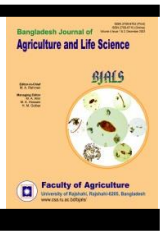
Original Article

Bangladesh Journal of Agriculture and Life Science
Faculty of Agriculture, University of Rajshahi
Rajshahi-6205, Bangladesh
Vol. 04 (1&2): 35-43, 2023
http://csa.ru.ac.bd/bjals/



Isolation and characterization of *Cryptosporidium parvum* in fresh foodstuff from Rajshahi district, Bangladesh

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ARTICLE INFO

Article History
Received: November 21, 2025
Accepted: December 7, 2025
Online: December 10, 2025

Keywords
Contamination
Cryptosporidium parvum
Fresh foodstuffs
Phylogenetic analysis
Zoonotic potential

ABSTRACT

Fresh foodstuff is vital to a healthy diet but can harbor foodborne pathogens with serious health risks. This study investigated the presence of the zoonotic parasite *Cryptosporidium* spp. in frequently consumed fresh foodstuff in Rajshahi, Bangladesh. A total of 450 samples (15 types; 30 each) were collected, processed, and screened using modified Ziehl–Neelsen staining. Overall, 6.0% (27/450) food samples were contaminated, with radish showing the highest Contamination (26.7%), followed by red amaranth (16.7%), water spinach and spinach (13.3% each), cabbage (10%), green amaranth (6.7%), and coriander leaves (3.3%). PCR analysis confirmed 4.88% (22/450) contaminated samples. PCR targeting the SSU rRNA/18S rRNA gene generated amplicons of 310 bp and 738 bp, which enabled species-specific identification of *Cryptosporidium* sp. and *C. parvum*. BLAST analysis of consensus sequences showed close similarity with global isolates, while phylogenetic analysis revealed clustering with human and non-human primate isolates. Bangladeshi isolates from other reservoirs also demonstrated evolutionary relationships with study isolates. These findings indicate geographic variation, adaptive mutation, and zoonotic potential of *Cryptosporidium*. The detection of this parasite in fresh foodstuff highlights a critical food safety concern and underscores the need for improved agricultural practices, surveillance, and public awareness to prevent zoonotic transmission and protect human health.

Introduction

Fresh foodstuff are fundamental components of the diet for humans and animals, supplying essential nutrients necessary for growth, development, and maintaining good health. Their diversity in taste, color, and texture enables the preparation of varied meals daily, supporting a well-balanced diet and a healthy lifestyle. Eating raw fruits and vegetables can expose people to harmful zoonotic parasites, since they are a common source of many parasitic infections. *Strongyloides stercoralis, Ascaris lumbricoides, hookworm, Hymenolepis* spp., *Enterobius vermicularis, Trichuris trichiura, Cryptosporidium* spp., *Balantidium coli, Entamoeba histolytica*, *Giardia intestinalis*, and others are important zoonotic parasites isolated from fresh foodstuff that can infect a wide range of hosts, including humans (Anisuzzaman, 2023; Duedu et al., 2014; Berger et al., 2010; Rzezutka et al., 2010). These parasites are responsible for foodborne outbreaks, as they spread through the feco–oral route when food or water becomes contaminated with parasitic eggs, larvae, cysts, or oocysts, posing significant risks to public health. Foodborne outbreaks are defined as incidents in which multiple individuals develop parasitic infections after consuming the same contaminated food source. These outbreaks are frequently linked to parasites acquired from inadequately cooked meat, such as *Toxoplasma gondii* and *Trichinella spiralis*, or from fresh foodstuff and water contaminated with parasites including Giardia spp. and Cryptosporidium spp. (Dawson, 2005). Furthermore, the practice of

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dumping raw muck or contaminated water directly into agricultural land might parasitize the growing vegetables and fruits (Hutchison et al., 2005). In addition, improper handling during irrigation with wastewater or contamination by humans and animals occurs through multiple pathways, including poor personal hygiene and improper handling practices by food handlers, the use of contaminated water during washing or processing of foods, direct contact with domestic animals in markets, and agricultural practices such as the application of untreated animal manure to crops increases the risk of parasite transmission. Further contamination can occur throughout harvesting, packing, transport, processing, distribution, marketing, and even at the consumer level (Al Nahhas and Aboualchamat, 2020; Gabre and Shakir, 2016).

