Online ISSN: 2588-3526

Journal of Wildlife and Biodiversity

Volume 9(3): 237-261 (2025) (http://www.wildlife-biodiversity.com/)

Research Article

Diversity of *Giardia intestinalis*: Comparative evaluation of AI stool analysis, traditional techniques, and molecular phylogeny

Lana Othman Kanabe[1*], Samir Jawdat Bilal[2]

[1]Department of Biology, College of Education, Salahaddin University-Erbil, Iraq
[1]Department of Biology, College of Education, Research Center, Salahaddin University-Erbil, Iraq
*Email: lana.kanabe2@gmail.com

Received: 11 August 2025 / Revised: 05 October 2025 / Accepted: 14 October 2025/ Published online: 14 October 2025.
How to cite: Kanabe, L.O.., Bilal, S.J. (2025). Diversity of Giardia intestinalis: Comparative evaluation of AI stool analysis, traditional techniques, and molecular phylogeny, Journal of Wildlife and Biodiversity, 9(3), 237-261. DOI: https://doi.org/10.5281/zenodo.17386580

Abstract

Giardia intestinalis is one of the most prevalent intestinal protozoan parasites causing Giardiasis, a disease that infects diverse people of any age and is characterized by abdominal cramps, diarrhea, and weight loss. Although traditional diagnostic techniques are still widespread, however, newer AI and molecular technologies have much to offer in the way of more precise and quicker detection. This research aimed to determine the prevalence of Giardiasis among patients presenting to the Public Health Laboratory in Erbil, Iraq, between January–December 2024, and to create a comparison of the effectiveness of traditional, immunological, molecular, and AI-based diagnostic techniques. A total of 25,460 stool specimens were first screened by microscopic direct wet mount. Positives were further analyzed with trichrome and acid-fast stains for morphological identification. Immunochromatographic assays and the KU-F600 AI-based automatic fecal analyzer were utilized. Molecular detection by real-time PCR and standard DNA sequencing was utilized for *G. intestinalis* infection confirmation. The prevalence of *G. intestinalis* was evaluated using direct wet mount, revealing 290 (1.14%) positive cases. The monthly gender-based diversity index with giardiasis was low (<0.5), indicating that the infections with males are dominant in each month. Immunochromatographic and AI-based methods markedly improved diagnostic speed and ease compared to conventional microscopy. Molecular techniques demonstrated the highest accuracy in detecting *G. intestinalis*. Although traditional microscopy remains a useful screening tool, it is less reliable than molecular and AI-based methods. The KU-F600 AI analyzer exhibited strong potential for rapid and accurate diagnosis. Further research is recommended to validate the broader application of AI technologies in parasitological diagnostics.

Keywords: AI stool analyzer; Molecular diagnostics; Conventional techniques

Introduction

Giardia intestinalis is a protozoan parasite responsible for Giardiasis, an intestinal infection with global distribution. This disease distribution is particularly prevalent in low-income countries and is characterized by symptoms such as diarrhea, bloating, and abdominal discomfort. Giardiasis frequently affects travelers, hikers in remote areas, and caregivers of young children. In certain situations, the infection leads to severe dehydration (Ryan et al., 2019). In Iraq, Giardiasis is an endemic illness, whose infection rates are significantly different in different areas. For instance, research done in Dohuk City stated a prevalence of 38.5% among children (Al-Saeed & Issa, 2006). However, others have reported considerably low infection rates of 0.86% and 1.04% in various populations (Zaki & Dhubyan, 2022). These differences highlight the uneven distribution of giardiasis in Iraq and emphasize its ongoing status as a public health problem. Consequently, identification of geographic 'hotspots' is critical for control and prevention strategies. Intestinal parasitic infection is a serious public health problem worldwide, especially where sanitation is poor and the health infrastructure is weak. Precise and timely diagnosis is required for the treatment, epidemiological surveillance, and application of control interventions (Reena Leeba Richard & Yusof, 2018). Diagnostic technology for intestinal parasites, in terms of sensitivity, specificity, and detection speed, has significantly advanced during the last decade (Soares et al., 2020).

Historically, Giardiasis was diagnosed using direct wet mount microscopy and antigen-antibody reaction tests. Although they are still widely used as standard methods, molecular biology, DNA sequencing, AI (artificial intelligence)-based analyzer, and imaging technologies have revolutionized diagnosis (Shetty et al., 2021; Soares et al., 2020). Molecular testing, like real-time PCR, provides higher sensitivity and specificity with the capability of specific detection of *G. intestinalis* even in low-intensity infections (Alharbi et al., 2020; Parčina et al., 2017). In parallel, the development of rapid diagnostic techniques is crucial, especially when modified for point-of-care application and designed with simple protocols that are amenable to the majority of clinical laboratory environments, especially in low-income countries (Hobbs et al., 2021). The combination of AI with diagnostic computer programs has enhanced diagnostic dependability and simplified the method of measurement and interpretation of data (Gurgitano et al., 2021).

One notable development is the AI-based stool analyzer, which has shown promising performance with high sensitivity and specificity in detecting *G. intestinalis* (Rosado et al., 2017). In comparison, molecular real-time PCR also demonstrated excellent diagnostic accuracy, whereas traditional microscopy exhibited lower sensitivity, especially in cases of low-intensity infections (Pritt, 2015;

Wambani & Okoth, 2022). The automated AI-based stool analyzer could be a valuable tool for rapid and reliable diagnosis of Giardiasis, potentially overcoming some of the limitations of traditional methods (Rosado et al., 2017). It offers rapid and reliable results, making it a preferred choice in clinical settings (Shin et al., 2018; Won et al., 2016; Zhang et al., 2022). While AI-based analyzers are promising, more research is needed to establish their efficacy compared to established molecular techniques. The objectives of this study were to assess the performance of diagnostic methods and link the proficiency of AI-based computerized stool examination with routine microscopic and molecular real-time PCR, as well as immunochromatographic methods for identifying *G. intestinalis.*

Materials and Methods

Sampling area and sample collection

The study was conducted on 25,460 visitors attending Public Health Laboratory-Erbil, Iraq over one year (January - December 2024), including both males and females. The ages ranged from 5 to over 45 years. Basic demographic and clinical information were collected from each participant using a structured questionnaire. Stool samples were collected from the visitors in a clean, disposable plastic container with tightly fitting screw caps without preservative and stored at -20°C. Samples from individuals undergoing anti-diarrheal treatment were excluded. Each container was labeled with a unique number, age, gender, date, and participant's name.

Diagnostic Techniques

The stool samples were examined macroscopically with the naked eyes to assess the consistency, color, mucus or blood in the stool samples. For identifying *G. intestinalis* trophozoite and cyst stages the following laboratory methods were employed:

Microscopic examination

Direct wet-mount examination

Fresh stool samples were examined under a light microscope using the saline (0.85%) and iodine (Lugol's) wet mount techniques to detect different stages of different parasite species as described by Zaman et al. (2017).

Staining techniques (Trichrome and Modified Acid-Fast)

All positive stool samples were smeared onto two slides, then fixed and preserved with Polyvinyl Alcohol (PVA)-Schaudinn and methanol solution. The former one stained with trichrome technique and the later one was stained with modified acid-fast stain for detecting diagnostic stages of *G. intestinalis*.

Immunochromatographic (IC) assay

The Biozek parasite combo3 (*Crypto*+*Giardia*+*Entamoeba*) test is based on the principle of a qualitative immunochromatic assay for the determination of *Cryptosporidium*, *Giardia*, and *Entamoeba* (*histolytica* and *dispar*) in stool samples. Test kit, stool samples, and controls were allowed to reach room temperature (15-30˚C) prior to testing. After that, with a wax pencil, the samples were labeled and examined.

Automated AI-Based Stool Analyzer (KU-F600)

An automated AI-based stool analyzer was used in this study. Homogenized stool samples were loaded into the system and analyzed using the pre-programmed *Giardia* detection mode. It delivered high-resolution images and AI-predicted *Giardia* positivity that may be beneficial in the interpretation of results. The KU-F600 auto-feces analyzer (KUBO Technology Co., Ltd., China) was employed to analyze the stool sample. Stool samples were taken in fully sealed sampling cups to safety and to avoid contamination. Samples were diluted according to the analyzer specifications. The analyzer was operated at a temperature of 2-40°C. The prepared samples were fed into the analyzer. Single-sample loading of the KU-F600 permitted efficient processing. The analysis cycle was commenced. The KU-F600 used a combination of high-powered and low-powered microscopy and AI identification based on deep learning algorithms to identify helminth eggs, stages of the protozoans, and other formed elements in the stool samples. After analysis, the results were reviewed as given by the analyzer. High-resolution imaging provided detailed visualization images that allowed diagnosis to be accurate. The analyzer was then serviced and washed as per the manufacturer's instructions to provide maximum performance.

Molecular characterization of *G. intestinalis*

Genomic DNA extraction

Genomic DNA was extracted from 45 *G. intestinalis* positive and 15 negative stool samples using the SORB-B nucleic acid extraction kit. Washed stool samples were processed following the manufacture's protocol involving lysis, interaction with sorbent, repeated wash, and elution of DNA. The procedure included incubation, vortexing, and centrifugation to ensure DNA purification. The purified DNA was kept at -20°C for subsequent use.

