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Prevalence and Molecular Diagnosis of *Cryptosporidium ssp.* and *Giardia lamblia* in Fresh Vegetables from Kurdistan Region/Iraq

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***Abstract*—*Giardia lamblia* and *Cryptosporidium* species are common intestinal parasites that contaminate fresh vegetables and create significant public health problems. This study investigated the prevalence and genetic diversity of six fresh vegetables sourced from agricultural fields and markets in the Kurdistan Region, Iraq. The vegetable samples (n = 210) were obtained from local farms and markets during summer and autumn, 2024, including garden cress (*Lepidium sativum*), leek (*Allium ampeloprasum var. porrum*), lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*), spinach (*Spinacia oleracea*), and rocket (*Eruca vesicaria*). The samples were examined microscopically, stained with acid-fast stain, and subjected to molecular identification through polymerase chain reaction, followed by nucleotide sequencing. Molecular analysis revealed that 75 samples (35.7%) tested positive for *Cryptosporidium*, whereas 16 samples (7.6%) tested positive for *Giardia*. *Cryptosporidium* exhibited a higher prevalence. Garden cress had the highest level of contamination, with 68.6% of its samples testing positive for both parasites. Lettuce and leek exhibited the lowest percentage, approximately 11%. Sequencing identified the isolates as *Cryptosporidium parvum* and *G. lamblia*. A significant correlation ($p < 0.05$) existed between the kind of vegetable and the incidence of *Cryptosporidium* cases; however, no such correlation was observed between the type of vegetable and the incidence of *Giardia* cases. Overall, this study provides important information on the frequency of *Cryptosporidium* spp. and *G. lamblia* detection in six common vegetables consumed in Kurdistan, Iraq. Moreover, it shows how important molecular identification is for correctly identifying species and coming up with good ways to stop the spread of these diseases through food.**

***Index Terms*—*Cryptosporidium* spp., *Giardia lamblia*, Polymerase chain reaction, Prevalence, Vegetables.**

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I. Introduction

Cryptosporidium species and *Giardia lamblia* are two of the most common intestinal protozoan parasites that cause zoonotic diarrhea illnesses worldwide. They typically spread through contaminated food and water, particularly in places with bad sanitation, poor hygiene, and inadequate safe drinking water (Li, et al., 2020). *Giardia* spp. produces cysts that can survive harsh environmental conditions and continue feasible intended for several months; they are encased in a thick cyst wall. A mature infective cyst contains four nuclei (Gaona-López, et al., 2023). *Cryptosporidium* species are distributed through oocysts, which are spherical to oval in shape, with a residual body, and they contain four sporozoites (Helmy and Hafez, 2022). Fruits and vegetables can become contaminated with *Cryptosporidium* oocysts and *Giardia* cysts when irrigated with sewage water. Diarrhea remains an important cause of sickness and death among children in low- and middle-income countries. Furthermore, diarrhea caused by *Cryptosporidium* spp. and *Giardia* spp. is a major cause of malnutrition in young babies, which can make them more likely to get repeated diarrhea (Bitilinyu-Bangoh, et al., 2024). Currently, *Cryptosporidium* includes 41 documented species and over 60 recognized genotypes of these; 21 species and genotypes have been described in humans, with *C. parvum and C. hominis* being the key pathogenic species that cause more than 90% of human infections. Other pathogenic species have sporadically caused zoonotic outbreaks in humans, especially after contact with sick animals (Holubová, et al., 2019). The *G. lamblia* multifaceted has eight morphologically comparable genotypes with genetically separate lineages, classified as assemblages A–H. Assemblages A and B cause illness in humans and many other animals. Assemblages C–H are typically present in canines (C, D), ungulates (E), felids (F), rodents (G), and marine species (H). Recent studies show that assemblages once thought exclusive to animals, such as assemblage E, can also infect humans. This indicates that host specificity among certain assemblages may be less strict, allowing those adapted to animals to potentially infect humans (Hashemi-Hafshejani, et al., 2022).