This phenomenon suggests that there are enough oocysts in the environment to contaminate fresh foodstuff and raises the possibility that susceptible hosts would consume the infectious oocysts, which could be harmful to public health (Smith et al., 2007). *Cryptosporidium parvum* is a protozoan parasite of major public health importance that spreads from animals to humans through contaminated food, water, or surfaces and causes cryptosporidiosis—a disease characterized by severe watery diarrhea, intestinal inflammation, and adverse effects on growth and development in children as well as illness in animals (Bouzid et al., 2018; Squire and Ryan, 2017). In underdeveloped nations, up to 20% of pediatric instances of diarrhea and over 8 million cases of foodborne illness occur globally each year due to Cryptosporidium (Mosier and Oberst, 2000; Ryan et al., 2018). Hospitalized children aged 1–2 years with diarrheal illness have more than double the risk of death, and cryptosporidiosis remains the leading diarrheal killer of children under five, causing over 50,000 deaths annually in the world (Gerace et al., 2019; Kotloff et al., 2013; Troeger et al., 2017).Cryptosporidiosis in livestock leads to significant health challenges and increased mortality. The disease also imposes major financial losses through higher veterinary costs, additional labor, and reduced growth performance (Santin, 2013). Although Cryptosporidium includes over 30 species, only *C. parvum* and *C. hominis* commonly infect humans, with most zoonotic infections caused by *C. parvum* (Ryan et al., 2014; Thomson et al., 2017).

Growing awareness of foodborne parasitic contamination is largely driven by the recognition of its serious health risks. At the same time, fine detection tools, particularly PCR-based methods, have been developed to accurately identify and differentiate oocysts from diverse sources (Slifko et al., 2000; Verweij et al., 2004). To the best of our knowledge, no molecular studies have yet examined parasite contamination in fresh foodstuff sold in Bangladeshi local markets. Therefore, this study aimed to investigate the presence of *C. parvum* in fresh foodstuff using modified Ziehl-Neelsen staining and PCR targeting the small subunit ribosomal RNA/18S rRNA gene.

Materials and methods

Collection and processing of samples

Samples were collected from the Rajshahi districts of Bangladesh for a period from December 2021 to November 2022. Fresh foodstuff samples were purchased from local retail markets and transported to the research laboratory. Each collected sample (450 samples; 15 types), weighing about 125 g, was individually cleaned in 1000 ml of phosphate buffered saline (PBS) with 0.01% Tween 80 solution. To separate the oocysts, it was agitated with an electric shaker for 15 minutes. The washed solution was sifted on eight layers of medical gauze for sieving and left to stand overnight for sedimentation. The supernatants were carefully disposed of, and the leftover sediments were centrifuged for 10 minutes at 2500 rpm. The resulting pellet was resuspended in 2 to 5 ml of PBS, depending on the sample concentration, and stored for further analysis (Abbas et al., 2022; Dixon et al., 2013; Lass et al., 2017).

Isolation and identification of *Cryptosporidium* oocyst

Modified Ziehl–Neelsen staining was used to detect *Cryptosporidium* oocysts under 40× and 100× microscope objectives. Briefly, a thin smear was prepared from the processed sediment sample, air-dried, and fixed in methanol for 2–3 minutes. The slide was stained with carbol fuchsin for 15-20 minutes and then thoroughly rinsed in tap water. The sample was decolorized with acid alcohol (1% HCl in methanol) for 15–20 seconds, followed by a thorough rinse with tap water. The slide was counterstained with 0.4% methylene blue for 30–60 seconds, then thoroughly rinsed in tap water and air-dried. Finally, it was examined under a light microscope fitted with a camera (Optica®, Ponteranica, Bergamo, Italy). *Cryptosporidium* oocysts were identified following the keys and descriptions described previously (Healy, 1996; Nawa et al., 2015; Williams, 1992).

DNA extraction

The suspended pellet solution from each processed sample was taken in separate centrifuge tubes and washed three times with a PBS-Tween 80 solution. The samples were centrifuged at 2500 rpm for 10 minutes, the supernatants were discarded, and pellets were collected. The pellets were mixed with 500 µl of PBS-Tween 80 buffer and transferred to a 1.5 ml Eppendorf tube. It was centrifuged at 12,000 rpm for 10 minutes, then 500

µl of PBS-Tween 80 buffer was added to each tube. The pellets were resuspended by vortexing as the final suspension. Finally, DNA was extracted using the QIAamp® DNA mini kit (QIAGEN, Hilden, North Rhine-Westphalia, Germany) with the following modifications: The final pellet suspensions (100 µl) were transferred to 1.5 ml of Eppendorf tubes, and 300 µl of ATL buffer was added to them. All tubes underwent five cycles of freezing and thawing for 15 minutes at -20°C in a refrigerator and 2 minutes at 90°C in a water bath, respectively. Following the addition of 20 µl of the supplied proteinase K, the samples were mixed properly by vortexing and incubated at 56°C overnight. Then, DNA was eluted in 100 µl of AE buffer following the Qiagen protocol and stored at -20°C for PCR amplification.

