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Original Article

Circulating miR-21-5p as a Potential Diagnostic Biomarker in Symptomatic *Giardia lamblia* Infection in Tikrit, Iraq

Raghda Mahmood Hamad[1], Mohammed Mohsen Sharqat Ali Al-Jumaili[2], Zaher Salem Omar Musleh Al-Jaata[3], Youssef Shakuri Yasin[*3]

[1]*Department of Science, College of Basic Education, Shirqat, Tikrit University, Iraq.*
[2]*Ministry of Education Directorate General of Salahuddin Education, Iraq.*
[3]*Bilad Alrafidain University, Iraq.*

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ABSTRACT

Giardia lamblia is one of the predominant intestinal protozoans and is primarily responsible for global diarrheal illness, especially in areas with scarce sanitation. Conventional diagnostic measures are not highly sensitive, and so non-invasive biomarkers are indispensable. In this respect, circulating microRNAs, the miR-21 molecule (especially miR-21-5p), could act as signal waves of the host immune response to infection. In the current study, aimed to investigating an expression of circulating miR-21-5p in patients with symptomatic *Giardia lamblia* infection and considering its diagnostics. Case–control study of Tikrit General Hospital, Iraq, January–November 2025. A microscopically confirmed sample consisting of 44 giardiasis cases was included, and 40 healthy control participants were also included. Direct microscopy and concentration methodologies were performed for stool samples. Plasma miRNA was extracted and quantitated to calculate using qRT-PCR assay with U6 as an internal control. Relative ratios of expressions were determined by the 2^-ΔΔCt method. Statistical analysis employed a student t-test and ROC curve. Circulating miR-21-5p expression increased significantly in patients relative to controls ($p < 0.001$) with a fold change of 6.38. The ROC analysis showed remarkable diagnostic performance, an AUC of 0.90; a sensitivity of 94%, and a specificity of 90%. Strong correlation between miR-21 expression ($r = 0.72$, $p < 0.001$) and inverse correlation with PCR Ct ($r = -0.78$, $p < 0.001$). Circulating miR-21-5p is markedly enriched in giardiasis and is an excellent candidate as a non-invasive diagnostic biomarker.

Keywords: Giardia lamblia, Circulating microRNA, miR-21-5p, Diagnostic biomarker, qRT-PCR.

1 Introduction

Giardia lamblia is one of the most prevalent intestinal protozoan parasites that infect humans across the world and continues to be a major contributor to diarrheal disease, particularly in children and those exposed to contaminated water. Infection is primarily occurs through the fecal-oral route and is more common in tropical and developing countries with poor sanitation and unsafe drinking water. Clinically, giardiasis presents with a spectrum ranging from asymptomatic infection to fatty diarrhea, abdominal pain, malabsorption, and growth disturbances [1-2-3]. Despite its clinical significance, the diagnosis of giardiasis is difficult. The conventional microscopic assessment is still predominant but owing to the intermittent removal of cysts and operator dependability, the sensitivity has been compromised. Recent developments of immunoassays have made diagnostic performance better, presenting superior specificity and inter-platform sensitivity [4]. The low-quality assay performance, the cost of the assay, and differences found between symptomatic and asymptomatic individuals point to the use of higher quality diagnostic biomarkers [1-5]. *Giardia lamblia* has distinct biological properties such as simple cellular organization and variable regulatory systems. The presence of short regulatory RNAs and RNA interference pathways in *Giardia*, which are involved in gene expression, parasite differentiation, and adaptability, have recently been highlighted in studies. Studies

* Corresponding author
E-mail address dryoussef@bauc14.edu.iq (Instructor).
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have improved the understanding of parasite biology and have provided new opportunities for molecular diagnostics [6-7-8]. Because of their stability in bodily fluids and their role in hosting immunological responses, circulating microRNAs have become appealing non-invasive indicators in infectious disorders. Previous studies have demonstrated that parasite-derived and host-related microRNAs can be detected during infection and may reflect host–parasite interactions. In particular, miR-21-5p is known to be involved in inflammatory signaling pathways and immune regulation and has been reported to be dysregulated in several parasitic infections, suggesting its potential as a diagnostic biomarker [9-10]. Therefore, the present study aimed to evaluate the expression of circulating miR-21-5p in patients with symptomatic *Giardia lamblia* infection and to assess its diagnostic value as a non-invasive diagnostic biomarker.