Real-Time Polymerase chain reaction (qPCR) and DNA amplification

Extracted parasite DNA was analyzed using Bosphore Gastroenteritis Panel Kit v3 (Real-Time PCR Kit, Anatolia Geneworks). About 5 µl of DNA template was added to 20 µl of PCR master mix.

Conventional PCR Process

The PCR process was carried out for the target *β-giardin* gene**.** Forward *β-giardin* sequence was 5'-GCGAGGAGGTCAAGAAGTC-3' (F: 19 mer), and reverse sequence was 5'-GAGCGTGTTGACGATCTTGT-3' (R: 20 mer). For each sample, 10µl of PCR master mix was dispensed into a 0.2 ml PCR tube, then 1µl of each primer, and then 3 µl of the sample (template DNA) was added, and 5µl of dH_2O to complete the volume of the PCR mixture, which was 20 µl, and placed it in the thermocycler machine. The PCR amplification was performed under the following cycling conditions (Eppendorf master cycle, Germany). The cycling profile consists of an initial denaturation of 5 minutes at 94˚C followed by 35 cycles, 30 seconds at 94˚C, 30 seconds at 58˚C, 30 seconds at 63˚C and final extension 10 minutes at 72˚C.

Post-PCR process

The quality of genomic DNA (6 µl PCR product) was assessed by determining DNA extracts on 2.0% agarose gel electrophoresis with a power supply condition (120V for 40 minutes) (Biomax, European Economic Community). Bands stained with ethidium bromide (1.75 µl) and visualized under a UV transilluminator using GeneReguler 50 bp DNA ladder (Thermo Scientific, Germany). The 100bp ladder was supplied in a ready-to-use format containing the fluorescent DNA stain and tracking dyes. The expected size of the PCR amplicon was 450 bp.

DNA sequencing for *G. intestinalis*

Post PCR Cleaning

After the PCR process, there was a post-PCR clean up from the reaction mixtures so that those unincorporated primers and dNTPs wouldn't interfere with the results. The magnetic bead purification was used to isolate the purified DNA only. About 20 µl of PCR product was added to magnetic beads, and well mixed, then put the tubes in the magnetic separator until all beads were collected at the magnetic side of the tube and the solution became clear. The beads were washed by adding 200 µl of 80% ethanol (this step was repeated twice), and after the second wash, the ethanol was completely discarded. Air dried for 15 min. Then 22.5 µl of nuclease-free water was added and mixed by pipette, then the tube was returned to the magnetic separator for one min. until the solution became clear. Finally, 20 µl of purified PCR product was transferred into a new tube.

Cycle Sequencing

A partial region of *β-giardin* gene was amplified by PCR using forward and reverse primers, designed to specifically amplify *G. intestinalis*. For DNA sequencing, the ABI 3130X genetic analyzer (SINGAPORE) was used to find the nucleotide order of *β-giardin* gene*.* The PCR fragments of the parasite were excised from the agarose gel and used as a source of DNA template for sequence-specific

PCR amplification and sent to the private Exogene Laboratory, Zhene International Hospital, Erbil-Iraq.

Molecular Phylogeny

Phylogenetic tree

The phylogenetic tree was constructed using MEGA11 software based on the maximum-likelihood (ML) approach, and the query sequences were acquired from NCBI BLASTN. The sequence of *Cryptosporidium parvum* (G35341.1) was included as an outgroup.

Photomicrograph and Software

The Samsung Galaxy S24 Ultra was used to capture a photomicrograph. The stages of *G. intestinalis* were measured by applying the ImageJ software.

Diversity Indices

To measure the parasite diversity based on the months of the study, both Shannon (H) and Simpson € indices were calculated based on Roswell et al. (2021)

Sensitivity and Specificity of the diagnostic techniques

To evaluate the diagnostic performance of various detection methods for *G. intestinalis*, sensitivity and specificity were calculated by comparing test results against established reference standards. The results were compared to the direct wet mount to determine its detection accuracy. These measurements were employed in determining each technique's diagnostic validity for the diagnosis of *G. intestinalis*. Sensitivity and specificity were calculated using the following equations:

Sensitivity = True Positives / (True Positives + False Negatives)

Specificity = True Negatives / (True Negatives + False Positives)

Statistical analysis

Data analysis was carried out using Statistical Package for Social Sciences (SPSS, version 21) and Graph Pad-Prism (version 10.0). The Chi-Square (X^2) test and one-way ANOVA test were used to analyze the data. The results were expressed as P< 0.05 was considered statistically significant.

Results

Direct Microscopic Examination

The results of the present study demonstrate the prevalence of intestinal parasites among individuals attending the Public Health Laboratory in Erbil. A total of 25,460 stool samples were microscopically examined, revealing a positivity rate of 4.65% for a diverse group of parasitic species. Notably, *G. intestinalis* was detected in 290 cases, accounting for 1.14% of the total examined samples. The microscopic examination using direct wet mount (both saline and iodine preparations) proved to be an effective initial diagnostic tool for detecting *Giardia* trophozoites and cysts **(Fig. 1)**.

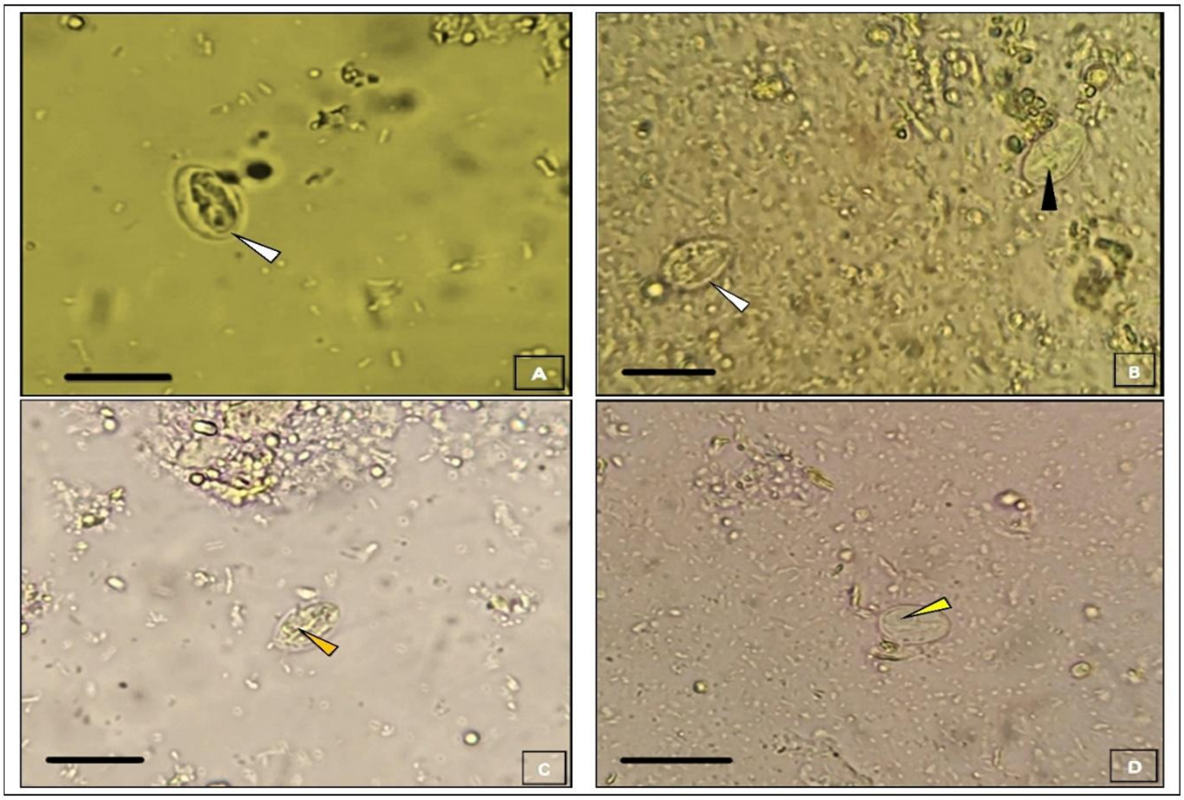

Figure 1. Wet-Mount preparations of *G. intestinalis* cystic stage (A and B representing Iodine-wet mount, C and D representing Saline-wet mount) (Magnification Power: 400X; scale-bar = 10 µm) (White-arrows: *G. intestinalis* cystic stage; Black-arrow: Median body; Orange-arrow: Axostyle, and Yellow-arrow: Nucleus)

The application of permanent staining techniques, including trichrome and acid-fast staining, further enhanced the confirmation of positive cases **(Fig. 2)**. Technically, it was confirmed that in the most positive cases with *G. intestinalis*, the stool consistency in parallel with the presence of the parasites, appeared as fatty diarrhea (*i.e.,* steatorrhea). The phenomenon was clearly reported in **Figure 3).**

