employing statistical tools to discern patterns relevant to the *Cryptosporidium* incidence over the observed period. Drafted initial sections of the manuscript, particularly the introduction and results, and revised the document for critical content. Provided final approval of the version to be published.
4. M D (Corresponding Author): Led the project and supervised the study design, execution, and coordination. Condensed research findings, offered critical insights for data interpretation, and led the manuscript writing process, incorporating contributions from co-authors while maintaining coherence and academic integrity. Served as the guarantor for the work, provided final approval for publication, and addressed any post-publication issues, ensuring the accuracy and integrity of the study

Declarations

We appreciate the opportunity to clarify this aspect and affirm our commitment to maintaining the highest standards of academic integrity throughout the research process. Should there be any further inquiries regarding the funding or support of this study, please feel free to reach out.
Authors' Contributions: Mansour Dabirzadeh guided the research as a supervisor and worked with the other authors. Reza Shahraki and Mahdi Khoshsima Shahraki gathered and analyzed the samples, and Mohammadreza Beheshtizadeh helped with the molecular method, analyzed the molecular data, and gave final consent for publishing the manuscript.

Availability of data and materials

Upon a reasonable request, the corresponding author will share the datasets of this study.

Funding sourse

Not applicable.

Ethical statement

This study involved harvested oocysts from water, ensuring no unethical practices. It was approved by the University Research and Ethics Committee (code: http://ethics.research.ac.ir/IR.ZBMU.RE*CRYPTOSPORIDIUM*1398.159), and all procedures followed institutional guidelines for the care and use of humans and animals.
All participants in this study provided informed consent before participating. They were fully informed about the study's purpose, procedures, potential risks, and benefits.
This consent process ensured that participants were aware and agreeable to the terms of their involvement in the study.

Conflicts of interest

There are no conflicts of interest.

Acknowledgments

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Appendix 1. Nucleotide sequence of the SSU-rRNA gene obtained in this study.

CGCCCTTATTCTCATTCACTTTACAGATCACTTAAATGTGACATATCATTCAGTTTCTGACCTATCAACTTTACAC
GGTAGGGTATTGGCCTACCGTGCCAAACACGGTAAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAG
AAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTAATACAGGGAGGTAGTGACAA
GAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGTATCAATTG
GAGGGCAAGTCTGGGGCCAGCAGCCGCGGTAATTCCAGCTCCACTAACGTATATTAAAGTTGTTGCAAAAAAAA
AGCCCGTAATTGGATTTCCAACAAAAGTTATATAAAAAATATTGTGGATAATAGAAACAAAAAAAACAAACTTC
CCATTCCAATCAATAATA

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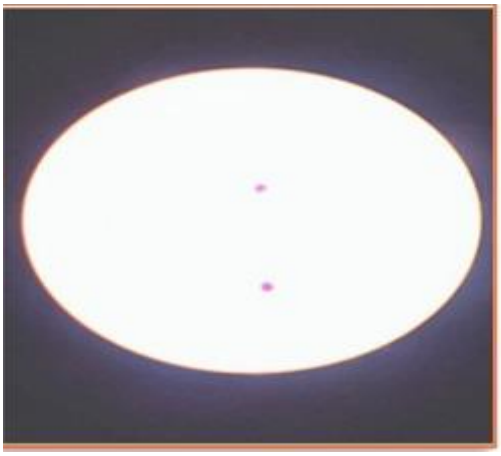
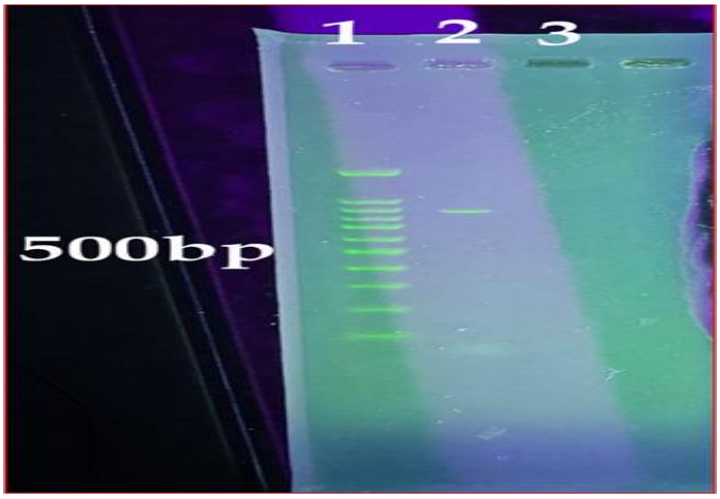


Fig 1. *Cryptosporidium* oocyst samples isolated from water and stained by the modified Ziehl-Neelsen method

Fig. 2. Agarose gel electrophoresis (1% agarose, ethidium bromide staining) of the PCR products amplified from the ITS1-rRNA gene. Lane L: 100 bp DNA ladder; Lane 1: positive control (*Cryptosporidium parvum*); Lane 2: PCR-positive water sample; Lane 3: negative sample.