2 Materials and Methods

2.1 Study Design and Population

This case-control study was conducted from January 2025 to November 2025 at Tikrit General Hospital, Iraq. Among 146 suspected cases that were screened, 44 positive cases with Giardia lamblia were included as patients (patient group). The age of participants ranged from 5 to 60 years. Microscopic examination confirmed 44 positive cases for *Giardia lamblia*, which were included as the patient group. Among them, 29 were males, and 15 were females. A control group of 40 apparently healthy individuals was also included. Controls had no history of diarrhea or gastrointestinal disorders and tested negative for *Giardia lamblia*. Inclusion criteria: Patients with symptomatic *Giardia lamblia* infection confirmed by microscopy and PCR, aged 5–60 years, who provided informed consent. Exclusion criteria: Individuals with chronic inflammatory diseases, autoimmune disorders, malignancy, liver or kidney diseases, pregnancy, other parasitic, bacterial, or viral infections, and those receiving antibiotics or anti-inflammatory medications within the previous two weeks.

2.2 Stool Sample Collection and Microscopic Examination

Fresh stool was obtained from all suspected patients in clean, sterile, wide-mouthed containers. Samples were processed immediately or stored properly when delays were anticipated. Each stool sample was examined macroscopically (Olympus Corporation, Tokyo, Japan) for consistency and presence of mucus or fat droplets. Direct wet mount (saline and iodine preparations) and the formalin-ether sedimentation (FES) method were employed to increase detection sensitivity through microscopic examination [21]. For *Giardia lamblia* cysts, slides were examined under light microscopy at 10× and 40× magnifications. The samples included within the study were thus selected as the test group only if they presented with confirmed cysts.

2.3 Blood Sample Collection

5 mL of venous blood from each confirmed patient and each control subject was obtained in aseptic conditions with sterile syringes. Blood samples were moved into EDTA tubes (AFICO, Amman, Jordan), followed by centrifugation (Hettich, Germany) at 3000 rpm for 10 min to separate plasma. The plasma samples were then stored at −80°C until further molecular analysis.

2.4 ELISA Technique for *Giardia* Detection

Using the commercial ELISA kit for *Giardia* antigen (Ridascreen Giardia, R-Biopharm, Germany), an enzyme-linked immunosorbent assay (ELISA) was performed to identify *Giardia lamblia* coproantigens in stool samples according to the manufacturer protocol. Put simply, stool samples were diluted, transferred to microplate wells coated with anti-Giardia antibodies, incubated and washed. Next, enzyme-conjugated antibodies were added, then a chromogenic substrate was applied to induce a color phase. For the measurement of the optical density (OD) a microplate reader was used. Results were interpreted according to the cut-off values provided by the kit.

2.5 PCR Technique for *Giardia* Detection

PCR based conventional polymerase chain reaction (PCR) was used for the molecular detection of *Giardia lamblia* using glutamate dehydrogenase (*GDH*) gene, which is one of the most widely used markers during the studies in this regard for *Giardia*. Genomic DNA from stool samples was extracted using a commercial DNA extraction kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions. Amplification of the GDHgene with the appropriate GDH primers under optimized thermal cycling conditions was performed. PCR products were analyzed by agarose gel electrophoresis and visualized under ultraviolet illumination. A band at the expected size confirmed a positive result for *Giardia lamblia*. Following extraction, DNA concentration and purity were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, USA) prior to PCR amplification.

2.6 MicroRNA Extraction

Total circulating microRNA was extracted from plasma samples using a commercial microRNA extraction kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA). Samples with an A260/A280 ratio ranging from 1.8 to 2.2 were considered suitable for downstream cDNA synthesis and qRT-PCR analysis. RNA samples were immediately stored at -80°C until further use to preserve RNA integrity.

2.7 cDNA Synthesis

Complementary DNA (cDNA), obtained from the extracted microRNA, was synthesized according to the manufacturer's instructions using a miRNA-specific reverse transcription kit (miScript II RT Kit, QIAGEN, Germany). For this conversion of

microRNA into complementary DNA, the reverse transcription reaction was performed in a thermal cycler (Sacace, Italy), under optimized conditions.