Microscopic examination and molecular diagnostic assays continue to serve as the main approaches for identifying this illness. The inability to notice infection is worsened by the frequently low concentrations of pathogens and the complexity of biological or environmental samples. Despite their limitations in terms of sensitivity and quantification accuracy, researchers have used both conventional and quantitative polymerase chain reaction (qPCR) (Lotz, et al., 2025). Microscopic discovery of *Cryptosporidium* oocysts necessitates exact techniques, such as Ziehl–Neelsen or Heine staining, due to the small size of the oocysts (Henriksen and Pohlenz, 2021). Conversely, the microscopic identification of developmental forms (cysts and trophozoites) in feces is the main diagnostic approach for *Giardia* infection (Barrera, et al., 2024; Vicente, et al., 2024). The current study is an attempt to investigate the contamination rate of the fresh vegetables and molecular characterization of the *Cryptosporidium* spp. and *Giardia* species isolated from the vegetables in Kurdistan, Iraq.

II. Materials and Methods

A. Sample Collection

A cross-sectional study, which consists of 210 fresh vegetable samples, was conducted during summer and autumn, 2024, in different places of Erbil and Sulaymaniyah governorates. The samples included garden cress (*Lepidium sativum*), leek (*Allium ampeloprasum var. porrum*), lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*), spinach (*Spinacia oleracea*), and rocket (*Eruca vesicaria*). One hundred five samples were collected directly from agricultural areas, whereas another 105 were obtained from local markets. Field samples were collected from farms utilizing diverse irrigation sources, whereas randomly selected market samples represented items available to consumers. This sampling technique aimed to provide a comprehensive assessment of the impact of irrigation water quality and distribution methods on the safety and quality of fresh vegetables, as well as the occurrence of two intestinal protozoa.

B. Sample Processing and Microscopic Examination

Two hundred grams of each vegetable sample were sanitized and thereafter placed in a sterile container containing 150 mL of physiological saline solution (0.85% NaCl). The contents were shaken vigorously by an automated shaker for 15 min to eliminate any parasitic stages. The vegetables were fully submerged in the solution and permitted to settle overnight at 4°C. After meticulously decanting the upper layer of saline solution, 5 mL of sediment was procured the next day and subjected to centrifugation at 2000 rpm for 15 min (Bilgiç, et al., 2023). The sediment was divided into two sections: One for microscopy and acid-fast stain (AFS) examination, and the other, stored in absolute ethanol, for molecular studies and smear preparation on microscope slides. The specimens were subsequently stained with Lugol's iodine to identify *G. lamblia* cysts and then subjected to AFS stain/modified Zihel–Neelsen stain (Cat.No. 20307100, Microxpress® Tulip Diagnostics [P] Ltd., India) using the manufacturer's protocol to detect *Cryptosporidium* oocysts (Al-Jarjary, Hasan, and Hussain, 2024). A light microscope was employed to analyze the stained smears at different magnifications (Aboelsoued, Toaleb, and Abdel Megeed, 2025). Densely red bodies of *Cryptosporidium* measuring 4–6 μm in diameter allow its clear identification against a blue background. The positive samples were preserved in absolute ethanol until DNA extraction occurred.

C. Molecular Analysis

DNA extraction

The DNA was isolated from the conserved sediments of each vegetable. The sediments were rinsed twice with physiological saline to eliminate any residual ethanol that could interfere with subsequent procedures. The ethanol was meticulously isolated and extracted through centrifugation following each wash. Genomic DNA was extracted using the Soil and Stool DNA Kit (Cat. No. CW2091S, CWBIO, Jiangsu, China) following the manufacturer's instructions. We preserved the extracted genomic DNA at −20°C for further molecular analysis.

