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Research Article

Diagnosis of Clinically Suspected Amoebic Liver Abscess Using Conventional PCR and Antigen ELISA in Liver Aspirate and Serum

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Abstract

Introduction: *Entamoeba histolytica*, a protozoan parasite causes the infectious disease amoebiasis. The majority of amoebiasis-related death are caused by extra-intestinal pathology, the most prevalent of which is an amoebic liver abscess. Here we analyzed the difference between the serological detection of antigens using ELISA and the molecular method using PCR in liver abscess patients.

Aim: To validate the diagnosis of clinically suspected cases of Amoebic liver abscess using PCR and/antigen ELISA in liver aspirate and serum samples.

Materials and Methods: Liver aspirate and blood samples were collected from 45 clinically suspected ALA patients. Those samples were subjected to microscopy, antigen ELISA, and PCR. The patient's demographic details along with clinical findings were noted and co-related. Here we considered liver aspirate PCR was gold standard and diagnostic accuracy of liver aspirate ELISA, serum ELISA, and serum PCR for identifying *E. histolytica* was evaluated.

Results: It was found that liver aspirate showed positive for *E.histolytica* DNA in 32 patients (71.1%) by PCR and for antigen in 45 patients (100%) by ELISA respectively. Serum sample showed positive for *E. histolytica* DNA in 4 patients (8.8%) by PCR and for antigen in 42 patients (93.3%) by ELISA. Our study found that abdominal pain was the chief complaint found in 44 patients (97.7%) followed by fever was seen in 27 patients (60%). Diabetes mellitus was the common co-morbidity (n = 13, 28.8%) followed by hypertension (n = 8,17.7%). Additionally found that 71.1% (n = 32) of patients had a habit of alcohol consumption and 35.5% (n = 16) were chronic smokers.

Conclusion: Our study found the importance of utilizing liver aspirate PCR and antigen ELISA for accurate diagnosis of Amoebic liver abscess, especially in patients presenting with typical symptoms and relevant co-morbidities.

Keywords: *Entamoeba histolytica*; Amoebiasis; Polymerase Chain Reaction; Serology

Abbreviations

ALA: Amoebic Liver Abscess; ALT: Alanine Transaminase; ALP: Alkaline Phosphatase; AST: Aspartyl Transaminase; ELISA: Enzyme Linked Immunosorbent Assay; PCR: Polymerase Chain Reaction

Introduction

Amoebic liver abscess is a secondary result of amoebiasis caused by *Entamoeba histolytica*, affecting over 50 million people annually and causing 100,000 fatalities [1]. Amoebiasis-related deaths are primarily caused by extra-intestinal pathology, with ALA being the most prevalent, accounting for 3-9% of total amoebiasis cases [2]. The US National Institute of Allergy and Infectious Diseases has designated *E. histolytica* as a category B priority biodefense pathogen due to its devastating consequences [3].

Various diagnostic techniques, including microscopy, antigen detection, serology, molecular tests, and imaging, are suggested for accurate diagnosis of ALA [4]. The current diagnostic techniques lack sensitivity, making it difficult to make an accurate diagnosis of *E. histolytica* infections in the laboratory. The laboratory diagnosis of amoebiasis is influenced by various factors such as the operator's experience, the chosen testing method, and the stage at which the test is conducted [5]. Unfortunately, diagnosis of ALA can be challenging for most clinical testing laboratories.

The domonstration of *E. histolytica* trophozoite in liver aspirate by microscopy confirm the diagnosis of ALA, however many studies quoted that only 15% of the liver aspirate were found to have amoebic trophozoite by direct microscopy [6]. In addition, an accurate diagnosis of ALA can be achieved by detecting amoebic antigens in the liver pus. A study from Bangladesh demonstrates the effectiveness of serum lectin antigen detection from liver aspirate with a sensitivity of 78%, the study provides a non-invasive and potentially more reliable alternative to conventional diagnostic technique [7]. Therefore, reliable identification of amoeba in clinical specimens is crucial for diagnostic purposes, patient care management, and avoiding unnecessary treatment of non pathogenic parasite-infected individuals with anti-amoebic drugs.

Materials and Methods

A cross-sectional study was conducted on clinically suspected ALA patients at a tertiary care hospital in South India from March 2022 to March 2023 (12 months). Ethics approval for this study was obtained from the institute ethics committee (JIP/IEC/2021/347). Informed consent was obtained from all patients before they were recruited into the study. The sample size for this study was calculated using a software OpenEpi version 3.01, by considering the sensitivity of ELISA to find out the liver abscess is 96.9%, absolute precision of 5% and confidence level of 95%.