Primer design and PCR amplification

The small-subunit rRNA/18S rRNAgene sequences of *Cryptosporidium* sp. and *C. parvum* were used to design the primers (GenBank accession numbers AF442484 and AF108865, respectively). The Primer BLAST tool was used to align this target sequence (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and an in silico analysis was performed to explore the complementarity of each primer by using the PCR Primer Stats tool (https://www.bioinformatics.org/sms2/pcr_primer_stats.html**).** The designed primers for *Cryptosporidium* sp. are *C.* sp. SS rRNA F: ACGAGACCTTAACCTGCTAA and *C.* sp. SS rRNA R: ACAAAGGGCAGGGACGTAA, and for *C. parvum*, they are Cp 18S rRNA F: TGAGAAACGG CTACCACA and Cp 18S rRNA R: GCCCTTC CGTCAATTCCT. A conventional PCR was performed using a Biorad T100 thermal cycler® (Hercules, California, USA). The PCR mixture volume was 25 µl containing 6.5 µl nuclease-free water, 12.5 µl PCR master mix (Vivantis®, Subang Jaya, Selangor, Malaysia), 2µl forward primer, 2µl reverse primer, and 2µl template DNA. The reaction conditions were similar for both target genes: initial denaturation at 94°C for 4 minutes, denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The amplicons were separated on a 1.5% (W/V) agarose gel stained with ethidium bromide solution (0.005%) using gel electrophoresis and visualized on a UV transilluminator. Finally, it was photographed by the gel documentation system.The distinct band region of each gene within the gel was cut into small pieces and purified using the FavorPrep GEL Purification kit (FAVORGEN, Biotech Corp, Taiwan) as per the manufacturer's instructions.

DNA sequencing and phylogenetic analysis

DNA sequencing was conducted at the National Institute of Biotechnology (Dhaka, Bangladesh) using the Sanger method based on dideoxy chain termination. The obtained DNA sequences were analyzed using Bioedit Sequence Alignment Editor Software (version 7.2.5.0), and the consensus sequences were subjected to a nucleotide BLAST (https://blast.ncbi.nlm.nih.gov). Multiple alignments of every sequence were carried out using ClustalW of MEGA 11 software version 51.52.0.0 (Molecular Evolutionary Genetics Analysis, New York, USA). Phylogenetic analysis was performed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), and the robustness of the result was assessed using the bootstrap method with 1,000 replications.

Results

Significant occurrence of *Cryptosporidium* oocysts detected by conventional microscopy

Initially the presence of *Cryptosporidium* oocysts in the examined samples was examined using MZN staining, which demonstrated the contamination of vegetables with this foodborne parasite. In MZN staining and subsequent microscopic examination showed *Cryptosporidium* oocysts as small (4.1–5.8 µm), round structures with light pink coloration and distinct internal black granules (Fig.1). Among 450 samples examined, 27 (6.0%) samples were positive for *Cryptosporidium* oocysts, with radish (26.7%) showing the highest contamination, followed by red amaranth (16.7%), water spinach (13.3%), spinach (13.3%), cabbage (10.0%), green amaranth (6.7%), and coriander leaves (3.3%). *Cryptosporidium* oocysts were not detected in other vegetables such as jute leaves, arum leaves, and Malabar nightshade, nor in salad vegetables such as carrots, cucumbers, tomatoes, and green peppers, as well as fruits like guava (Table 1).