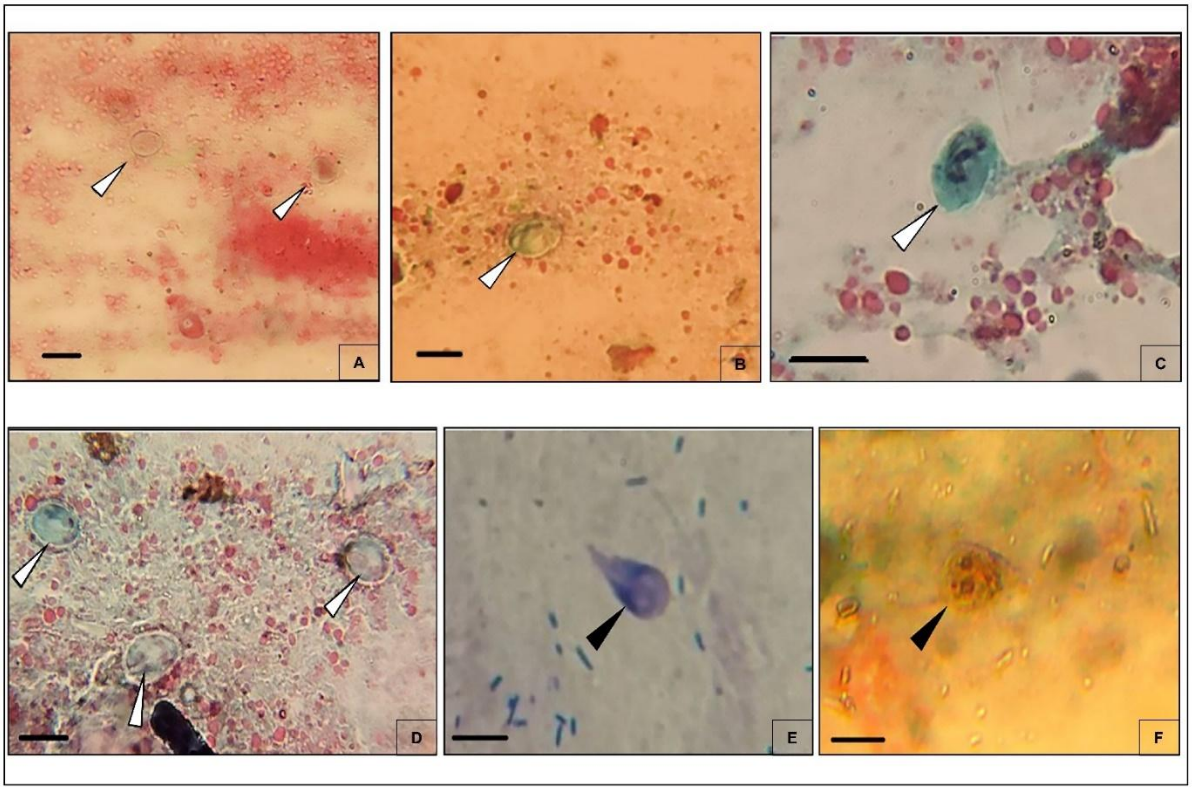

Figure (2): Stool staining smears with *G. intestinalis* cystic (White arrows: A-D) and trophozoite (Black arrows: E and F) stages (A, B and E representing Acid-Fast staining, C, D and F representing Trichrome staining) (Magnification power: 400X; scale-bare = 10 µm).

Fig. (3): Photomicrograph of direct stool smear with the presence of *G. intestinalis* cystic stage and different sized refractile round fat droplets arround the hair artifact (Magnification Power: 400X).
A: Representg refractile round-dodies fat droplets surrounding hair artifact.
B: Representing the presence of *G. intestinalis* cystic stage after a small fine microscopic focussing beside the fat droplets and hair artifact.
(White-arrows: *G. intestinalis* cyst; Yellow-arrows: Fat droplets; Black-arrows: Hair artifact).

Seasonal variations in *G. intestinalis* infection rates were observed in the current study, with the highest infection rate recorded in March 2024 (2.20%), and the lowest in May 2024 (0.82%).

Additionally, gender-related diversity indices were noted, as male visitors exhibited a higher infection rate (1.03%) compared to female visitors (0.11%), as shown in **Table (1)**.

Table 1: Monthly Shannon and Simpson gender-based diversity indices with *G. intestinalis* positivity among examined male and female individuals during 2024

Months (2024)	Examined No.	Positive No.	*G. intestinalis*		Infected male visitors		Infected female visitors		Shannon diversity index (H)	Simpson diversity index (D)
			+Ve No.	%	No.	%	No.	%		
January	817	62	12	1.47	10	1.22	2	0.24	0.500	0.278
February	1812	95	19	1.05	16	0.88	3	0.17	0.426	0.239
March	1180	89	26	2.20	25	2.12	1	0.08	0.145	0.074
April	1753	49	16	0.91	14	0.80	2	0.11	0.337	0.156
May	3405	98	28	0.82	24	0.70	4	0.12	0.367	0.167
June	1644	83	17	1.03	15	0.91	2	0.12	0.328	0.146
July	1667	89	21	1.26	18	1.08	3	0.18	0.376	0.181
August	2603	129	27	1.04	26	1.00	1	0.04	0.122	0.069
September	2853	138	30	1.05	27	0.95	3	0.11	0.286	0.150
October	2511	103	38	1.51	36	1.43	2	0.08	0.191	0.102
November	2410	101	25	1.04	23	0.95	2	0.08	0.237	0.117
December	2805	149	31	1.10	29	1.03	2	0.07	0.189	0.099
Total	**25,460**	**1185**	**290**	**1.14**	**263**	**1.03**	**27**	**0.11**		

*Chi-square= 20.21; P-value = 0.042

Overall monthly Shannon gender-based diversity index with giardiasis was low (<0.5), denoting those infections with males are dominate compared to females in each month. The analysis of diversity of *G. intestinalis* infections among male and female visitors in 2024 showed that the diversity value was consistently low. Shannon diversity index (H) per month was between 0.12 and 0.50 and the Simpson diversity index (D) was between 0.07 and 0.28. These are significantly lower than what should have been in balanced distributions, and show that infection in males was unevenly distributed during the study period, reciprocating in favor of the female. The low Shannon values indicate that the distribution was very skewed, that is, the distribution was dominated by one category (male infections). In the same way, the low Simpson index supports the fact that there was a low chance of randomly choosing two members of different gender groups of infections, yet once again male dominance is depicted. Despite the slight differences, the trend of the monthly indices was similar; that is, males were contributing more than 85-90 % of the overall number of positive cases, and females were a minor percentage. The highest diversity values were seen in January (H' = 0.50; D = 0.28), and February (H' = 0.43; D = 0.24), when female infections were relatively more. Conversely, maximum diversity was recorded in March (H' = 0.15; D = 0.07), August (H' = 0.12; D = 0.07), and October (H' = 0.19; D = 0.10) when few females were infected compared to males.

Gender-wise distribution indicated a significantly higher prevalence in males (90.69%) compared to females (9.31%). Monthly trends demonstrated fluctuations in infection rates, with the highest number of cases recorded in October (38 cases, 1.51%), whereas the lowest number was observed in January (12 cases, 0.49%). A seasonal pattern was evident, with a gradual increase in cases from March to October, peaking in October, followed by a decline in November and December. Statistical analysis using the Chi-square test assessed the association between gender and *G. intestinalis* infection. The results yielded a Chi-square value of 20.21 with a p-value of 0.042, indicating a statistically significant relationship between gender and infection rates. This suggests that males were significantly more affected than females. Additionally, an ANOVA test was conducted to evaluate variations in infection rates across different months. Age-related differences in *Giardia* infections were also observed. Age-wise analysis showed that the majority of infections occurred in individuals aged 15-44 years (83.79%), followed by those ≥45 years (6.55%) and 10-14 years (1.38%). The highest number of positive cases was reported among individuals aged 15-44 years, with 238 cases in males and 25 cases in females. In the 45 years and older group, only 21 male cases were recorded. Meanwhile, among the 10-14 years age group, only 4 males and 2 females tested positive for the parasite (**Table 2**).

Table (2): Monthly distribution of *G. intestinalis* infections by age and gender during 2024

Months (2024)	Examined No.	Positive No.	*G. intestinalis*				
			Age (Y)	Male		Female	
				No.	%	No.	%
January	817	12	15-44	10	1.22	2	0.24
February	1812	19	15-44	15	0.83	3	0.17
			>45	1	0.06	0	0.00
March	1180	26	15-44	23	1.95	1	0.08
			>45	2	0.17	0	0.00
April	1753	16	10-14	2	0.11	0	0.00
			15-44	10	0.57	2	0.11
			>45	2	0.11	0	0.00
May	3405	28	10-14	0	0.00	2	0.06
			15-44	20	0.59	2	0.06
			>45	4	0.12	0	0.00
June	1644	17	10-14	1	0.06	0	0.00
			15-44	14	0.85	2	0.12
July	1667	21	15-44	17	1.02	3	0.18
			>45	1	0.06	0	0.00
August	2603	27	15-44	25	0.96	1	0.04
			>45	1	0.04	0	0.00
September	2853	30	15-44	24	0.84	3	0.11
			>45	3	0.11	0	0.00
October	2511	38	15-44	34	1.35	2	0.08
			>45	2	0.08	0	0.00
November	2410	25	15-44	21	0.87	2	0.08
			>45	2	0.08	0	0.00
December	2805	31	10-14	1	0.04	0	0.00
			15-44	25	0.89	2	0.07
			>45	3	0.11	0	0.00
Total	**25,460**	**290**	**263**		**1.03**	**27**	**0.11**

Immunochromatographic (IC) assay

For confirmation and comparison among different diagnostic techniques, 60 positive and 20 negative stool samples identified with direct microscopic examination were randomly selected and subjected to immunochromatographic (IC) assay. The results demonstrated that 56 out of 60 microscopically positive samples were confirmed positive, while 3 out of 20 negative samples also tested positive **(Fig. 4)**. Over all 59 out of 80 samples were identified as positive using IC technique. These findings suggest that IC assays offer approximately similar sensitivity in detecting *Giardia* infections compared to direct microscopy.