PCR

Conventional PCR employing species-specific primers that target the *18S rRNA* gene detected *G. lamblia* and *Cryptosporidium* spp. The primers 5'-CATCCGGTCGATCCTGCC-3' (forward) and 5'-AGTCGAACCCTGATTCTCCGCCCAGG-3' (reverse) amplified *G. lamblia*, producing a 292 bp amplicon (Rayani, Unyah, and Hatam, 2014). The amplification protocol commenced with an initial denaturation at 96°C for 2 min, followed by 35 cycles comprising 96°C for 20 s (denaturation), 59°C for 20 s (annealing), and 72°C for 30 s (extension). A concluding final extension occurred at 72°C for 7 min. For *Cryptosporidium* ssp. Home-designed primers in the current study were utilized: 5'-TCAGCTTTAGACGGTAGGGT-3' (forward) and 5'-TGGTTAAGACTACGACGGTATCT-3' (reverse), which amplified a 700 bp fragment, providing this (PX129240) accession number. The PCR protocol commenced at 94°C for 3 min (initial denaturation), followed by 35 cycles of 94°C for 30 s (denaturation), 58°C for 30 s (annealing), and 72°C for 30 s (extension), a and g extension phase at 72°C for 5 min. Each 20 μL PCR reaction comprised 10 μL of HotStarTaq DNA polymerase master mix buffer (Qiagen GmbH, Hilden, Germany), 1 μL of each primer, 5 μL of DNA template, and 3 μL of nuclease-free water. PCR products were separated using agarose gel electrophoresis on a 1.5% gel stained with ethidium bromide, run at 85 V for 40 min, and subsequently visualized using a UV transilluminator (Emisiko, et al., 2020).

DNA sequencing

For nucleotide sequencing, 15 μL of PCR product from each sample was transferred into 1.5 mL microcentrifuge tubes. After that, the samples were sent to Macrogen Inc. in Korea for sequencing using the Sanger method. The nucleotide sequences were submitted to the GenBank database and provided the following accession numbers

(PX088737) for *G. lamblia* and (PX129240, PX129241, PX129242, PX129243, PX129244, PX129245, PX129246, and PX022656) for *Cryptosporidium* ssp.

Phylogenetic analysis

The National Center for Biotechnology Information database and the BLASTn algorithm identified homologous sequences of *G. lamblia* and *Cryptosporidium* spp. from the obtained nucleotide sequences. The Clustal X version 2.1 (University of Dublin, Dublin, Ireland) was used to perform multiple sequence alignments, and then BioEdit version 7.2 (Informer Technologies, Los Angeles, CA, USA) was used to refine them. Then, phylogenetic trees were constructed in Molecular Evolutionary Genetics Analysis (MEGA) version 12 (MEGA, New York, NY) to examine the genetic similarity and differences between the isolates over time.

D. Statistical Analysis

Data were analyzed using GraphPad Prism software (version 9.5.1). A Chi-square test for independence was utilized to assess the correlation between the occurrence of parasites (*Cryptosporidium* ssp. and *G. lamblia*) and various vegetable types. Statistical significance was defined at $p < 0.05$, indicating a 95% confidence level.

III. Results

Microscopic examination was utilized to identify *G. lamblia* and *Cryptosporidium* spp. *Giardia* spp. were identified directly using iodine and methylene blue simple stains (Fig. 1), whereas *Cryptosporidium* spp. were found via modified Ziehl–Neelsen staining (Fig. 2). Among the 210 vegetable samples, 7.6% (16/210) were found to be contaminated with *G. lamblia*, whereas 35.7% (75/210) exhibited contamination with *Cryptosporidium* ssp. A contamination rate of 68.6% was recorded for both protozoan parasites in garden cress (*L. sativum*). Lettuce (*L. sativa*) and leek (*A. ampeloprasum*) exhibited the lowest contamination rates, with approximately 28.6% testing positive (Table I).

Cryptosporidium spp. was the most common parasite in all 210 vegetable samples, which included 105 from fields and 105 from markets. The study of the samples revealed that veggies from Erbil demonstrated a higher contamination rate than those from Sulaymaniyah. The field samples from Erbil exhibited a higher incidence of *Cryptosporidium* ssp. and *Giardia* ssp. compared to the other samples. Garden cress, lettuce, leeks, rocket, and spinach from the field always had a higher ratio of contamination; the difference was statistically significant ($p < 0.05$), whereas parsley showed the same level in both field and market samples. On the other hand, detection of *G. lamblia* was less frequent. It mostly showed up in field samples, and rockets had the most cases. There were also a few cases in market spinach, parsley, and garden cress, though the differences between the samples were not always statistically significant ($p > 0.05$ for some vegetables). In general, the results show that vegetables from the field had more protozoan contamination than those from markets (Fig. 3). Microscopically, a greater number of positive *Cryptosporidium ssp*. were detected in samples by PCR (Table II). The microscopic examination method detected a higher contamination rate of 40.5% (85/210), whereas the contamination rate of vegetables with *Cryptosporidium* spp. was 35.7% (75/210). Both methods yielded the same results for *G. lamblia*, with 7.6% (16/210) positive.