The inclusion criteria for the study were, all the adult patients with clinically suspected ALA and the exclusion criteria were confirmed cases of malignancies and pediatric cases. Liver aspirates were collected from 45 patients and sent to the Microbiology department for routine parasitology investigations in a sterile container. Blood samples were collected from these patients with written informed consent. Liver aspirates were divided in the laboratory into three parts for microscopy, ELISA, and PCR. Similarly, the blood samples were divided into two parts for ELISA, and PCR. Demographic details like Age, gender, occupation, socio-economic status, clinical findings, co-morbidities were also recorded for each participant. Figure 1 depicts the workflow of sample processing.

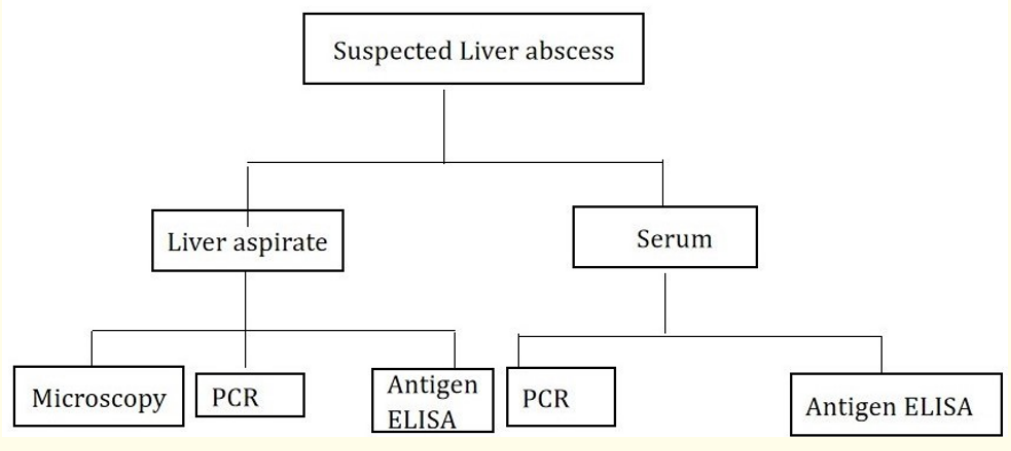

Figure 1: Workflow of Sample Collection and Processing.

Microscopy for trophozoites of *Entamoeba*

The liver aspirate samples were first centrifuged for 5 mins at 2500 g as soon as it was received in the laboratory. The supernatant was discarded and sediment was taken and mixed with a drop of warm saline on a microscopic slide. After making a fine suspension, a wet mount was prepared and observed microscopically using a high power (40x) objective and the results were noted [7].

Extraction and Nested PCR amplification of *Entamoeba* DNA from liver aspirates and serum samples

The *Entamoeba* genomic DNA was isolated from the liver aspirate samples using the QIAamp® fast DNA tissue Kit (QIAGEN-51404, Germany) and from serum samples using Qiagen blood mini kit (QIAGEN-51104, Germany). In the next step all the extracted samples were amplified by using the protocol which was based on the method optimized in the Department of Microbiology [6]. It involved two rounds of PCR both genus specific and species-specific PCR using set of primers targeted for an expected PCR product 16s rRNA gene extending from 174 bp to 553 bp (174 bp for *E. dispar,* 439 bp for *E. histolytica* and 553 bp for *E. moshkovskii*).

Primers used in the PCR

Entamoeba genus specific primer

Forward Primer 5' TAAGATGCACGAGAGCGAAA3'

Reverse Primer 5'GTACAAAGGGCAGGGACGTA3'

Entamoeba species specific primer

E. histolytica forward primer 50AAGCATTGTTTCTAGATCTGAG30

E. histolytica reverse primer 50AAGAGGTCTAACCGAAATTAG 30

E. dispar forward primer 50TCTAATTTCGATTAGAACTCT30

E. dispar reverse primer 50TCCCTACCTATTAGACATAGC 30

E. moshkovskii forward primer 50GAAACCAAGAGTTTCACAAC 30

E. moshkovskii reverse primer 50CAATATAAGGCTTGGATGAT 30

The amplification products were separated using electrophoresis through 1.5% agarose gel in Tris–borate-EDTA buffer at 120 V for 45 min and were visualized by ethidium bromide staining under UV light for bands of DNA of appropriate sizes ascertained in comparison to a standard 100 bp DNA ladder used as a molecular weight marker. Positive control (*E. histolytica* HM1: IMSS strain DNA) and negative control (Milli-Q water added instead of DNA) was included with each batch of samples analysed by nested-multiplex PCR.