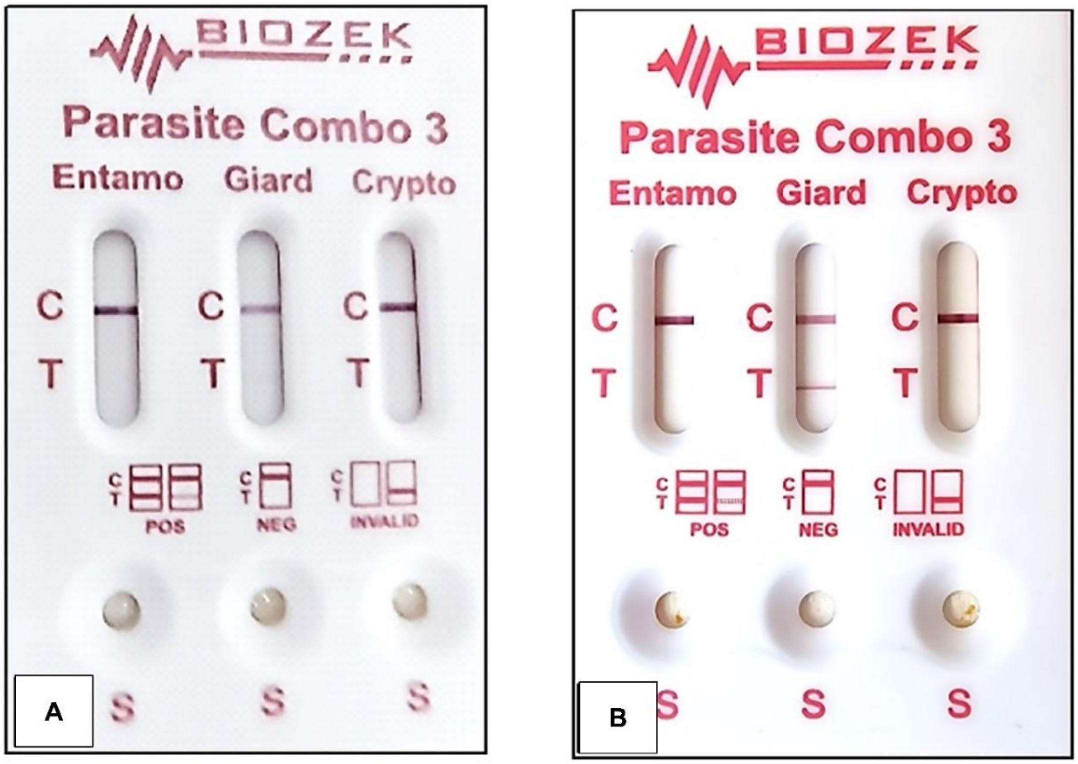

Figure (4): Immunochromatographic (IC) rapid test (BIOZEK parasite Combo 3) for diagnosing *Entamoeba histolytica*, *G. intestinalis*, and *Cryptosporidium* antigens in a stool sample. The presence of C-line (Control) confirms the test validity, whereas the T-line (Test) represents a positive result.
A: Representg a negative result for all parasites (line at C only).
B: Representing the positive result for *G. intestinalis* (two lines at C and T).

Automated AI-based stool analyzer (KU-F600)

The recent advancement of automated stool analyzers incorporating AI-assisted detection has significantly improved the efficiency and accuracy of parasitic identification. These systems rely on optical analysis combined with AI to rapidly identify and count stages of *G. intestinalis* in stool samples. In the current study, the KF-U600 stool analyzer was used to confirm the findings of *G. intestinalis*. A total of 15 positive and 10 negative stool samples (as control) identified using direct wet mount were subjected to AI-based analysis. The results demonstrated that 16 out of 25 samples were positive for *G. intestinalis*, including all 15 previously identified positive samples and one of the negative control samples. The parasite stages can be captured and analyzed efficiently, which depends on the optical analysis and AI combination to increase the speed of finding process **(Fig. 5)**. This indicates that the AI-assisted system exhibits high reliability in detecting *G. intestinalis*, although the single false-positive case suggests the need for further refinement and validation of the method.

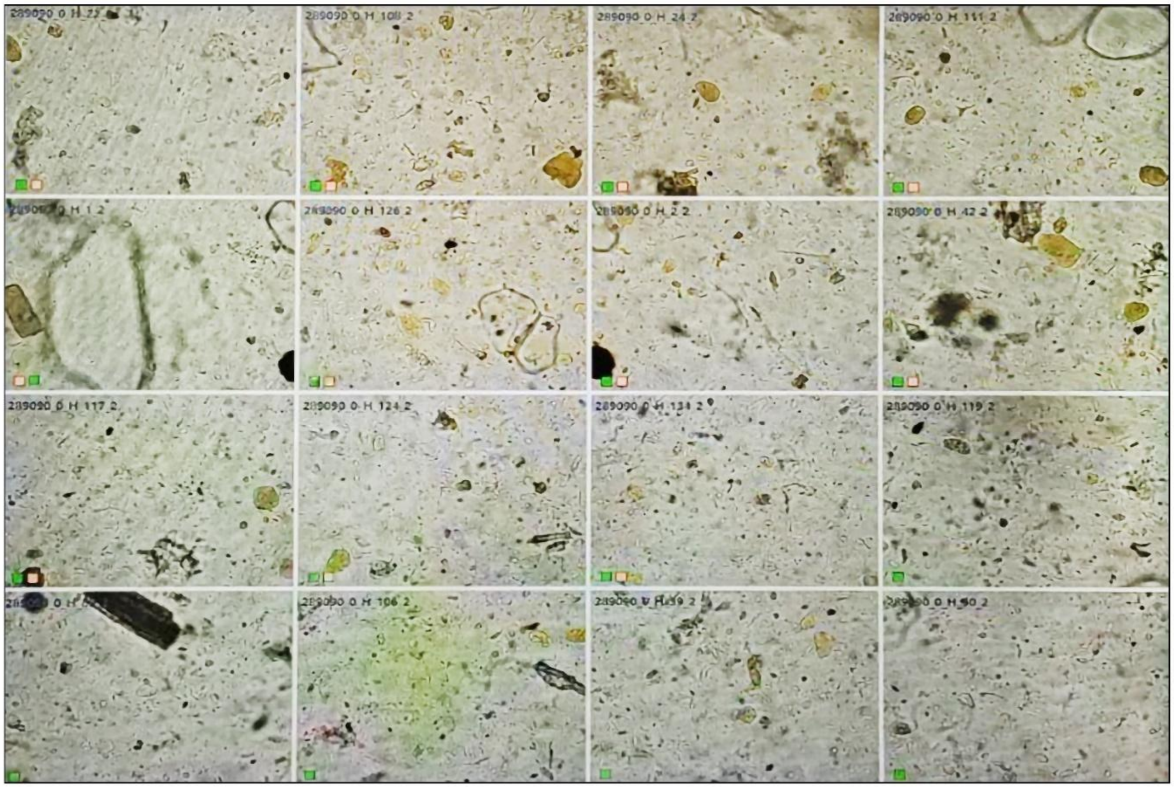

Figure (5): Automated KF-U600 AI-based stool analyzer. The image grid represents multi-field's microscopic view, using AI-assisted finding different components of the stool (including stages of the parasites, artifacts, and other components).

Confirmation of *G. intestinalis* by qPCR

In this study, real-time PCR targeting the *β-giardin* gene was employed to confirm *G. intestinalis* infections identified through direct wet mount microscopy. Out of 30 microscopically positive, 29 were confirmed positive by real time PCR. Additionally, 2 out of 10 samples that were negative by microscopy tested positive through PCR, resulting in an overall detection of 31 positive cases out of 40 samples. The cycle threshold ($C_{T)}$ values for the positive samples ranged from 28.0 to 35.0 (median: 30.0), as shown in **Figure (6)**.