Gel electrophoresis demonstrated the amplification of the *18S rRNA* gene in both *Giardia* and *Cryptosporidium*, revealing distinct bands at anticipated sizes: Approximately 700 bp for *Cryptosporidium* (Fig. 4) and 292 bp for *Giardia* (Fig. 5). The sequences and phylogenetic analysis of PCR-amplified *18S rRNA* gene fragments revealed zoonotic genotypes – *Cryptosporidium parvum* (Fig. 6) and *Giardia Assemblages A* and *B* (Fig. 7). A sequence similarity exceeding 98% with established strains capable of infecting humans indicates potential contamination of the sample

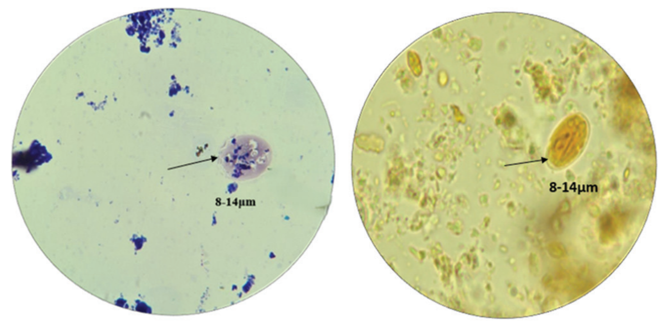

Fig. 1. Microscopic analysis reveals *Giardia lamblia* cysts (a: Cyst stained with methylene blue, b: Cyst stained with iodine) at ×100.

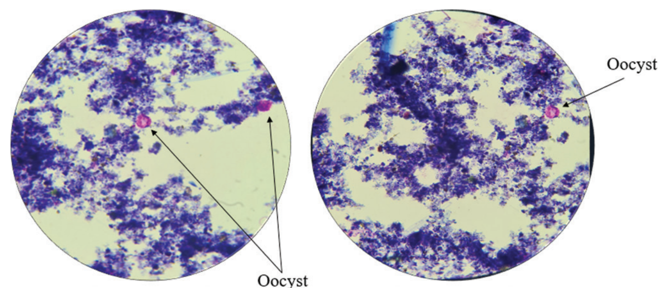

Fig. 2. A *Cryptosporidium* ssp. oocyst stained with ZN stain (acid-fast stain) ×100.

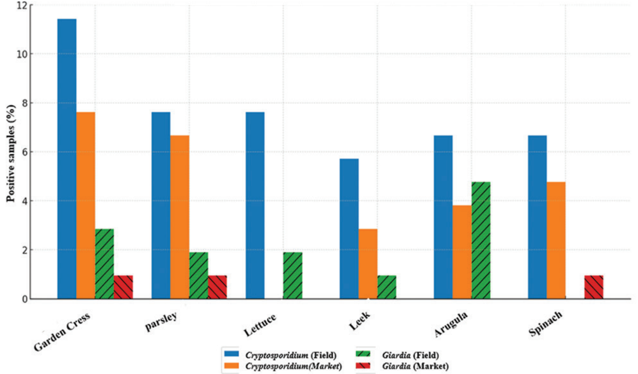

Fig. 3. The contamination rate of the vegetable with *Cryptosporidium* ssp. and *Giardia lamblia* from the field and the market.

TABLE I
The Contamination Rate of Each Parasite in the Different Vegetable Type n=210

Vegetable parasite		Garden cress n=35	Parsley n=35	Leek n=35	Lettuce n=35	Spinach n=35	Arugula n=35	Total n=210	Statistical analysis
Cryptosporidium spp.	N0.	20	15	9	8	12	11	75	$\chi^2 \approx 14.3$
	Percentage	57.1	42.9	25.7	22.9	34.3	31.4	35.7	p=0.011
Giardia lamblia	N0.	4	3	1	2	1	5	16	$\chi^2 \approx 5.9$
	Percentage	11.4	8.6	2.9	5.7	2.9	14.3	7.6	p=0.309
Total	N0.	24	18	10	10	13	16	91	
	Percentage	68.6	51.4	28.6	28.6	37.1	45.7	43.3	