2.8 Quantitative Real-Time PCR (qRT-PCR)

The expression of circulating miR-21 was determined by quantitative real-time PCR (qRT-PCR). miR-21-5p specific primers were developed and optimized in this study. Amplification was conducted using a real-time PCR system (SaCycler-96, Sacace, Italy). cDNA template, forward primer, universal reverse primer, and SYBR Green master mix were used as the reaction mixture. The qRT-PCR reaction was carried out in a final volume of 20 µL, consisting of 10 µL of SYBR Green Master Mix, 1 µL of cDNA template, 1 µL of miR-21-5p forward primer, 1 µL of universal reverse primer, and 7 µL of nuclease-free water. U6 small nuclear RNA serves as an internal control for normalization. Comparative expression levels of miR-21 were determined by the 2^(-ΔΔCt) method. The primer sequences in this study are described in Table 1. This Primer desined by this study.

Table 1: Primer sequencing for miR-21-5p**.**

Target	Sequence	Primer sequence (5′–3′)	Tm (°C)	GC%
miR-21-5p	Stem-loop	5’GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACTCAACA3′	78.0	54
	F	5’TAGCTTATCAGACTGATGTTGA3′	60.0	55.4
U6	F	5’CGCGCGTTTCCAGGTGTTT3′	62	57
	R	5’AATCCGTTGACTCCGACCTT3′	61.4	50
Universal	R	5’CACTAGGCGCTCACTGTTCTC3′	68.2	65

2.9 Statistical Analysis

The statistical analyses were performed using both SPSS version 26.0 and GraphPad Prism version 9.0. The mean and standard deviation were given during the analysis of the data. Data normality was evaluated using the shapiro-wilk test. for data with normal distribution, the independent samples t-test was used and the mann-whitney u test was used for data without normal distribution to compare the expression levels of miR-21 between patients and controls. association and diagnostic performance were evaluated by pearson's correlation coefficient and roc curve analysis. a p-value <0.05 was considered statistically significant.

2.10 Ethical Approval

The study protocol was reviewed and approved by the relevant scientific and ethical committees in Tikrit Province, Iraq, **(TU-BE-2025-0002).** Written informed consent was obtained from all participants prior to enrollment. All specimens and data were handled anonymously, and confidentiality was maintained in accordance with the ethical principles of the Declaration of Helsinki.

3 Results

3.1 Demographic distribution and clinical profile of the study group

The present study included 44 patients with *Giardia lamblia* infection confirmed by microscopic examination. Table 2 shows the patients' demographics and clinical profile. Most cases were from children ≤10 years, 24 (54.5%) were the number of cases, which is consistent with age distribution. Patients in the 11–20 years age range were 8 (18.2%) and patients 21–40 years and >40 years each accounted for 6 (13.6%). Regarding sex distribution, 23 (52.3%) were males and 21 (47.7%) were females. Although patients lived in rural areas (32; 72.7%) in urban areas (12; 27.3%). Fatty diarrhea was the main symptom reported by all patients at the clinic (100%). Abdominal pain was reported among 39 (88.6%) patients and 5 (11.4%) do not have abdominal pain.

Table 2: Demographic and clinical characteristics of patients (n = 44).

Variable	n	%
Age groups (years)		
≤10	24	54.5
11-20	8	18.2

21-40	6	13.6
>40	6	13.6
Gender		
Male	23	52.3
Female	21	47.7
Residence		
Rural	32	72.7
Urban	12	27.3
Clinical features		
Fatty diarrhea	44	100
Abdominal pain	39	88.6
No abdominal pain	5	11.4

3.2 Comparison of Diagnostic Methods for *Giardia lamblia*

Giardia lamblia diagnostic patterns were variable as a function of methods used, with the number of infected cases recorded from microscopic examination (61 cases, 41.8 per cent) being the highest, followed by ELISA diagnosis (55 cases, 37.7 per cent), and the lowest was obtained from PCR (44 cases, 30.1 per cent). The results reveal that microscopy detected more cases compared to ELISA and PCR, though ELISA and PCR may provide more precise confirmation of infection, as illustrated in Figure 1, where the distribution of positive cases differs clearly among the three diagnostic methods, with microscopy representing the largest proportion, followed by ELISA and then PCR.