ELISA (Enzyme linked immunosorbent assay) to detect Human *Entamoeba histolytica* Antigen (EH Ag)

ELISA was performed on both liver aspirates and serum samples using a commercial kit MYBIOSOURCE catalog number (MBS2607330) following the manufacturer's instructions. The collected whole blood and liver aspirate was centrifuged for 10 minutes at 1000-3000rpm and the supernatant was taken for assay. The optical density of each sample was obtained using an ELISA reader.

Statistics

The data collected were entered and analyzed in STATA software. The Continuous variables were summarized as mean/ median depending upon the distribution of data. Categorical variables were expressed as frequency with proportion. The outcome variables were summarized as proportions with a 95% confidence interval. The minimum level of statistical significance was set at a p-value <0.05%.

Results

In the current study, among 45 suspected ALA patients we found that 42 were male (93.33%), and 3 were female (6.67%). The study population had a mean age of 49.75 years and a standard deviation of 13.8 years. The majority were aged 25-74 years, with the most affected age group being 25-40 years (31.11%). Of these, 13 participants were primarily from Villupuram and Cuddalore (28.89%), followed by Puducherry (8.89%, n = 4) and Kallakurichi (6.67%, n = 3) respectively. It was found that 14 participants (31%) of the study's participants were agricultural laborers and 23 participants (51%) were daily laborers. Additionally, the study found that 32 participants (71.1%) had a habit of consumption of alcohol and 16 participants (35.7 %) were chronic smokers. Figure 2 depicts the age wise distribution of patients.

The study found that the majority of patients had abdominal pain (97.78%, n = 44), followed by fever (60%, n = 27), vomiting (31.11%, n = 14), cough (26.67%, n = 12), jaundice (22.22%, n = 10), and breathlessness (13.33%, n = 6) respectively. The predominant co-morbidity among patients was diabetes (28.88%, n = 13), followed by hypertension (17.77%, n = 8), chronic kidney disease (8.88%, n = 4), chronic liver disease (6.66%, n = 3) and chronic vascular disease (2.22%, n = 1) respectively. The mean (SD) value of liver enzymes such as Aspartyl transaminase (AST), Alanine transaminase (ALT), and Alkaline phosphatase (ALP) was 69.6 (73.8) U/L, 51.5 (51.06) U/L and 255.7 (155.9) U/L respectively.

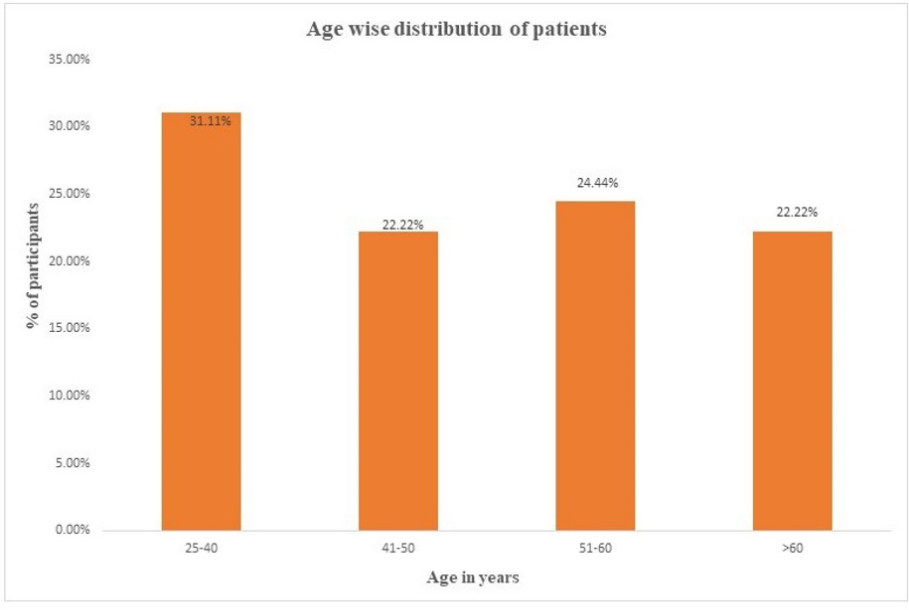

Figure 2 : Age-wise distribution of patients

In our study, out of 45 liver aspirate samples one was found to be positive for trophozoite of *Entamoeba* by microscopy. Figure 3 depicts the microscopic appearance of trophozoite in liver aspirate.