Validation of *Cryptosporidium* oocysts using PCR coupled with bioinformatics

PCR amplification of extracted DNA using a specific set of primers confirmed the presence of the *Cryptosporidium* oocysts.The amplified product**s** of SSU rRNA/18S rRNA gene of *Cryptosporidium* sp. and *C. parvum* were 310 bp and 738 bp, respectively **(**Fig. 2**)**. After PCR amplification and analysis, a total of 22 (4.88%) examined samples were confirmed positive. Radish was the highest contaminated vegetable (20.0%), followed by red amaranth and spinach (13.3% each), water spinach (10.0%), and green amaranth (6.7%). *C. parvum* was not detected in other vegetables, including jute leaves, arum leaves, and Malabar nightshade, or in salad vegetables such as carrots, cucumbers, coriander leaves, tomatoes, and green peppers, or in fruits like guava (Table 1).

Table 1: Comparative sensitivity of MZN staining and PCR technique for detection of *C. parvum* oocysts in fresh foodstuffs

Fresh foodstuffs (n = 30 samples for each)	***C. parvum* contaminated samples in MZN staining**	***C. parvum* contaminated samples in PCR**
Red amaranth	5 (16.7%)	4 (13.3%)
Water spinach	4 (13.3%)	3 (10.0%)
Spinach	4 (13.3%)	4 (13.3%)
Cabbage	3 (10%)	3 (10%)
Green amaranth	2 (6.7%)	2 (6.7%)
Coriander leaves	1 (3.3%)	0 (0%)
Jute leaves	0 (0%)	0 (0%)
Arum leaves	0 (0%)	0 (0%)
Malabar night Shade	0 (0%)	0 (0%)
Radish	8 (26.7%)	6 (20.0%)
Carrot	0 (0%)	0 (0%)
Cucumber	0 (0%)	0 (0%)
Tomato	0 (0%)	0 (0%)
Green pepper	0 (0%)	0 (0%)
Guava	0 (0%)	0 (0%)

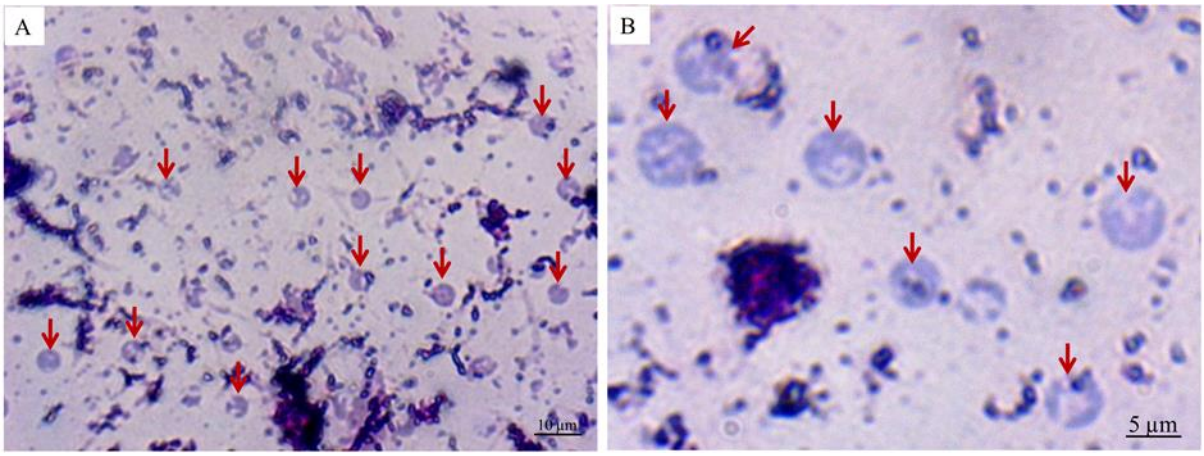

Fig. 1. Detection of *C. parvum* oocysts in MZN staining technique. Small (4.1–5.8 µm), round bodies with light pink coloration and distinct internal black granules were present in the fresh food sample under microscopic examination (A) Scale bars: 10 µm; 40× objectives. (B) Scale bars: 5 µm; 100× objectives.