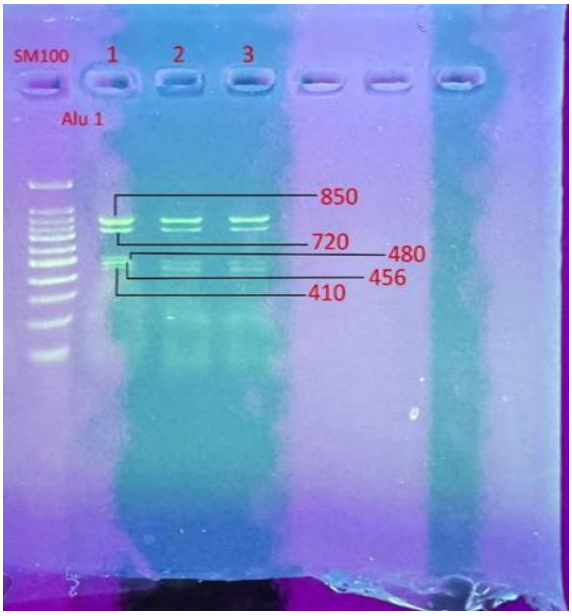
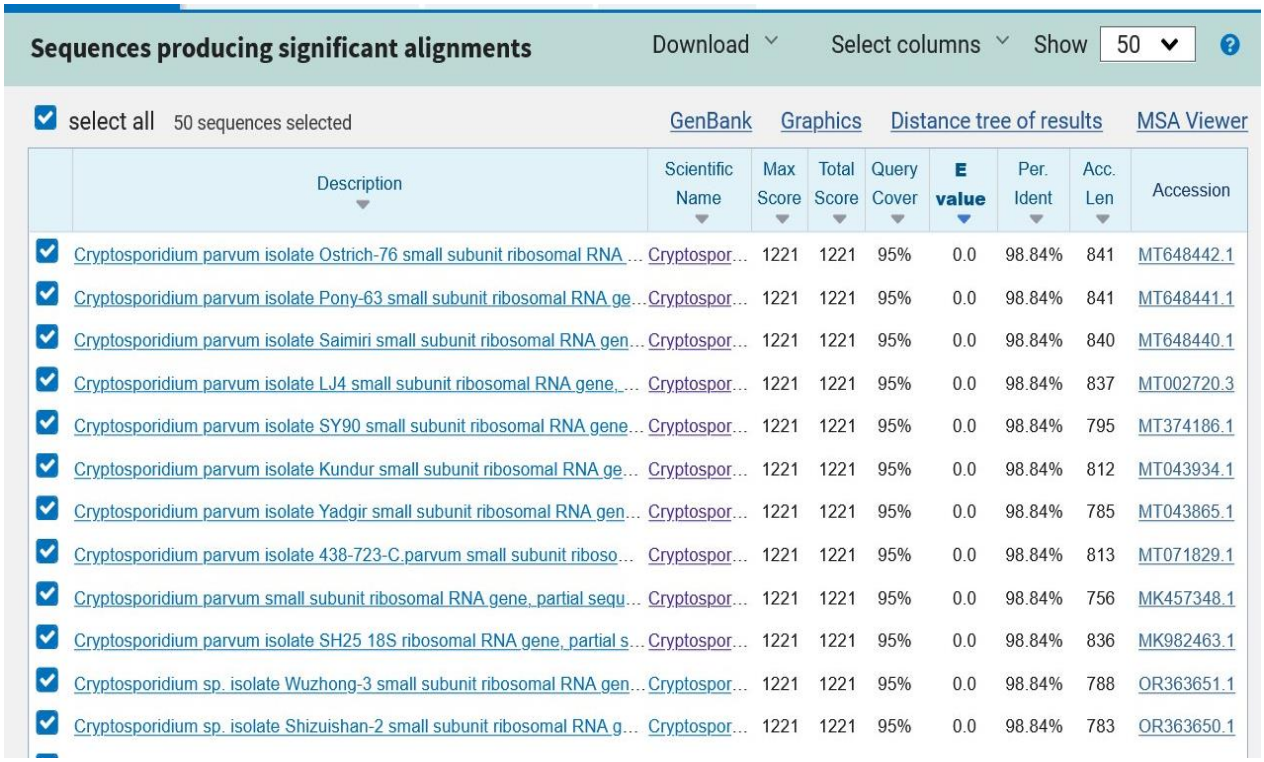


Fig 3. SM(Sample Marker)100 genetic marker (in the right). PCR-RFLP analysis ITS1 gene Band 1 to 3 on the right side of the marker cut with *Alu1* restriction enzyme. Enzymes *Alu1* yielded profile banding sizes of 410-457-480-760-850 bp.