TABLE II
Comparison of Microscopic and PCR Methods for Detecting *Cryptosporidium* spp. and *Giardia lamblia* in Vegetable Samples

Method	*Cryptosporidium* spp.		*Giardia lamblia*		Total contamination rate		Statistical analysis
	No.	Percentage	No.	Percentage	No.	Percentage	
Microscopic examination	85	40.5	16	7.6	101	48.1	$X^2 \approx 60.28$ p=0.001
PCR	75	35.7	16	7.6	91	43.3	$X^2 \approx 0.017$ p=0.89

PCR: Polymerase chain reaction

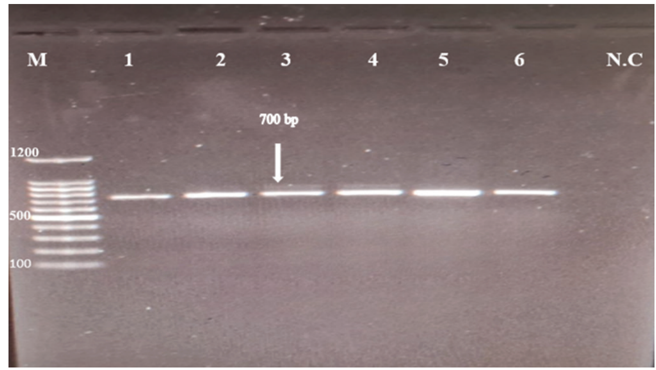

Fig. 4. Agarose gel electrophoresis of the polymerase chain reaction product of the *SSUrRNA* gene of *Cryptosporidium* spp. in vegetable samples. M=DNA ladder (100bp scale), 1–6: Samples, NC: Negative control.

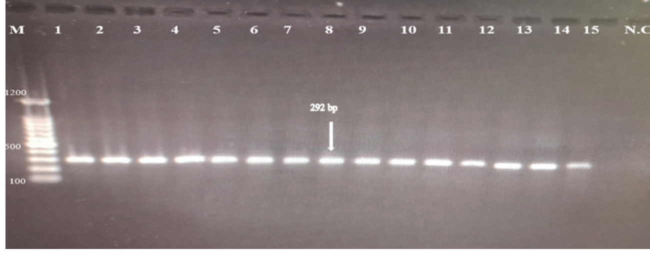

Fig. 5. The gel-electrophoresis of polymerase chain reaction of the *18srRNA* gene of *Giardia lamblia* on agarose (1.5% agarose). M=DNA ladder (100bp scale). 1–15: Samples, NC: Negative control.

by humans or animals. Phylogenetic analysis categorized local isolates within the same clade as zoonotic genotypes previously documented in India, China, and Iran (Das, Deka, and Lalrinkima, 2022; Jiang, et al., 2023; Mahdavi, Dalimi, and Far, 2024).

IV. Discussion

Intestinal protozoal infection is a prevalent food and waterborne disease globally. It is significantly endemic in Iraq owing to conducive weather and filthy conditions that promote food and water contamination. This study examines the total prevalence was 43.3%, which indicates that the contamination rate of vegetables was exceedingly high, corroborated by many investigations conducted in different regions (Al-Mozan and Dakhil, 2019). Contrary to our findings, previous investigations indicated that the contamination rates of several vegetables with intestinal parasites were lower than those seen in the current study in Kurdistan and Iraq (Latif, Al-Talib, and Al-Akely, 2020; Abdullah, 2021). The steady detection rates for *G. lamblia* imply that these constraints had a minimal impact on its identification in this study. Positive cases for *Cryptosporidium* spp. were detected in 75 (35.7%) of the 210 vegetable samples, and *G. lamblia* in 16 (7.6%). Significant disparities were seen in the contamination of *Cryptosporidium* spp. and *G. lamblia* across several vegetable types ($p < 0.05$), with certain varieties exhibiting elevated contamination levels. This significant disparity may be attributed to factors such as the surface morphology, cultivation methods, or post-harvest handling practices. Vegetables with higher contamination levels are more likely to transmit protozoa to humans. Garden cress showed the highest contamination rate, with 20 out of 35 (57.1%) samples testing positive for *Cryptosporidium* ssp. The overall positivity rate was 68.6% when both parasites were analyzed together. Arugula had the highest number of *G. lamblia* cases (14.3%). These numbers are higher than those from other parts of Iraq. Only 10.2% of vegetables in Duhok were contaminated, and 4.2% of them tested positive for *G. lamblia* by microscopy. (Sleman Ali, et al., 2018; Abdullah, 2021) found that 6.5% of the oocysts in raw vegetables in Koya were *Cryptosporidium*. According to a survey by Soran, 48.4% of the samples were contaminated, with cress (*L. sativum*) being the most positive (71.1%) (Mirzaei, et al., 2021). Contamination levels in Mosul varied from 26% to 39%, with the presence of both *Cryptosporidium* and *Giardia*. These results indicate that leafy greens, particularly garden cress, are highly vulnerable. The discrepancies in prevalence rates of certain harmful intestinal parasites from fresh vegetables noted in this study and those documented by previously cited researchers. Various variables may account for these discrepancies. Factors such as geographical location, type and number of