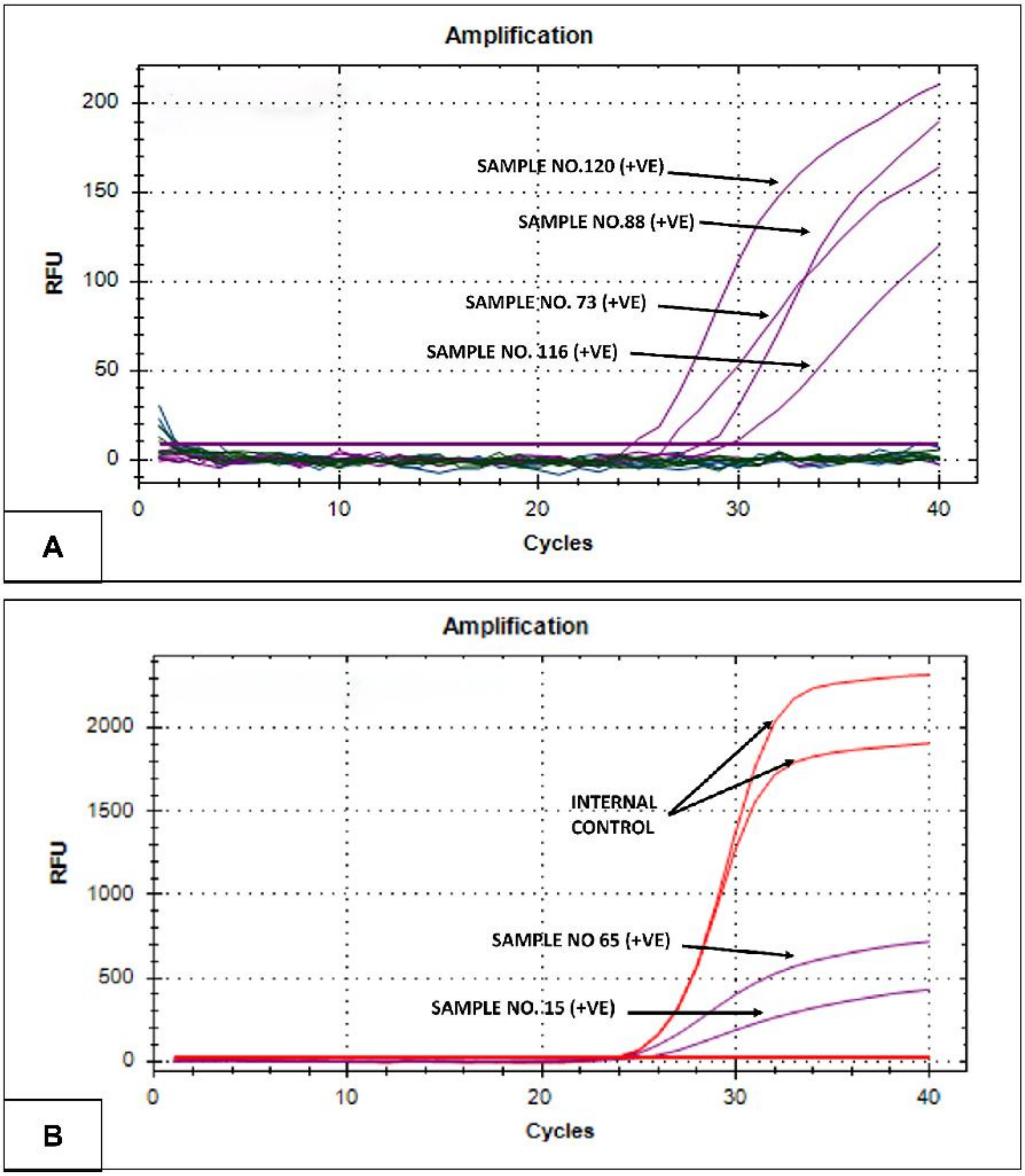

Figure (6): The graphs illustrate the relationship between Relative Fluorescence Units (RFU) and the number of cycles in a Polymerase Chain Reaction (PCR) of *G. intestinalis*. The x-axis represents the number of cycles (0-40), and the y-axis represents RFU (0-200).
A: Four specific samples are labeled with arrows indicating their curves: All the labeled samples are positives (73, 88, 116 and 120).
B: Three curves are displayed: internal control with a rapid increase in RFU starting around cycle 25, peaking at over 2000; sample number 65 (+VE) with a gradual increase starting around cycle 30, peaking at around 1000; and sample number 15 (+VE) with a gradual increase starting around cycle 30, peaking at around 750.

These findings underscore the enhanced sensitivity of real-time PCR over traditional microscopy in detecting *G. intestinalis*. Looking at the Real-time PCR amplification plot for the *β-giardin* gene. The graph showed amplification curves for samples 73, 88, 116, and 120, with the following observations:

Sample 120 showed the earliest amplification (lowest Ct value), crossing threshold around cycle 28-29. Sample 88 amplified next, crossing threshold around cycle 30-31. Sample 73 follows, crossing threshold around cycle 32. And sample 116 showed the latest amplification, crossing threshold around cycle 34-35. Regarding the curve characteristics: All positive samples showed typical sigmoidal amplification curves. The curves reached different RFU (Relative Fluorescence Units) plateaus, with maximum values ranging from about 120-200 RFU. The baseline was stable for all samples up to about cycle 25. Aforementioned all samples were positive for the *β-giardin* gene, indicating the presence of *Giardia* DNA. The different Ct values suggested varying initial template concentrations (Sample 120 had the highest initial concentration and sample 116 had the lowest initial concentration among positives). The clean baseline and well-defined exponential phases indicated good quality amplification without significant background noise. All positive samples showed good amplification efficiency as evidenced by the smooth sigmoidal curves.

Sensitivity and Specificity of the Diagnostic Techniques

The **Table (3)** summarizes the sensitivity and specificity of four diagnostic methods used for the detection of *G. intestinalis*. The KF-U600 Analyzer demonstrated the highest sensitivity at 100%, indicating its ability to detect all true positive cases identified in the sample set.

Table (3): Comparison of Sensitivity and Specificity of IC Assay, KF-U600 Analyzer, Real-Time PCR, and Direct Wet Mount Microscopy in Detecting *G. intestinalis*

Method	Sensitivity (%)	Specificity (%)
IC Assay	93.33	85.00
KF-U600 Analyzer	100.00	90.00
Real-Time PCR	96.67	80.0
Direct Wet Mount	93.55	88.89

The Real-Time PCR method also showed high sensitivity (96.67%) and is considered the most reliable reference standard. The IC Assay and Direct Wet Mount methods both exhibited comparable sensitivity levels (93.33% and 93.55%, respectively), suggesting strong performance in detecting positive cases. In terms of specificity, the KF-U600 Analyzer again outperformed the other methods (90.00%), followed closely by the Direct Wet Mount (88.89%). The IC Assay had a slightly lower specificity (85.00%), while the Real-Time PCR, although highly sensitive, showed a lower specificity (80.00%), likely due to detection of cases missed by microscopy. Overall, the results indicate that while all methods are reasonably accurate, the KF-U600 Analyzer and Real-Time PCR provide superior diagnostic performance, particularly for confirming positive cases.

Molecular Phylogeny of *G. intestinalis*

Regarding molecular based identification, the 99% identity and absence of significant gaps indicated that the query sequence was highly homologous to the *G. intestinalis β-giardin* gene. This strong similarity suggested that the sequence likely belongs to *G. intestinalis*, closely related to the STS-U isolate (Accession Number: DQ090542.1). The minimal genetic divergence suggested that the sequence was evolutionarily stable, with minor variations potentially useful for strain differentiation and transmission tracking. Given that *G. intestinalis* was a major cause of Giardiasis, its molecular characterization is crucial for understanding infection dynamics, drug resistance, and zoonotic transmission. The strong sequence identity highlights the *β-giardin* gene's reliability as a molecular marker, particularly for PCR-based diagnostic assays in clinical and environmental studies. The given phylogenetic tree is represented the evolutionary relationships among *G. intestinalis* sequences, including Iraqi isolates and reference sequences, using the Maximum Likelihood (ML) method as shown in **Figure (7).**

The Iraqi *G. intestinalis* sequences (blue circles) form distinct clusters with reference *Giardia* sequences, indicating their close genetic relationship. The Iraqi Seq. *G. intestinalis* 15-FBG, Consensus Seq., and 65-RBG cluster together with *Giardia* BG-gene reference sequences (e.g., OQ385210.1, LT603958.1), suggesting high similarity in the *β-giardin* (BG) gene. The Iraqi Seq. *G. intestinalis* 15-RBG appears slightly distant but still related to the BG-gene group. Separation of the BG and Triosephosphate Isomerase (TPI) Gene Clusters. The tree distinctly separated sequences based on their gene types, including the BG gene group was at the top, showing evolutionary relatedness among *Giardia* strains based on this structural protein. The TPI gene group (MH730627.1, MH730629.1, AB516353.1, MW344041.1, AB618783.1) forms a separated cluster, indicated a different evolutionary lineage compared to BG-gene sequences. Bootstrap values (numbers on branches) indicated the confidence level of the branching patterns. Values above 70% (e.g., 79, 88, 98, 99, 100) suggested strong support for the clustering of sequences.