Sequences producing significant alignments Download Select columns Show 50

select all 50 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cryptosporidium parvum isolate Ostrich-76 small subunit ribosomal RNA ...	Cryptospor...	1221	1221	95%	0.0	98.84%	841	MT648442.1
Cryptosporidium parvum isolate Pony-63 small subunit ribosomal RNA ge...	Cryptospor...	1221	1221	95%	0.0	98.84%	841	MT648441.1
Cryptosporidium parvum isolate Saimiri small subunit ribosomal RNA gen...	Cryptospor...	1221	1221	95%	0.0	98.84%	840	MT648440.1
Cryptosporidium parvum isolate LJ4 small subunit ribosomal RNA gene, ...	Cryptospor...	1221	1221	95%	0.0	98.84%	837	MT002720.3
Cryptosporidium parvum isolate SY90 small subunit ribosomal RNA gene...	Cryptospor...	1221	1221	95%	0.0	98.84%	795	MT374186.1
Cryptosporidium parvum isolate Kundur small subunit ribosomal RNA ge...	Cryptospor...	1221	1221	95%	0.0	98.84%	812	MT043934.1
Cryptosporidium parvum isolate Yadgir small subunit ribosomal RNA gen...	Cryptospor...	1221	1221	95%	0.0	98.84%	785	MT043865.1
Cryptosporidium parvum isolate 438-723-C.parvum small subunit riboso...	Cryptospor...	1221	1221	95%	0.0	98.84%	813	MT071829.1
Cryptosporidium parvum small subunit ribosomal RNA gene, partial sequ...	Cryptospor...	1221	1221	95%	0.0	98.84%	756	MK457348.1
Cryptosporidium parvum isolate SH25 18S ribosomal RNA gene, partial s...	Cryptospor...	1221	1221	95%	0.0	98.84%	836	MK982463.1
Cryptosporidium sp. isolate Wuzhong-3 small subunit ribosomal RNA gen...	Cryptospor...	1221	1221	95%	0.0	98.84%	788	OR363651.1
Cryptosporidium sp. isolate Shizuishan-2 small subunit ribosomal RNA g...	Cryptospor...	1221	1221	95%	0.0	98.84%	783	OR363650.1

Fig 4. BLAST output showing a strong alignment between the SSU rRNA sequence obtained in this study and reference Cryptosporidium parvum isolates, with 98.84% sequence identity and an E-value of 0.0.

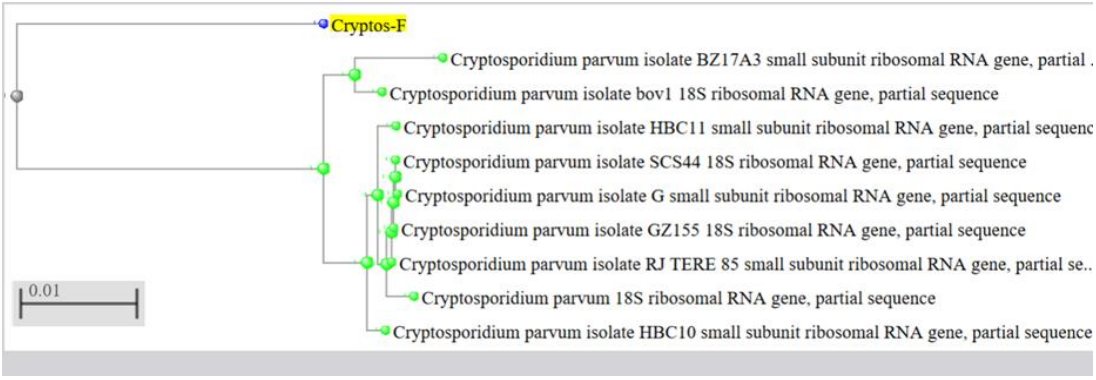


Fig 5. Phylogenetic tree generated using the BLAST distance-tree method based on pairwise sequence alignments of the SSU-rRNA gene. The sequence obtained in this study (Crypto-F / Query_3448041) clustered closely with Cryptosporidium parvum isolates, confirming its identity as C. parvum.