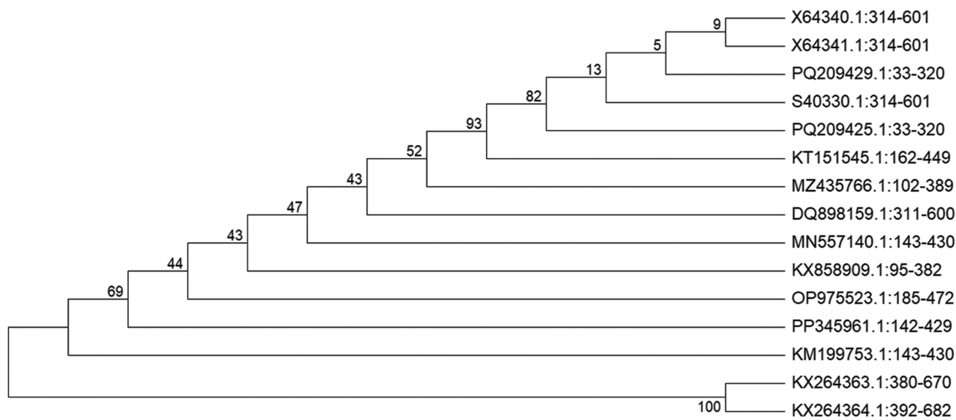

Fig. 6. Illustrates the evolutionary relationships between the SSU rRNA sequences of the *Cryptosporidium parvum* isolates identified in this investigation and a few isolates from GenBank using the neighbor-joining method.