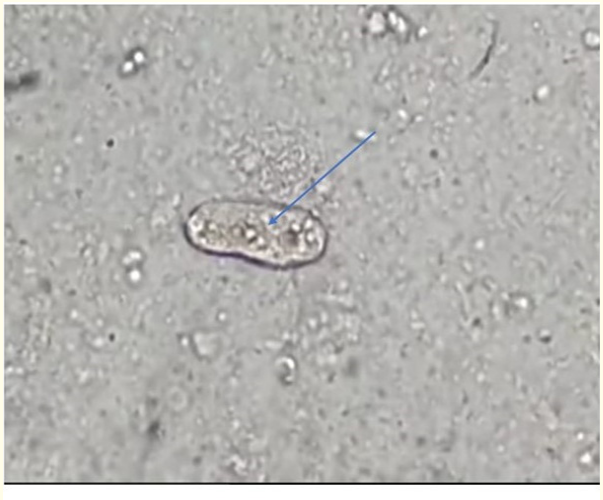

Figure 3:Trophozoite of *Entamoeba histolytica*

All 45 liver aspirate and serum samples underwent nested multiplex PCR and antigen ELISA. Among them, 32 liver aspirate samples (71.1%) and 4 serum samples (8.89%) were showed positive results by PCR. Antigen was detected in 45 liver aspirate samples (100%), and 42 serum samples (93.3%) respectively. Figure 4 depicts the gel images of PCR.

In our study Liver aspirate PCR was taken as the gold standard method and diagnostic accuracy of various methods was evaluated and expressed in terms of Sensitivity, Specificity, PPV, NPV, and 95 % confidence interval. Table 1 Showing diagnostic accuracy of different diagnostic methods. This study does not include the bacteriological workup and sequencing procedure; hence it was not carried out.

Discussion

Amoebic liver abscess is an extra-intestinal manifestation caused by protozoa called *E. histolytica*. It is predominantly seen in tropical countries due to poor hygiene, poor sanitation, and poor socio-economic status. The transmission is mainly via the fecal-oral route. Therefore, accurate diagnosis and proper treatment are required to reduce mortality associated with ALA.

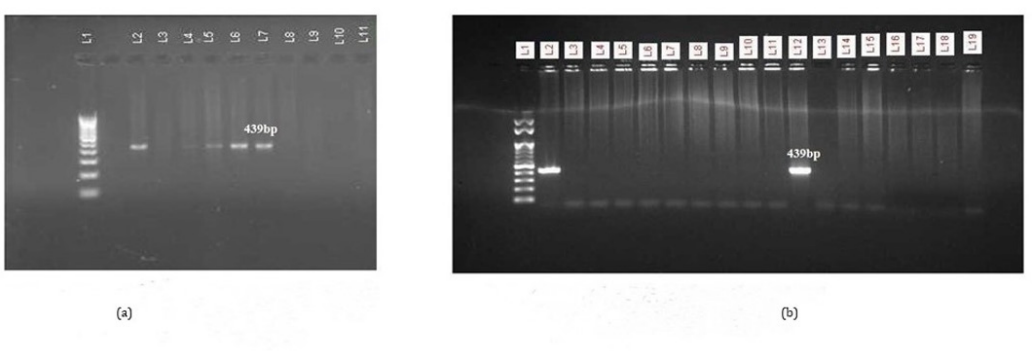

Figure 4: (a) Gel images of liver aspirate samples. Lane 6 & Lane 7 positive samples. (b) Gel images of serum samples Lane 12 positive samples. Lane 1 – 100 bp ladder, Lane 2- positive control, Lane 3- Negative extraction control (NEC), Lane 4- Negative template control (NTC)

	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)
Serum PCR	9.38% (0.86% -17.89%)	92.31% (84.52% -100.09%)	75% (62.35% -87.65%)	29.27% (15.97% -42.56%)
Serum antigen ELISA	93.75% (86.68% -100.82%)	7.69% (1%-15.48%)	71.43% (58.23% - 84.63%)	33.33% (19.56% - 47.11%)

		Liver aspirate PCR		Total
		Positive	Negative	(n = 45)
Serum ELISA	Positive	30	12	42
	Negative	2	1	3
Serum PCR	Positive	3	1	4
	Negative	29	12	41

Table 1: Diagnostic accuracy of different methods.