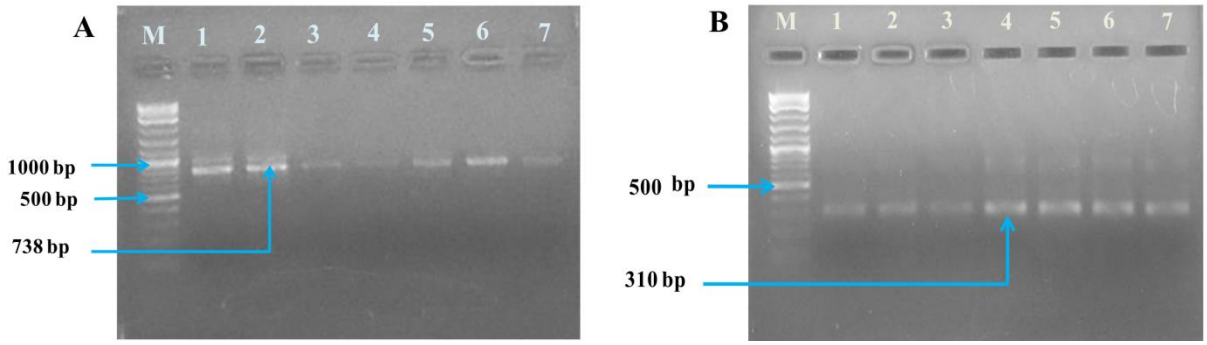

Fig. 2. PCR amplification of 18S rRNA/SSU rRNA gene. (A)*C. parvum* (738 bp). (B) *Cryptosporidium* sp. (310 bp). Lane 'M' indicates a 100 bp DNA marker; lanes 1–7 indicate positive samples from vegetable sources.

The amplified genes were further confirmed by DNA sequencing. The BLAST analysis of the nucleotide sequences revealed 99.68-99.04% similarities with *Cryptosporidium* sp. (AF442484, AY237630, DQ520950), and 99.73-99.32% similarities with *C. parvum* (AF108865, AY204234, AY204229, AY204232, AY204241, AY204229, AF112569). The nucleotide sequences found in this research were deposited into the GenBank database with the accession number PV789331 for *Cryptosporidium* sp. isolate VAS_2 RU and PV788691 for *C. parvum* isolate VAS_1 RU.

The phylogenetic analysis revealed that the representative 18S ribosomal RNA gene had evolutionary relationships between the isolates. From the tree, it was observed that our newly retrieved sequence (PV788691) appeared in one clade, which seems to be the descendant of *C. parvum* isolated from humans in Australia, England, and India (AF108865.1, AY20429.1, MF326947.1). Moreover, this clade appeared to be the ascendant of *C. parvum* isolated from dairy cows in China and rhesus monkeys in the USA (AJ567388.1, AF112569.1). In the case of *Cryptosporidium* sp., our subject sequence (PV789331.1) was found to be another clade forming a sister taxon with *Cryptosporidium* sp. azami in Iran (DQ520950.1) (Fig. 3A). On the other hand, the *Cryptosporidium* isolates obtained from vegetables clustered closely with *C. parvum* sequences previously identified in humans (children), poultry (chickens), and livestock (calves, cattle) in Bangladesh (Fig. 3B). This close genetic relationship suggests that vegetable isolates share common ancestry with strains infecting both humans and animals.