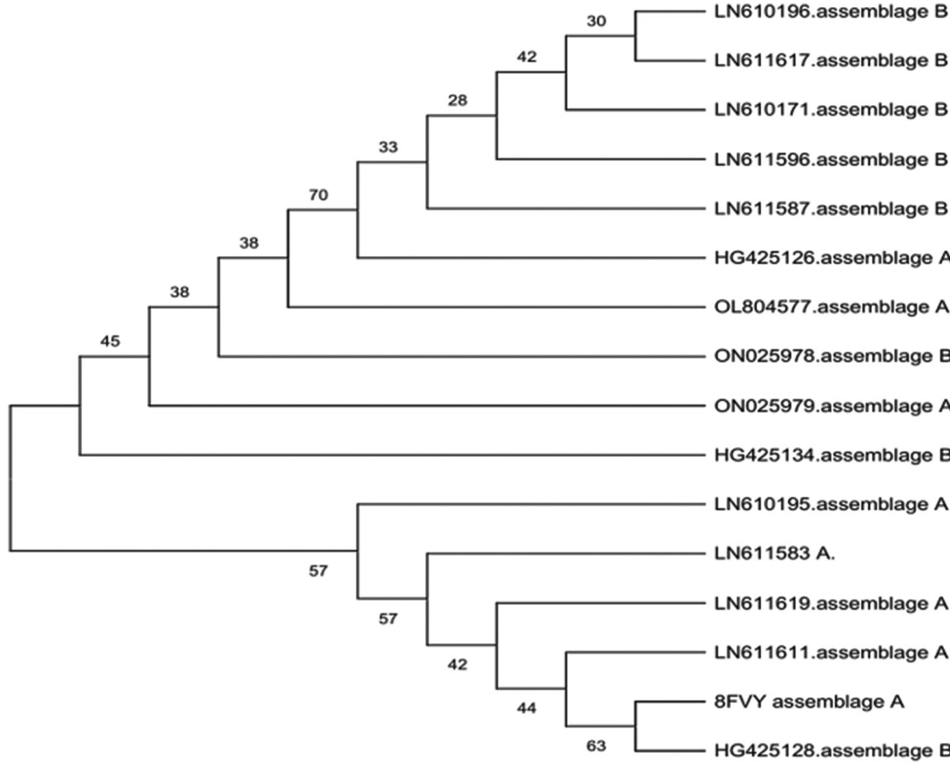

Fig. 7. Illustrates the evolutionary relationships between the SSU rRNA sequences of the *Giardia* Assemblage A and B isolates identified in this investigation and a few isolates from GenBank using the neighbor-joining method.

samples examined, methods employed for detecting intestinal parasites, the type of water utilized for irrigation, post-harvest handling techniques of vegetables, and the water used for washing vegetables can significantly influence the epidemiology of parasitic disease transmission (Hama, et al., 2022). Less contamination has been reported around the world. In Spain, 9.2% of leafy greens were contaminated with *Cryptosporidium* (Trelis, et al., 2022), and in China, 15.6% of vegetables were found to be contaminated (Li, et al., 2020). An Iranian study employing metagenomics and qPCR identified fewer than 15% of individuals infected with *Cryptosporidium* (Naushad, et al., 2025), in contrast (Nguyen, et al., 2023) reported *Giardia* and *Cryptosporidium* in under 20% of food and environmental samples from Vietnam. The elevated prevalence observed in the current study is likely attributable to the utilization of untreated sewage and river water for irrigation, in addition to the application of organic fertilizers. The occurrence of *G. lamblia* (7.6%) in this study is comparable to findings from China (10.9%) and Spain (5.3%) (Li, et al., 2020; Trelis, et al., 2022). The continued presence of *Giardia* cysts on leafy vegetables is a health concern, as they are resistant to numerous disinfectants and can lead to prolonged gastrointestinal illness (Barrera, et al., 2024). Microscopy identified an increased number of *Cryptosporidium*-positive samples in comparison to PCR, a discrepancy resulting from the intrinsic methodological

characteristics of each technique. The identification of oocyst morphological characteristics using microscopy following modified ZN staining is the standard method for diagnosing cryptosporidiosis; nevertheless, this technique is labor-intensive, less sensitive, and hence susceptible to errors. *Cryptosporidium* oocysts are diminutive and can therefore be readily misidentified in stool waste as artifacts. Furthermore, they may be readily mistaken for other oocysts, including those of *Cyclospora* species and yeast cells (Omoruyi, et al., 2014). On the other hand, PCR requires nucleic acids that are intact and of high quality. These nucleic acids can be compromised by environmental factors, prolonged storage in ethanol, or improper handling, particularly in samples with low parasite loads. Polysaccharides, phenolics, and residual ethanol are also present in plant matrices, which inhibit DNA polymerase activity (Naushad, et al., 2025). Some commercial DNA extraction kits exacerbate this problem because they fail to effectively break down the tough walls of *Cryptosporidium* oocysts, resulting in a lower DNA extraction yield (Valeix, et al., 2020). These factors together explain why microscopy found more positives than PCR. The *SSU rRNA* gene's effective amplification confirms that PCR-based methods allow for the sensitive detection of *G. lamblia* and *Cryptosporidium* spp. in fresh vegetables, even at low contamination levels. The high positive rate highlights how crucial it is for public health to prevent the spread of protozoa through raw vegetables, particularly in areas with poor irrigation or washing practices. Identifying zoonotic genotypes (*C. parvum* and *Giardia assemblages A* and *B*) increases the risk of foodborne transmission even further. Their resemblance to strains documented in China, India, and Iran indicates contamination sources associated with livestock waste and untreated wastewater (Xu, et al., 2022; Franco Abuín, et al., 2023).

V. Conclusion

The current study findings may significantly influence food safety protocols and clearly identify vegetable risk factors for public health related to the spread of *Cryptosporidium* ssp. and *G. lamblia*. Vegetables gathered from markets were usually less contaminated than those grown in fields, underscoring the importance of soil contact, irrigation water, and cultivation methods as primary pathways for protozoan transmission.

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