The study found that the most affected age group was 25-40 years (31.11%), with the oldest being 74 years old and the youngest being 25 years. Similar results were found in Aradhana., *et al*. study were the most affected age group was 35 to 55 (54.5%) years of age [8]. A study from Srilanka also found that the most common age group infected was 31-50 years (94.2%) [9].

The study involved 45 participants, of which 42 were men and 3 were women. In our study, men constituted the majority, (93.3%) of which 32 participants (71.1%) had a habit of alcohol consumption. Several studies have shown that men were more affected than women, a study by Chaudhary., *et al*. showed 86.8% of the study population were men [10]. In another study done in Qatar, it was found that 9% of ALA patients were women [11]. This significant male predominance may be explained by the high levels of alcohol consumption among male participants which lead to liver damage, a major contributing factor to the development of ALA. Furthermore, iron is a mineral found in alcohol, which makes it more invasive in adult men [12].

It was shown that the majority of study participants were day labourers (51%) and agricultural labourers (31%). Almost all patients were from lower socioeconomic backgrounds. The main cause of acquiring ALA is due to poor hygiene practices and poor sanitary conditions, which are frequently observed among those from low socioeconomic backgrounds. A study from north India found that amoebiasis is prevalent in the country due to poor sanitation, inadequate urban services and poor socio-economic status with 3-9% cases linked to ALA [13].

The study found that most patients presented with complaints of abdominal pain (97.78%, n = 44), followed by fever (60%, n =

27) and loose stool (11.11%, n = 5). This findings is consistent to a study conducted by Ghosh., *et al*. in North India, where abdominal pain (99%, n = 144) and fever (94%, n = 188) were the predominant complaints [2]. Another uncommon complaint was vomiting (31.11%, n = 14), which may be due to medication. Pulmonary amoebiasis, an extra-intestinal manifestation, affects 7-20% of patients, with cough, dyspnea, and hemoptysis being the most typical symptoms [14]. In this study, cough was present in 12 patients (26.67%). Studies conducted in north India showed that cough was presented in (3.5%, n = 2) and (16%, n = 32) cases respectively [2,13].

The study found that jaundice was the least common complaint among patients with amoebic liver abscess, with 10 cases (22.22%). Another study conducted in Kolkata showed that jaundice was seen in 11 patients (14.67%) [15]. This occurrence may be attributed to damage in the bile duct and various vascular structures. Loose stool was the least common complaint, with 5 patients [11.11%] experiencing it. A study by Sharma., *et al*. reported 10.5% [n = 9] cases of diarrhea [13]. Diarrhea is one of the clinical symptoms which was seen in intestinal amoebiasis and also present in amoebic liver abscess.

The study found that diabetes mellitus (28.8%, n = 13) was the most common co-morbidity among participants with ALA, followed by hypertension (17.7%, n = 8). A similar study by Jha., *et al*. reported diabetes mellitus was the predominant co-morbidity among 33.64 % (n = 37) patients [16]. A study showed that diabetes mellitus was more often related to amoebic liver abscess (33.5%, n = 443) than pyogenic liver abscess (19.3%, n = 36) [17]. Therefore, appropriate glycaemic management is necessary to minimize infection severity and improve clinical results. One patient had tuberculosis as a co-morbidity, while in another study tuberculosis was present in 25% (n = 40) patients [18].

The study found varying levels of aspartate transaminase (AST) and alanine transaminase (ALT), with AST having a mean of 69.6 IU/L and ALT having a mean of 51.5 IU/L. Alkaline phosphatase (ALP) had a mean of 255.5 IU/L. A study revealed that ALP, AST, and ALT were elevated in 60 %, 50%, and 50% of ALA patients respectively [19]. ALP elevation was predominant among most patients, making it as a reliable indicator for diagnosing amoebic liver abscess.

Our study found that only 2.2% (n = 1) of liver aspirate samples tested positive for *Entamoeba* trophozoites using a microscopic technique. The low sensitivity of microscopy to detect trophozoites of *Entamoeba* may be due to a delay in the collection and transport of samples. When samples come in contact with air during aspiration, the trophozoites lose their viability and motility. Similar results were found in Puducherry and Aradhana Singh., *et al*. study where 1.5% (n = 63) & 5.2% (n = 115) liver aspirate were positive for trophozoite of *Entamoeba* by microscopy [8,20].