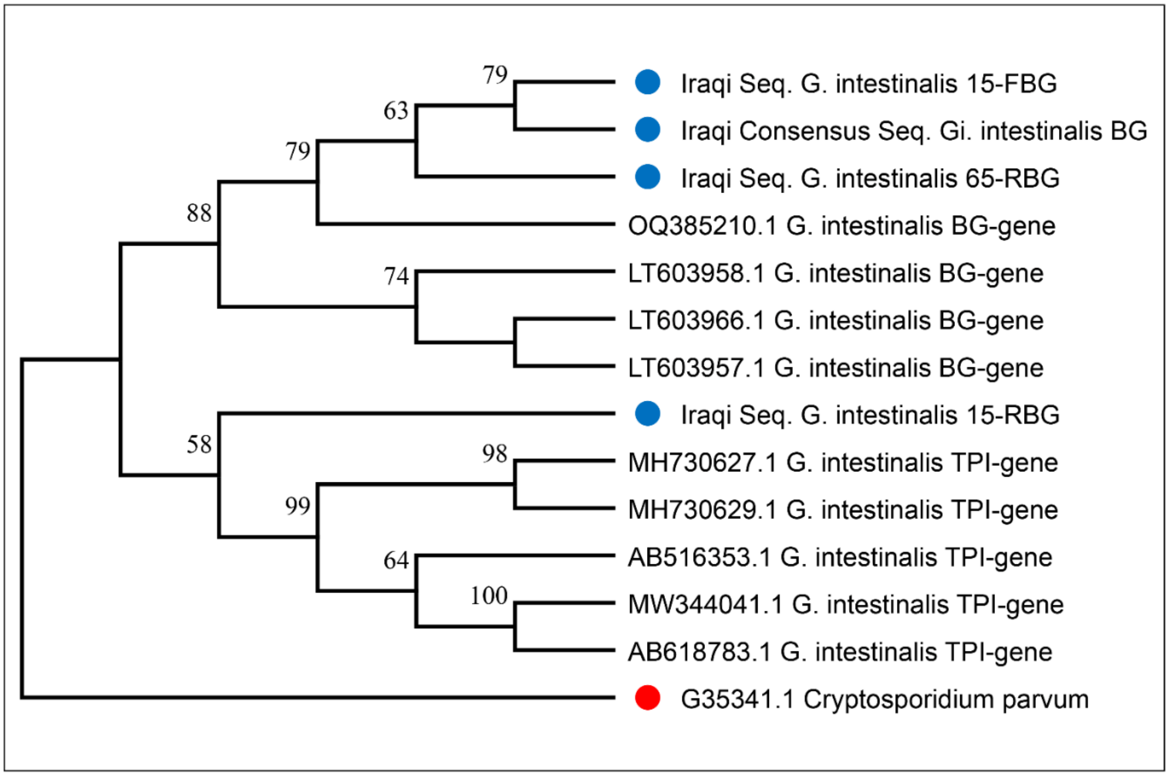

Figure (7): The evolutionary history of *G. intestinalis* was inferred by using the Maximum Likelihood method and Tamura-Nei model (K. & M., 1993). The tree with the highest log likelihood (-3572.16) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 14 nucleotide sequences. There was a total of 671 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021)

The TPI gene cluster showed high bootstrap values (99-100%), reinforcing its evolutionary distinctiveness from the BG-gene sequences. *Cryptosporidium parvum* (G35341.1, red circle) serves as an outgroup, helping to root the tree and providing a reference for divergence. Its placement at the farthest branch confirms its evolutionary distance from *G. intestinalis*. The ML-based phylogenetic tree effectively distinguished Iraqi *G. intestinalis* isolates from reference sequences while maintaining clear gene-based clustering. The BG and the TPI genes form distinct evolutionary groups, supporting their molecular classification. The high bootstrap values provided confidence in the evolutionary relationships inferred. This analysis can aid in understanding genetic diversity, strain differentiation, and epidemiological tracking of *Giardia* in Iraq.

Discussion

In line with previous researches, the microscopic findings of the current study in line with the results reported in Iraq and other regions, indicating the persistence of diverse intestinal parasitic infections as a public health concern (Hasan et al., 2020; Qadir et al., 2022). Trichrome staining is particularly useful for visualizing protozoan trophozoites and cysts, while acid-fast staining aids in identifying coccidian parasites, which may coexist with *Giardia* in some infections (Garcia et al., 2017; Mathison & Pritt, 2022). The prevalence of *Giardia* (1.14%) in this study is comparable to other regional reports but may vary due to differences in sample size, population demographics, and diagnostic methodologies. Factors such as hygiene practices, water quality, and socio-economic conditions significantly influence the occurrence of *Giardia* infections (Damitie et al., 2018; Nisar et al., 2024). However, the overdependence of microscopic study in repetitive clinical practice serves to necessitate newer and more automated techniques. The conventional methods are direct slide preparation, concentration techniques, and application of the cellophane tape method. Handheld microscopy is currently considered the gold standard presently, and entails visual inspection for the identification of various parasitic stages. However, the technique is time-consuming, tedious, and requires expert skill (Kumar et al., 2023). The association between steatorrhea and *Giardia* infection is established, since the parasite is responsible for disruption of intestinal absorption as well as lipid metabolism. The discovery brings evidence for clinical relevance of infection with *G. intestinalis*, highlighting the need for proper diagnosis and treatment (Halliez & Buret, 2013). Microscopic method is the classical gold standard for detecting *G. intestinalis*. It is very specific and can detect more than one parasite in a sample but is time-consuming and less sensitive if single stool samples are employed (Schuurman et al., 2007; Van Lint et al., 2013; Verweij et al., 2004). Conventional techniques, like microscopy are readily available but have restricted sensitivity and specificity as opposed to molecular techniques (Alharbi et al., 2020; Parčina et al., 2017). Microscopy is operator-dependent and has the ability to also miss low parasite density (Parčina et al., 2017). On the other hand, seasonal variation could be caused by environmental conditions, water quality changes, or differences in individual hygiene and food eating habits on a seasonal basis (Abate et al., 2024; Hajare et al., 2022). The results support possible behavioral or occupational exposure contrasts between males and females, that need further exploration (Gautam et al., 2024). Since the data distribution was of a type not amenable to significant results, the test was not significant. The monthly differences indicate that there could be a possible function for environmental or behavioral causes of transmission of *Giardia*, which should be explored. Epidemiologically, the low gender-based variation in infection rates is consistent with earlier literature that found greater susceptibility or exposure of males to intestinal protozoan infections. This could be attributed to variations in occupational exposure, personal hygiene patterns or health seeking behaviour by gender. On the other hand, the relatively low percentage of infections in females indicates a relatively lower risk or more preventive actions, even though underreporting or lesser attendance in the laboratory by females cannot be ruled out. Altogether, diversity indices demonstrate that, despite the presence of *G. intestinalis* infections in both genders, males bore an unequal epidemiological burden, which resulted in low diversity and uneven gender distribution in all months under investigation (Sarkari et al., 2016). The results have significant epidemiological implications in demonstrating monthly and demographic patterns of *G. intestinalis* infection and informing enhanced public health measures in Erbil City. The Giardiasis with age indicates that people in the 15-44-year age group could be at greater risk due to higher exposures concluded occupational, social, or lifestyle exposure. The decreased rate in children and elderly groups could be a sign of variation in immune response, hygiene, or environmental exposure. In Iraq, intestinal parasites are a significant public health problem, as attested by the large fluctuations in rate of incidence within and between communities and across regions. Prevalence rates in certain research have been reported to range from 7.36% to 84.67%. The significant cause of morbidity and mortality is related to the frequency of these parasites (Harb et al., 2020). In Iraq, different species of parasites are recognized, for instance, *Entamoeba histolytica and G. intestinalis* which often cause diarrhoea (Hussein, 2022). On the other hand, much research has been done to evaluate the frequency and species of parasites among people suffering from diarrhoea. For example, the study investigated the parasite infection rates between June and September of 2018 in Babylon province, in which 10.80% of the cases were with Giardiasis (Al-Taei, 2019). The false-positive cases among negative samples may indicate cross-reactivity or limitations of the assay, warranting further validation through molecular methods. This reinforces the need for integrating multiple diagnostic approaches to improve accuracy and reliability in *Giardia* detection (Alharbi et al., 2020; Goni et al., 2012). The use of AI-assisted stool analyzers presents a promising advancement in parasitology diagnostics, offering a faster and potentially more accurate alternative to traditional microscopic methods (Kenneth et al., 2024). Previous research has demonstrated that PCR-based methods can identify *Giardia* DNA even in samples where microscopy fails, due to low parasite loads or intermittent shedding (Van Lint et al., 2013). The identification of more positive cases from earlier reported negative samples proved the possibility of false negatives of routine techniques and the need for molecular confirmation. The Ct values generated are as anticipated in predicted patterns of amplification in Giardiasis. Lower Ct values were shown to correlate with increased initial target DNA concentrations, implying increased parasitic burden in such samples (Guy et al., 2004). The heterogeneity of patient-to-patient infection intensity was highlighted by distribution of Ct values from this study. The use of real-time PCR for the diagnosis of routine cases has several benefits, such as enhanced specificity and sensitivity, and quantification of the parasite burden. In addition, detection of the *β-giardin* gene offers a reliable marker for the identification of *Giardia* since it is conserved and high reiterated within the parasite's genome (Ghosh et al., 2000). Nevertheless, the appearance of PCR-positive results from microscopically negative samples should be interpreted with caution as it could reflect low-level infections or contamination. Molecular analysis in this research confirmed that the native sequence of *G. intestinalis* was 99% homologous with the *β-giardin* gene of a well-documented reference strain (STS-U, Accession No. DQ090542.1), which demonstrates its high specificity and genetic stability. The *β-giardin* gene, with its structural function as a part of the parasite cytoskeleton, is conserved in molecular diagnosis because it is highly conserved sequence and ubiquitous in the genome (Cacciò Sm et al., 2002; Lalle M et al., 2004). Having a very high value of alignment score (bit score of 874) and an E-value of 0.0, it was indicated that this alignment is statistically significant and not random (Altschul Sf et al., 1990). The small 1% variation indicates the potential of strain-level discrimination, since this is helpful in deciphering how the parasite grows and spreads (Read Cm et al., 2004). The Maximum Likelihood phylogenetic tree showed that the Iraqi *G. intestinalis* isolates clustered highly with other identified *β-giardin* sequences (like OQ385210.1 and LT603958.1), which suggesting a common evolutionary background or route of transmission (Feng Y & L., 2011; Zahedi A et al., 2017). On the other hand, triosephosphate isomerase (TPI) gene-based sequences groups with strong bootstrap values (99–100%), further evidence that different genes can report different evolutionary histories (Sulaiman Im et al., 2003). Using *Cryptosporidium parvum* as an outgroup rooted the evolutionary tree and validated the presented relationships (Ryan Um et al., 2005). Overall, these findings support that the *β-giardin* gene is a dependable target for detecting *G. intestinalis*, differentiating strains, and supporting epidemiological studies, both in Iraq and internationally (Foronda P et al., 2008; Wielinga Cm et al., 2011).