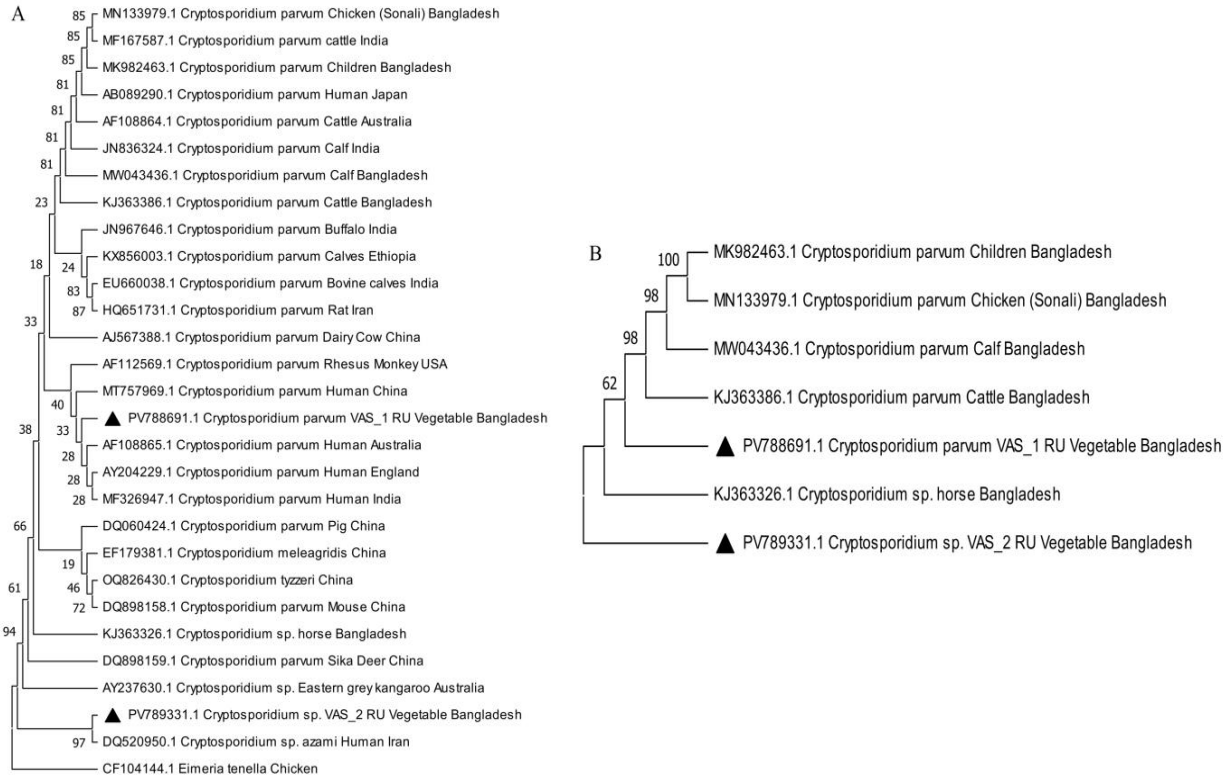

Fig. 3. Phylogenetic relationship of *Cryptosporidium*. (A) The tree was constructed based on SSU rRNA/18S rRNA gene sequences. *Cryptosporidium* sp. (AF442484.1) and *C. parvum* (AF108865.1) genes were used as reference sequences, while *Cryptosporidium* sp. isolate VAS_2 RU (PV789331.1) and *C. parvum* isolate VAS_1 RU (PV788691.1) were used as the subject sequences. *Eimeria tenella* (CF401144.1) was used as an outgroup. (B) This tree indicated the evolutionary relationship among Bangladeshi isolates. Both trees were constructed using the UPGMA method with 1000 bootstrap replicates. Black up-pointing triangles (▲) represent the isolates from fresh foodstuffs of Rajshahi, Bangladesh.

Discussion

Safe food is vital for health, but parasites in food can pose serious risks. In this study, *C. parvum* was detected in fresh vegetables. The presence of *C. parvum* was confirmed by MZN staining, and further validated through PCR, DNA sequencing, and phylogenetic analysis to assess its occurrence and evolutionary relationships.

This research finding showed 6.0% of *C. parvum* in fresh vegetables in MZN staining and 4.88% in PCR. In some previous studies the prevalence of *Cryptosporidium* oocysts in fresh vegetables by MZN staining was 6% in India (Utaaker et al., 2017), 6.5% in Iraq (Sleman Ali et al., 2018), 6.6% in Iran (Ranjbar-Bahadori et al., 2013), 7% in Nigeria (Saidu et al., 2023), 16% in Bangladesh (Asaduzzaman et al., 2016), and 34.7% in Peru (Lucas et al., 2023). The prevalence of *C. parvum* in some past studies by PCR was 2.5% in China (Li et al., 2020), 5.5% in Pakistan (Abbas et al., 2022), 5.6% in Nepal (Bhattachan et al., 2017), 5.9% in Canada (Dixon et al., 2013), and 7.7% in Korea (Sim et al., 2017). Variation in *C. parvum* prevalence in fresh vegetables is influenced by factors such as climate, water source quality, farming hygiene, vegetable surface structure, and diagnostic methods. Higher contamination occurs where sanitation is inadequate, livestock are nearby, and untreated water is used, while advanced molecular tools often reveal more cases (Efstratiou et al., 2017; Thompson et al., 2002).