The study found that *Entamoeba histolytica* DNA was detected in 32 (71.11%) liver aspirate samples using nested multiplex PCR. Previous studies by Jaiswal., *et al*. and Parija., *et al*. showed that *Entamoeba* DNA was detected from 83.5 % o & 80.4% liver aspirate [7,8]. So we can say that many studies have utilised nested multiplex PCR to detect *Entamoeba* DNA and which is considered as one of the most sensitive and specific methods.

In the current study, *Entamoeba* DNA was detected in 4 (8.8%) serum samples. Among the 4 positives, 3 were positive by liver aspirate PCR also. The difference in PCR results between serum and liver aspirate in ALA may occur due to variations in sample timing, site specificity, or the presence of low parasitic load. PCR in serum may detect circulating DNA, while liver aspirate PCR targets the abscess site directly [20]. Here our study found that PCR in serum had high specificity (92.3%) and low sensitivity (9.4%) when compared to PCR in liver aspirates.

The study found that ELISA had high sensitivity in detecting *E. histolytica* antigen in liver aspirate and serum samples, with detection rates of 95.5% and 100%, respectively. This was in line with previous studies, where antigen detection was found in 99.4% and 100%, respectively using an *E. histolytica/E. dispar* antigen detection ELISA kit from Diagnostic Automation/Cortez Diagnostics, Inc. (California, USA) [9]. A study in China revealed that 97.5% of ALA cases tested positive for amoebic antigen [21]. Antigens found in serum and liver aspirate suggest an ongoing infection. ELISA offers advantages over other techniques for diagnosing amoebiasis, such as microscopy, culture, and antibody detection assays [22].

In our study we aimed to evaluate the effectiveness different diagnostic methods for detecting *Entamoeba histolytica.* Here we considered PCR conducted on liver aspirate as gold standard method. We also assessed the diagnostic accuracy of PCR in serum, antigen ELISA in serum, and liver aspirate respectively with liver aspirate PCR. Unfortunately, our evaluation of the diagnostic accuracy of ELISA in liver aspirate was limited. This was because all liver aspirate samples tested positive for amoeba antigen using ELISA. As a result, we were unable to obtain negative results for comparison. Therefore, we could not fully determine the diagnostic accuracy of ELISA in liver aspirate compared to PCR.

Here we found that antigen ELISA in serum showed high sensitivity (93.7%) with a 95% confidence interval of 86.6 -100.8 & PCR in serum was more specific (92.3%) with a 95% confidence interval of 84.5-100.09 respectively. A study by Parija., *et al*. on liver abscess pus specimen, urine, and saliva using different laboratory methods such as nested multiplex PCR, IHA, and ELISA. He found that *E. histolytica* DNA was detected in 80.4% of liver abscess pus specimens and 39.6% urine samples, respectively by PCR. The ELISA for the detection of lectin antigen in liver abscess pus showed a sensitivity of 50% and the indirect haemagglutination (IHA) for detecting antibodies in the serum showed a sensitivity of 76.8% respectively [7]. Hence using a combination of PCR analysis in serum and liver aspirate, along with ELISA assays, creates a strong diagnostic strategy for accurately identifying ALA. This method improves the reliability of the diagnosis, giving a deeper insight into the diseases in both blood and liver aspirate samples and adding valuable information to ALA diagnostics.

During our study, one patient passed away, despite being negative by liver aspirate PCR and serum PCR, but positive by both serum and liver aspirate antigen ELISA. A study revealed that 5 patients died (7.9%), three of whom tested positive for *E. histolytica* and one had *Escherichia. coli* infection [20]. The ALA can lead to severe complications, including abscess rupture into the peritoneum, pleural space, or pericardium, which can be fatal [23].

Conclusion

Our study on diagnosis of amoebic liver abscess among clinically suspected patients found a high prevalence of risk factors like alcohol consumption and smoking, as well as clinical findings like abdominal pain and fever. These findings emphasize the need for targeted preventive strategies and health interventions. The diagnostic test PCR and antigen ELISA showed promising results, these methods could be valuable, especially where invasive procedures are limited.

However, the study's limitations, including the lack of bacteriological workup and sequencing procedures, emphasize the need for further research to validate and refine the diagnostic algorithms proposed in this study. Future studies incorporating microbiological and molecular analyses could provide a more comprehensive understanding of the etiology and pathogenesis of ALA, paving the way for improved diagnostic and therapeutic strategies.

Our findings emphasize the importance of early diagnosis and intervention, especially in regions with high ALA prevalence and among individuals with specific risk factors.

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Conflict of Interest

The authors declare no conflicts of interest.

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