Conclusion

This investigation casts some light on the persistent status of *Giardia intestinalis* as a public health problem, with infection detected in 1.14% of over 25,000 fecal specimens. Standard microscopy was useful for screening purposes, but newer methods such as real-time PCR, rapid immunochromatographic assay, phylogenetic analysis, and AI stool analyzers were more suitable to detect infections, especially low-parasite infections. The evidence revealed that infections were more common among males and adults aged 15 to 44 years and presented with overtly discernible seasonal patterns that reflect potential relationships with hygiene-related factors, environmental exposures, or lifestyle. Genetic testing confirmed the presence of *G. intestinalis* and showed strong similarities to strains seen globally, suggesting possible shared transmission routes. By using both traditional and advanced diagnostic tools, this study provides a clearer picture of Giardiasis in Erbil and highlights the importance of modern, reliable testing in guiding better prevention and control efforts.

Acknowledgements

We thank the visitors and their families for their participation in this study.

References

Abate, M. A., Robbins-Hill, A., Lawler, S., Assefa, Y., & Reid, S. (2024). A scoping review of modifiable and behavioural drivers of infectious gastroenteritis among children in high-income countries. Archives of Public Health, 82(1), 145. https://doi.org/10.1186/s13690-024-01375-5.

Al-Saeed, A. T., & Issa, S. H. (2006). Frequency of Giardia lamblia among children in Dohuk, northern Iraq. Eastern Mediterranean Health Journal, 12(5), 555-561.

Al-Taei, A. H. O. (2019). The prevalence of intestinal parasite among the attending peoples to Al-Hashimyah hospitals for seven years, Babylon province, Iraq. Journal of Physics: Conference Series, 1294(6), 062022. https://doi.org/10.1088/1742-6596/1294/6/062022.

Alharbi, A., Toulah, F., Wakid, M., Azhar, E., Farraj, S., & Mirza, A. (2020). Detection of *Giardia lamblia* by Microscopic Examination, Rapid Chromatographic Immunoassay Test, and Molecular Technique. Cureus, 12. https://doi.org/10.7759/cureus.10287.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Cacciò, S. M., De Giacomo, M., & Pozio, E. (2002). Sequence analysis of the β-giardin gene and development of a PCR–restriction fragment length polymorphism assay to genotype *Giardia lamblia* cysts from human faecal samples. International Journal for Parasitology, 32(8), 1023-1030. https://doi.org/10.1016/S0020-7519(02)00068-1.

Damitie, M., Mekonnen, Z., Getahun, T., Santiago, D., & Leyns, L. (2018). Molecular epidemiology of *Giardia duodenalis* infection in humans in Southern Ethiopia: a triosephosphate isomerase gene-targeted analysis. Infectious Diseases of Poverty, 7(1), 1-10. https://doi.org/10.1186/s40249-018-0397-4.

Feng, Y., & Xiao, L. (2011). Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clinical Microbiology Reviews, 24(1), 110-114. https://doi.org/10.1128/CMR.00033-10.

Foronda, P., Bargues, M. D., Abreu-Acosta, N., Periago, M. V., Valero, M. A., Valladares, B., & Mas-Coma, S. (2008). Identification of genotypes of *Giardia intestinalis* of human isolates in Egypt. Parasitology Research, 55(5), 444-447. https://doi.org/10.1111/j.1550-7408.2008.00348.x.

Garcia, L. S., Arrowood, M., Kokoskin, E., Paltridge, G. P., Pillai, D. R., Procop, G. W., Ryan, N., Shimizu, R. Y., & Visvesvara, G. (2017). Practical guidance for clinical microbiology laboratories: laboratory diagnosis of parasites from the gastrointestinal tract. Clinical Microbiology Reviews, 31(1), e00025-00017. https://doi.org/10.1128/cmr.00025-17.

Gautam, J., Parajuli, R. P., & Pandey, K. (2024). Prevalence and associated factors of intestinal parasitic infections in the Badi indigenous communities of Western Nepal. Journal of Health, Population and Nutrition, 43(1), 211. https://doi.org/10.1186/s41043-024-00694-1.

Ghosh, S., Debnath, A., Sil, A., De, S., Chattopadhyay, D. J., & Das, P. (2000). PCR detection of *Giardia lamblia* in stool: targeting intergenic spacer region of multicopy rRNA gene. Molecular Cellular Probes, 14(3), 181-189. https://doi.org/10.1006/mcpr.2000.0302.

Goni, P., Martin, B., Villacampa, M., Garcia, A., Seral, C., Castillo, F., & Clavel, A. (2012). Evaluation of an immunochromatographic dip strip test for simultaneous detection of *Cryptosporidium* spp, *Giardia duodenalis*, and *Entamoeba histolytica* antigens in human faecal samples. European Journal of Clinical Microbiology and Infectious Diseases, 31, 2077-2082. https://doi.org/10.1007/s10096-012-1544-7.

Gurgitano, M., Angileri, S. A., Rodà, G. M., Liguori, A., Pandolfi, M., Ierardi, A. M., Wood, B. J., & Carrafiello, G. (2021). Interventional Radiology ex-machina: Impact of Artificial Intelligence on practice. La Radiologia Medica, 126, 998-1006. https://doi.org/10.1007/s11547-021-01351-x.

Guy, R. A., Xiao, C., & Horgen, P. A. (2004). Real-time PCR assay for detection and genotype differentiation of *Giardia lamblia* in stool specimens. Journal of Clinical Microbiology, 42(7), 3317-3320. https://doi.org/10.1128/jcm.42.7.3317-3320.2004.

Hajare, S. T., Chekol, Y., & Chauhan, N. M. (2022). Assessment of prevalence of *Giardia lamblia* infection and its associated factors among government elementary school children from Sidama zone, SNNPR, Ethiopia. PLoS ONE, 17(3), e0264812. https://doi.org/10.1371/journal.pone.0264812.

Halliez, M. C., & Buret, A. G. (2013). Extra-intestinal and long term consequences of *Giardia duodenalis* infections. World Journal of Gastroenterology, 19(47), 8974-8985. http://dx.doi.org/10.3748/wjg.v19.i47.8974

Harb, A., Abraham, S., O'Dea, M., Hantosh, H. A., Jordan, D., & Habib, I. (2020). Sociodemographic Determinants of Healthcare-Seeking Options and Alternative Management Practices of Childhood Diarrheal Illness: A Household Survey among Mothers in Iraq. American Journal of Tropical Medicine and Hygiene, 104(2), 748-755. https://doi.org/10.4269/ajtmh.20-0529.

Hasan, T. A. H., Muhaimid, A. K. A., & Mahmoud, A. R. (2020). Epidemiological study of *Giardia lamblia* in Tikrit city, Iraq. Systematic Reviews in Pharmacy, 11(9), 102-106. https://dx.doi.org/10.31838/srp.2020.9.17.

Hobbs, E. C., Colling, A., Gurung, R. B., & Allen, J. (2021). The potential of diagnostic point of care tests (POCTs) for infectious and zoonotic animal diseases in developing countries: Technical, regulatory and sociocultural considerations. Transboundary and Emerging Diseases, 68(4), 1835-1849. https://doi.org/10.1111/tbed.13880.

Hussein, S. R. (2022). Prevalence of Diarrhea Caused by Intestinal Parasites in Children in AL-Kufa City, Iraq. The Egyptian Journal of Hospital Medicine, 89(1), 4723-4727. https://dx.doi.org/10.21608/ejhm.2022.260585.

Kenneth, M. H., Joseph, T., & Rose, N. (2024). AI Methods and Algorithms for Diagnosis of Intestinal Parasites: Applications, Challenges and Future Opportunities. East African Journal of Information Technology, 7(1), 366-379. https://doi.org/10.37284/eajit.7.1.2282.

Kumar, S., Arif, T., Ahamad, G., Chaudhary, A. A., Khan, S., & Ali, M. A. M. (2023). An Efficient and Effective Framework for Intestinal Parasite Egg Detection Using YOLOv5. Diagnostics, 13(18), 2978-2991. https://doi.org/10.3390/diagnostics13182978 .

Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., & Cacciò, S. M. (2004). Genetic heterogeneity at the β-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. International Journal for Parasitology, 35(2), 207-213. https://doi.org/10.1016/j.ijpara.2004.10.019.

McPherson, R. A., & Pincus, M. R. (2022). *Henry's Clinical Diagnosis and Management by Laboratory Methods.* (24th ed.). Elsevier, 1618pp.

Nisar, M., Khan, F., Ahmad, N., Ullah, S., Ullah, A., & Farooqi, M. W. (2024). Geographic And Demographic Influences on The Epidemiological Patterns of *Giardia lamblia* Infection in Rural Sites District Swat. Indus Journal of Bioscience Research, 2(02), 103-110. https://doi.org/10.70749/ijbr.v2i02.121.

Orfanou, D. C., Papadopoulos, E., Cripps, P. J., Athanasiou, L. V., & Fthenakis, G. C. (2011). Myiasis in a dog shelter in Greece: Epidemiological and clinical features and therapeutic considerations. Veterinary Parasitology, 181(1), 374-378. https://doi.org/10.1016/j.vetpar.2011.04.006.

Parčina, M., Reiter-Owona, I., Mockenhaupt, F., Vojvoda, V., Gahutu, J., Hoerauf, A., & Ignatius, R. (2017). Highly sensitive and specific detection of *Giardia duodenalis*, *Entamoeba histolytica*, and *Cryptosporidium* spp. in human stool samples by the BD MAX™ Enteric Parasite Panel. Parasitology Research, 117, 447-451. https://doi.org/10.1007/s00436-017-5720-7.

Pritt, B. S. (2015). Chapter 4 - Molecular Diagnostics in the Diagnosis of Parasitic Infection. In A. Sails & Y.-W. Tang (Eds.), *Methods Microbiol* (Vol. 42, pp. 111-160). Academic Press. https://doi.org/https://doi.org/10.1016/bs.mim.2015.05.001

Qadir, M. S., Hssein, Y. T., Karim, S. I., Rasheed, M. K., Palani, Z. M. R., Mohammed, A. B., Satar, H. M., Hassan, K. A., Omer, K. D., & Karim, A. K. (2022). Prevalence of *Giardia lamblia* among children in Sulaimani city, Iraq. International Journal of Health Sciences, 6(S2), 14827-14834. https://doi.org/10.53730/ijhs.v6nS2.8941.

Read, C. M., Monis, P. T., & Thompson, R. C. (2004). Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR–RFLP. Infection, Genetics and Evolution, 4(2), 125-130. https://doi.org/10.1016/j.meegid.2004.01.002.

Richard, R. L., & Yusof, H. (2018). Advancements in Parasite Diagnosis and Challenges in the Management of Parasitic Infections: A Mini Review. Regional Conference on Science, Technology and Social Sciences, 22, 667–677. https://doi.org/10.1007/978-981-13-0074-5_64.

Rosado, L., Da Costa, J. M., Elias, D., & Cardoso, J. S. (2017). Mobile-based analysis of malaria-infected thin blood smears: automated species and life cycle stage determination. Sensors, 17(10), 2167-2188. https://doi.org/10.3390/s17102167.

Roswell, M., Dushoff, J., & Winfree, R. (2021). A conceptual guide to measuring species diversity. Oikos, 130(3), 321-338. https://doi.org/10.1111/oik.07202.

Ryan, U., Hijjawi, N., Feng, Y., & Xiao, L. (2019). *Giardia*: an under-reported foodborne parasite. International Journal for Parasitology, 49(1), 1-11. https://doi.org/10.1016/j.ijpara.2018.07.003.

Ryan, U. M., Monis, P. T., Enemark, H. L., Sulaiman, I. M., Fayer, R., & Thompson, R. C. (2004). *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). Journal of Parasitology, 130(3-4), 197-207. https://doi.org/10.1016/j.vetpar.2005.03.002.

Sarkari, B., Hosseini, G., Motazedian, M. H., Fararouei, M., & Moshfe, A. (2016). Prevalence and risk factors of intestinal protozoan infections: a population-based study in rural areas of Boyer-Ahmad district, Southwestern Iran. BMC Infectious Diseases, 16(1), 703-707. https://doi.org/10.1186/s12879-016-2047-4.

Schuurman, T., Lankamp, P., Belkum, A., Kooistra-Smid, M., & Zwet, A. (2007). Comparison of microscopy, real-time PCR and a rapid immunoassay for the detection of *Giardia lamblia* in human stool specimens. Clinical Microbiology and Infection, 13(12), 1186-1191. https://doi.org/10.1111/j.1469-0691.2007.01836.x.

Shetty, O., Gurav, M., Bapat, P., Karnik, N., Wagh, G., Pai, T., Epari, S., & Desai, S. (2021). Moving next-generation sequencing into the clinic. Indian Journal of Medical and Paediatric Oncology, 42(03), 221-228. https://doi.org/10.1055/s-0041-1732854.

Shin, J.-H., Lee, S.-E., Kim, T. S., Cho, S.-H., Chai, J., & Shin, E. (2018). Development of Molecular Diagnosis Using Multiplex Real-Time PCR and T4 Phage Internal Control to Simultaneously Detect *Cryptosporidium parvum*, *Giardia lamblia*, and *Cyclospora cayetanensis* from Human Stool Samples. Korean Journal of Parasitology, 56(5), 419-427. https://doi.org/10.3347/kjp.2018.56.5.419.

Soares, F. A., Benitez, A. d.-N., Santos, B. M. d., Loiola, S. H. N., Rosa, S. L., Nagata, W. B., Inácio, S. V., Suzuki, C. T. N., Bresciani, K. D. S., & Falcão, A. X. (2020). A historical review of the techniques of recovery of parasites for their detection in human stools. Revista da Sociedade Brasileira de Medicina Tropical, 53, e20190535. https://doi.org/10.1590/0037-8682-0535-2019.

Sulaiman, I. M., Fayer, R., Bern, C., Gilman, R. H., Trout, J. M., & Schantz, P. M. (2003). Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerging Infectious Diseases, 9(11), 1444-1452. https://doi.org/10.3201/eid0911.030084.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10(3), 512-526. https://doi.org/10.1093/oxfordjournals.molbev.a040023.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution, 38(7), 3022-3027. https://doi.org/10.1093/molbev/msab120.

Van Lint, P., Rossen, J., Vermeiren, S., Elst, V., Weekx, S., Van Schaeren, J., & Jeurissen, A. (2013). Detection of *Giardia lamblia*, *Cryptosporidium* spp. and *Entamoeba histolytica* in clinical stool samples by using multiplex real-time PCR after automated DNA isolation. Acta Clinica Belgica, 68(3), 188-192. https://doi.org/10.2143/acb.3170.

Verweij, J., Blangé, R., Templeton, K., Schinkel, J., Brienen, E., Van Rooyen, M., Van Lieshout, L., & Polderman, A. (2004). Simultaneous Detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in Fecal Samples by Using Multiplex Real-Time PCR. Journal of Clinical Microbiology, 42(3), 1220-1223. https://doi.org/10.1128/jcm.42.3.1220-1223.2004.

Wambani, J., & Okoth, P. (2022). Impact of Malaria Diagnostic Technologies on the Disease Burden in the Sub-Saharan Africa. Journal of Tropical Medicine, 2022(1), 1-8. https://doi.org/10.1155/2022/7324281.

Won, E., Kim, S., Kee, S.-J., Shin, J. H., Suh, S., Chai, J., Ryang, D., & Shin, M. (2016). Multiplex Real-Time PCR Assay Targeting Eight Parasites Customized to the Korean Population: Potential Use for Detection in Diarrheal Stool Samples from Gastroenteritis Patients. PLoS ONE, 11(11), 1-14. https://doi.org/10.1371/journal.pone.0166957.

Zahedi, A., Ryan, U., Monis, P. T., & Oskam, C. (2017). Molecular typing of *Giardia duodenalis* in humans in Queensland–first report of assemblage E. Parasitology, 144(9), 1154-1161. https://doi.org/10.1017/S0031182017000439.

Zaki, M., & Dhubyan, Z. (2022). Prevalence of *Entamoeba histolytica* and *Giardia lamblia* Associated with Diarrhea in Children referring to lbn Al-Atheer Hospital in Mosul, Iraq. Archives of Razi Institute, 77(1), 73-79. https://doi.org/ 10.22092/ARI.2021.356312.1820.

Zaman, R. F., Khanum, H., Nargis, S., & Das, P. K. (2017). Comparison of saline, iodine and koh wet mount preparations for occurrence of parasites in stool samples from patients attending ICDDR, B. Bangladesh Journal of Zoology, 45(2), 159-170. https://doi.org/10.3329/bjz.v45i2.35711.

Zhang, Y.-G., Chen, J., Pan, H., Xiao, J., Jiang, L., Zhu, Q., Wu, H.-Y., & Wang, Z.-Y. (2022). Development and Preliminary Application of a Triplex Real-Time Quantitative PCR Assay for the Simultaneous Detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum*. Frontiers in Microbiology, 13, 1-9. https://doi.org/10.3389/fmicb.2022.888529.