3.3 Expression Level of Circulating miR-21-5p

The expression extent of miR-21-5p in the circulating blood of *Giardia lamblia* infected patients was assessed and compared with healthy controls, as shown in Table 3. Differences between patients and controls showed that miR-21 mRNA expression levels were higher in-patient group, where the mean Ct value for that mRNA was lower (30.47 ± 2.64) than in controls (32.38 ± 1.42). Hence, ΔCt values in patients (7.95 ± 2.78) were significantly lower than controls (10.50 ± 1.90) ($p < 0.001$). Additionally, the relative expression tests conducted in (2^(-ΔΔCt)) revealed a significantly enhanced expression of miR-21 in the patients (3.68 ± 7.66) when compared to the controls (0.38 ± 0.65) ($p < 0.001$). For all studies, the fold change analysis reported a statistically significant upregulation of circulating miR-21 in patients (6.38 vs. 1.00 in control group ($p < 0.001$). U6 Ct values were not significantly different from controls in patients ($p < 0.05$), indicating that the internal reference used for normalization is stable.

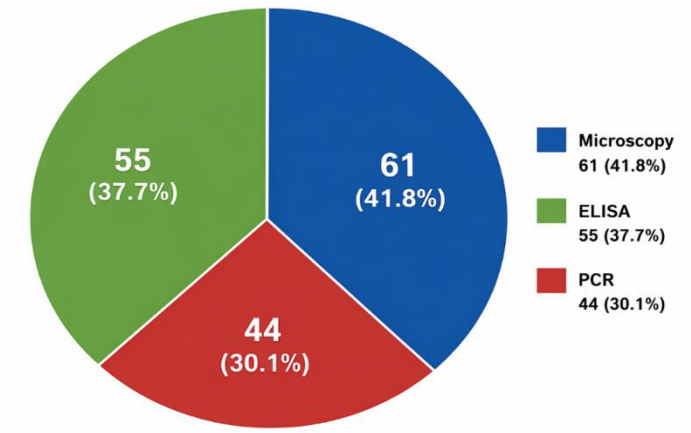

Figure 1: Distribution of positive *Giardia lamblia* cases detected by different diagnostic methods. Microscopy accounted for the highest proportion, followed by ELISA and PCR.

Table 3: Relative expression levels of circulating miR-21-5p in patients with *Giardia lamblia* infection and healthy controls.

Parameter	**Patients (n=44)**	**Controls (n=40)**	***P*-value**
U6 Ct (Mean ± SD)	22.50 ± 0.96	21.91 ± 1.08	<0.05
miR-21-5p Ct (Mean ± SD)	30.47 ± 2.64	32.38 ± 1.42	<0.001
ΔCt (Mean ± SD)	7.95 ± 2.78	10.50 ± 1.90	<0.001
ΔΔCt (Mean ± SD)	0.90 ± 5.63	2.75 ± 1.88	<0.001
2^-ΔΔCt (Mean ± SD)	3.68 ± 7.66	0.38 ± 0.65	<0.001
Fold Change	6.38	1.00	<0.001

P-values were calculated using Student's t-test. A p-value < 0.05 was considered statistically significant.

3.4 Distribution of Circulating miR-21-5p Expression

The distribution of circulating miR-21-5p expression levels in individuals with Giardia lamblia infection and controls was as shown in Figure 2. Patients exhibited markedly higher fold change values compared with controls, indicating significant upregulation of miR-21-5p in infected individuals. The box plot demonstrates a clear separation between the two groups, with most patient values distributed above those of the control group, despite the presence of some variability within the patient cohort. Circulating miR-21-5p expression levels were significantly higher in patients compared to controls ($p < 0.001$), indicating a strong upregulation associated with *Giardia lamblia* infection.

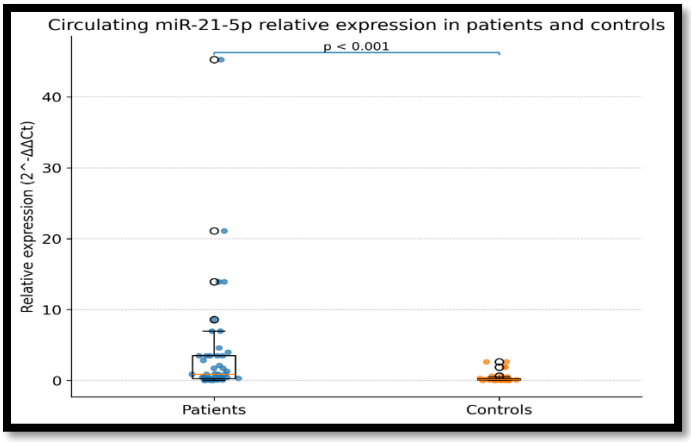

Figure 2: Relative expression of circulating miR-21-5p (2^-ΔΔCt) in patients with *Giardia lamblia* infection and healthy controls, showing a significant increase among the patients group ($p < 0.001$).