In this study, radish showed the highest level of contamination (26.7% by MZN staining and 20% by PCR), whereas coriander leaves had the lowest contamination by MZN staining (3.3%) and green amaranth by PCR (6.7%). *Cryptosporidium* contamination was absent in jute leaves, arum leaves, Malabar nightshade, carrots, cucumbers, tomatoes, green peppers, and guavas. *Cilantro roots* were the most contaminated samples (8.7%), and *lettuce* was the least contaminated sample (2.5%) in the Qinghai–Tibetan Plateau of China (Li et al., 2020). Chives showed the highest contamination (17.8%), while sprouts had the lowest (1.4%) in Korea (Sim et al., 2017). Lettuce exhibited the greatest contamination (47.1%), whereas basil and parsley were the least affected (20.0%) in the Yazd District of Iran (Bafghi et al., 2020). Lettuce was the top contaminated sample (40.0%), and mint leaves were the bottom contaminated sample (22.8%) in Bangladesh (Hossain, 2014). Radish was the most contaminated with *C. parvum* likely due to its underground growth, which exposes edible roots to contaminated soil, manure, and irrigation water, all potential oocyst reservoirs. The rough and irregular root surface further facilitates oocyst attachment and persistence. In contrast, jute leaves, arum leaves, Malabar nightshade, cucumber, tomato, green pepper, and guava showed no contamination, possibly due to smoother surfaces and limited soil contact (Duedu et al., 2014; Eraky et al., 2014; Robertson and Gjerde, 2001).

Phylogenetic analysis using the SSU/18S rRNA gene showed that our isolate (PV788691.1) clustered with sequences from other countries rather than with Bangladeshi isolates. This finding does not indicate that the isolate originated abroad; rather, it reflects the highly conserved nature of the SSU rRNA/18S rRNA gene, which is valuable for species identification but has limited ability to distinguish local genetic variants (Cunha et al., 2019; Robinson et al., 2025). Moreover, *C. parvum* populations often exchange genes and recombine, and research shows they tend to cluster by host type or subtype rather than by geographic location (Carey et al., 2023; Kimball et al., 2024). When analyzed with Bangladeshi sequences alone, PV788691.1 grouped within a local clade containing human, poultry, and cattle isolates, highlighting zoonotic circulation across hosts and the contamination of vegetables via manure, irrigation water, or handling (Hira et al., 2011; Karim et al., 2024). The detection of another *Cryptosporidium* sp. (PV789331.1) further indicates diverse species in the environment and suggests that multiple *Cryptosporidium* taxa contaminate fresh vegetables. Together, these findings emphasize a One Health perspective and the need for high-resolution sub typing, such as gp60, to trace transmission pathways more accurately. A limitation of the present study is that stool analysis was not conducted to assess the infectivity of the detected oocysts in humans or animals. Additionally, quantification of infectious oocysts in the tested samples was not performed, and the influence of seasonal variation on contamination levels of fruits, salad vegetables, and other produce was not evaluated. Future studies are planned to address these aspects, providing a more comprehensive understanding of parasitic contamination and associated public health risks.

Conclusion

The detection of *C. parvum* in fresh vegetables from Rajshahi district, Bangladesh, using MZN staining and PCR indicates a notable food safety issue at the pre-consumption stage. Although vegetables and fruits are generally washed before consumption in Bangladesh, potential risks may persist when washing is insufficient or when contamination occurs during handling and marketing. This zoonotic parasite can cause gastrointestinal illness, particularly in vulnerable groups such as children, the elderly, and immuno compromised individuals. Contamination at production or market levels may also affect consumer trust and livelihoods. These findings stress the need for clean irrigation, hygienic handling, effective washing practices, routine monitoring, and continued public awareness to reduce zoonotic transmission and protect public health.

Acknowledgement

The authors gratefully acknowledge the Bangladesh Bureau of Educational Information and Statistics (BANBEIS), the Grant for Advanced

Research in Education (GARE), and the Ministry of Education for financial support (Project ID: LS20201467). Partial funding from the Ministry of Science and Technology (MoST) through the NST Fellowship is also acknowledged. Special thanks to the Department of Veterinary and Animal Sciences, University of Rajshahi, Bangladesh, for laboratory and infrastructural support.

Ethics approval

This research was conducted under the ethical approval granted to the overall project by the Institutional Animal, Medical Ethics, Biosafety, and Biosecurity Committee (IAMEBBC), Institute of Biological Sciences, University of Rajshahi, Bangladesh, under approval number 41/320(47)/IAM EBBC/IBSc. The molecular analyses presented in this paper involved only fresh food samples and did not include human participants, human data, or human tissue; therefore, informed consent was not required.

Authors' contributions

Conceptualization: MR, LN, MGA and MZU; Methodology: MZU, MMH, MMR and MSU; Statistical analysis: MZU; Investigation: MZU, MMH, MEA, MMR and MSU; Data curation: MZU MMH and MSU; Data visualization: MZU, MMH, MMR, MSU, MEA and MGA; Draft preparation, review and editing: MZU; Supervision: MR and LN. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interests.

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