3.5 Diagnostic Performance of Circulating miR-21-5p

The reliability and accuracy of circulating miR-21 for the distinction between *Giardia lamblia* infected and non-infected controls was evaluated using receiver operating characteristic (ROC) curve analysis (Figure 3). The test overall performed better, with an AUC of 0.90 (95% CI 0.82–0.96; $p < 0.001$). For the optimal cut-off value, miR-21 has a sensitivity of 94% and specificity of 90%, showcasing a good sensitivity/specificity ratio when identifying infected patients from the control group.

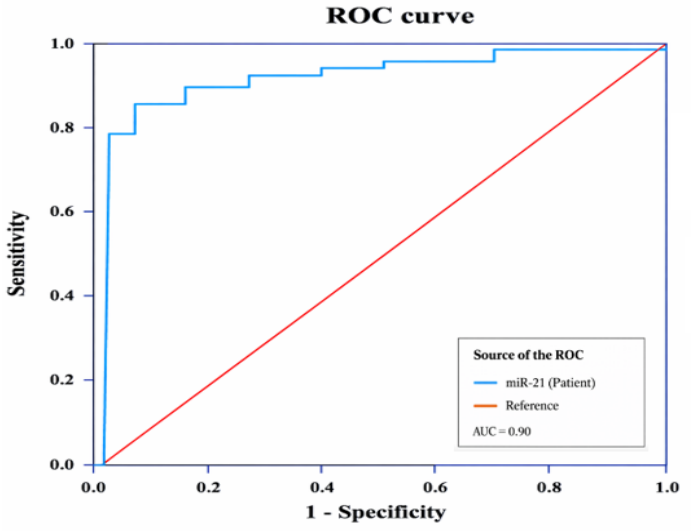

Figure 3: Receiver operating characteristic (ROC) curve of circulating miR-21-5p for discrimination between patients with *Giardia lamblia* infection and healthy controls.

Table 4: Diagnostic performance of circulating miR-21-5p.

microRNA	AUC	95% Confidence Interval	Sensitivity (%)	Specificity (%)	P-value
miR-21-5p	0.90	0.82 – 0.96	94	90	<0.001

3.6 Correlation Analysis Between Diagnostic Methods and Circulating miR-21 Expression

A Pearson correlation analysis was conducted to examine the relationships between different diagnostic methods and circulating miR-21 expression. As shown in Figure 4, ELISA optical density values exhibited a strong positive correlation with miR-21 expression ($r = 0.72$, $p < 0.001$), indicating that increased immunological response is associated with higher molecular expression levels. In contrast, PCR Ct values showed a strong inverse correlation with miR-21 expression ($r = -0.78$, $p < 0.001$), which reflects the expected negative relationship between Ct values and gene expression. Moderate yet statistically significant positive correlations were also observed between miR-21 expression and microscopy ($r = 0.55$, $p < 0.001$) as well as serology ($r = 0.60$, $p < 0.001$), suggesting that conventional diagnostic methods partially reflect the underlying molecular changes. Microscopy showed a relatively strong relationship with serological data ($r = 0.70$, $p < 0.001$), suggesting a reasonable

level of correspondence between these conventional methods. However, weaker negative correlations were found between PCR and microscopy (r = -0.40, p = 0.005) and between PCR and serology (r = -0.45, p = 0.003), which may be attributed to differences in sensitivity and detection capabilities between molecular and conventional diagnostic techniques.

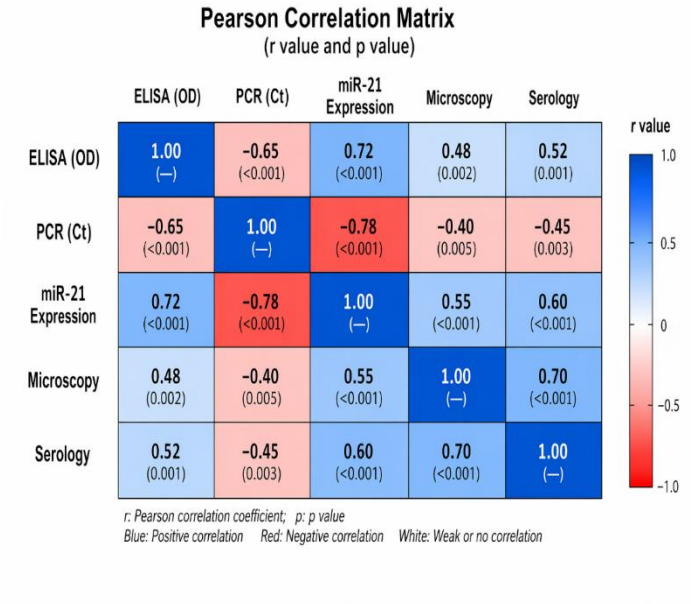

Figure 4: Pearson correlation heatmap illustrating the relationships between ELISA, PCR, circulating miR-21 expression, microscopy, and ELISA methods. Blue indicates positive correlation, while red indicates negative correlation. Values represent correlation coefficients (r) with corresponding p-values.

4 Discussion

The present study demonstrated a significant upregulation of circulating miR-21-5p in patients with symptomatic Giardia lamblia infection compared with healthy controls, with a fold change of 6.38 and excellent diagnostic performance (AUC = 0.90, sensitivity = 94%, specificity = 90%). These findings suggest that circulating miR-21-5p may serve as a promising non-invasive biomarker for the diagnosis of giardiasis and support the hypothesis that host microRNA responses are altered during parasitic infection. The observed increase in miR-21-5p expression may be attributed to activation of inflammatory and immune pathways induced by Giardia infection. Previous experimental studies have shown that Giardia lamblia activates the NOD2-Rip2-ROS signaling pathway and promotes the production of pro-inflammatory cytokines, which are known regulators of microRNA expression [11]. Therefore, the elevated expression of miR-21-5p observed in the present study may reflect a host regulatory mechanism involved in intestinal inflammation and maintenance of mucosal homeostasis.[12]

Our findings are in agreement with previous reports demonstrating the diagnostic value and stability of circulating microRNAs in infectious diseases. Meningher et al. [13] reported that Giardia-associated small RNAs may provide a novel molecular approach for the diagnosis of human giardiasis. Similarly, studies on other infectious diseases have highlighted the utility of circulating miRNAs as reliable biomarkers because of their stability in plasma and their association with host immune responses [9,10]. These observations support the present findings and further emphasize the clinical applicability of miRNA-based diagnostics. Regarding diagnostic performance, the present study demonstrated that circulating miR-21-5p showed high sensitivity and specificity and exhibited diagnostic accuracy comparable to, and potentially superior to, conventional immunological methods [14]. Differences among microscopy, ELISA, and molecular markers are expected because each method targets different biological components of the infection. Microscopy depends on the presence and concentration of cysts in stool samples, ELISA detects parasite antigens, whereas circulating miR-21-5p reflects host immune responses. These differences may explain the variation in sensitivity and specificity observed among the diagnostic approaches. The role of miR-21-5p is not restricted to giardiasis. Previous studies have demonstrated dysregulation of miR-21 in several parasitic and infectious diseases, suggesting that this microRNA plays an important role in host–pathogen interactions and immune modulation [15]. Thus, the elevated expression observed in the present study may represent a common host response to parasitic infection rather than a phenomenon exclusive to Giardia lambl. Several limitations should be considered. Variations in fold change values among patients may be influenced by parasite burden, disease stage, and individual immune status. In addition, the relatively limited sample size and the single-center design may affect the generalizability of the findings. Therefore, larger multicenter studies are required to validate the diagnostic value of circulating miR-21-5p and to investigate its potential role as a marker of disease severity and prognosis.[19,20]

In conclusion, the present findings indicate that circulating miR-21-5p is significantly elevated in symptomatic Giardia lamblia infection and may represent a valuable non-invasive biomarker for diagnosis. These results contribute to the growing evidence supporting the use of circulating microRNAs in infectious diseases and may provide a basis for future studies exploring their clinical applications in parasitic infections.

Conclusion

Elevated levels of circulating miR-21-5p in *Giardia lamblia* patients relative to controls indicate that one of its main roles is as an infection-related pathology marker. Given the large difference in expression levels and high diagnostic quality, miR-21-5p could represent a potential noninvasive diagnostic index in giardiasis. Although the clear positive results are evident, more expansive investigation in a different population, including a broad range of individuals, is recommended to confirm and further clarify the clinical significance of miR-21-5p.

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Authors' Contribution

All authors contributed equally to this work.

Conflict of Interest

The authors declare no conflict of interest.

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