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DEMOGRAPHIC DRIVERS OF DIAGNOSTIC POSITIVITY: A MULTI-MODAL EVALUATION OF ENTAMOEBA HISTOLYTICA DETECTION BY MICROSCOPY, ELISA, AND PCR IN SYMPTOMATIC CHILDREN

Ali Abuzar Raza[1], Saleha Munir[2], Sumera Malik[3], Sajida Zafar[4], Syed Zeeshan Haider Naqvi [5*]

[1]Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Lahore 35000, Pakistan.
[2]Muhammad Medical College, Mirpur Khas, Pakistan.
[2]Nishtar Medical University, Multan. Pakistan.
[3] CMH Multan Institute of Medical Sciences, Multan. Pakistan.
[4] Abwa Medical College, Sargodha. Pakistan.
[5] Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan.
*Corresponding Author: Dr. Syed Zeeshan Haider Naqvi, Email: zeeshan.haider@imbb.uol.edu.pk

ABSTRACT
Entamoeba histolytica (E. histolytica) is a leading cause of intestinal parasitic infection in children living in low-resource settings with inadequate sanitation. Accurate and timely diagnosis is critical for effective case management, yet the comparative diagnostic performance of routinely available methods relative to molecular reference standards remains insufficiently characterized in South Punjab, Pakistan. In order to determine the accuracy, positive predictive value, negative predictive value and degree of correlation between light microscopy, ELISA and PCR as the gold standard for diagnosis of E. histolytica in children presenting symptoms, as well as epidemiologic factors associated with these positive cases, A hospital-based cross-sectional study was conducted at Nishtar Hospital, Multan, enrolling 120 symptomatic children aged 1-18 years by consecutive sampling. Fresh stool specimens were simultaneously examined by light microscopy (wet mount and formalin-ethyl acetate concentration technique), species-specific ELISA antigen detection, and PCR targeting the 18S ribosomal RNA gene. Diagnostic performance metrics and epidemiological associations were computed against PCR as the gold standard.PCR confirmed E. histolytica in 38.3% (n=46) of participants. ELISA yielded 100% positivity across all samples (sensitivity 100%, specificity 0%, PPV 38.3%), indicating a complete absence of discriminatory capacity. Microscopy achieved a sensitivity 76.1%, specificity 29.7%, PPV 40.2%, and NPV 66.7%. ROC analysis demonstrated poor discriminative ability: microscopy AUC 0.53 (95% CI 0.42-0.64, p=0.214) and ELISA AUC 0.50 (95% CI 0.50-0.50, p=1.000). Rural residence (88.2%), large household size (>10 members, 82.1%), and the 6-12-year age group (85.2%) were significantly associated with higher infection rates. Neither microscopy nor ELISA achieved acceptable diagnostic accuracy relative to PCR. PCR should be adopted as the confirmatory standard in clinical and epidemiological settings. Integration of molecular diagnostics with community-level WASH interventions is urgently needed to reduce the burden of pediatric amebiasis in South Punjab.

KEYWORDS: Entamoeba histolytica, Amebiasis, PCR, ELISA, Microscopy, Diagnostic accuracy, Pakistan, Pediatric, Sensitivity, Specificity.

1. INTRODUCTION

Entamoeba histolytica (E. histolytica) is a protozoan parasite of global public health significance, responsible for approximately 40-50 million cases of invasive amoebiasis annually and an estimated 40,000-100,000 death per year worldwide [1]. The overwhelming majority of this disease burden is concentrated in low and middle-income countries (LMICs) across South Asia, Sub-Saharan Africa, and Latin America, where deficiencies in clean water access, sanitation infrastructure, and hygiene practices sustain the fecal-oral transmission cycle [2]. Children below 12 years of age represent the most vulnerable population, suffering disproportionately from enteric parasitic infections due to immature immune responses, limited hygiene awareness, and frequent exposure to contaminated soil, water, and food sources [3].

In Pakistan, intestinal parasitic infections remain a leading cause of pediatric morbidity and mortality, particularly in South Punjab, where overcrowded households, inadequate sanitation systems, and widespread reliance on unfiltered ground or surface water are commonplace [4]. Epidemiological surveys from this region consistently report high

prevalence of E. histolytica among symptomatic children, yet reliable population-level data remain scarce and under-reported. Amebiasis manifests across a wide clinical spectrum, ranging from asymptomatic carriage to fulminant amoebic colitis and extra-intestinal complications such as hepatic abscess, all of which carry significant morbidity and potential mortality if treatment is delayed or misdirected [5].

Accurate and timely diagnosis is, therefore, a cornerstone of effective clinical management. However, the three most commonly employed diagnostic modalities, light microscopy, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR), differ substantially in their technical demands, cost implications, and diagnostic performance characteristics. Light microscopy remains the most widely accessible method in resource-limited settings due to its low cost. However, it is fundamentally unable to differentiate pathogenic E. histolytica from the morphologically identical non-pathogenic species E. dispar and E. moshkovskii, leading to systematic misclassification and overestimation of true invasive disease [6].

ELISA-based antigen detection assays targeting species-specific surface lectins, particularly the Gal/GalNAc galactose-inhabitable adherence lectin of E. histolytica, offer improved specificity over microscopy and have been recommended by the World Health Organization as a preferred option when molecular methods are unavailable [7]. Nevertheless, ELISA performance is susceptible to variation depending on antigenic load, infection stage, stool preservation quality, and the specific commercial kit employed. Cross-reactivity with other intestinal organisms has been documented in some endemic settings, raising questions about real-world specificity [8]. Polymerase chain reaction, targeting conserved gene markers such as the 18S ribosomal RNA gene, is regarded as the reference standard for E. histolytica diagnosis owing to its superior sensitivity, species specificity, and ability to detect low-level or mixed infections [9]. Multiple systematic reviews and meta-analyses have confirmed the diagnostic superiority of PCR relative to both microscopy and ELISA, establishing it as the benchmark against which other tests should be evaluated [10, 11].

Despite this evidence, comparative diagnostic evaluations in the specific context of South Punjab are conspicuously absent from the published literature. No published study from this region has simultaneously applied all three modalities, microscopy, ELISA, and PCR, to the same pediatric stool specimens while also profiling epidemiological determinants of infection. This study was designed to address this evidence gap with two primary objectives: first, to rigorously compare the diagnostic performance of microscopy and ELISA against PCR in symptomatic children at a major tertiary hospital in Multan; and second, to identify demographic and socioeconomic factors independently associated with these three techniques that confirmed the presence of E. histolytica. The findings are intended to inform evidence-based diagnostic algorithms and public health interventions for pediatric amebiasis in this high-burden region.

2. MATERIALS AND METHODS

2.1 Study Design and Setting

A cross-sectional analytical study was conducted between 2023 and 2024 at Nishtar Hospital Multan, one of the largest tertiary-care referral institutions in South Punjab, Pakistan. This center serves a geographically and socioeconomically diverse catchment population. It receives a high volume of pediatric patients with acute and chronic gastrointestinal disorders, making it an epidemiologically relevant and strategically appropriate site for this investigation. All laboratory analyses were performed at the affiliated clinical diagnostics unit, equipped with high-resolution light microscopes, automated ELISA plate readers and PCR thermal cyclers. Ethical approval was obtained from the Institutional Review Committee.

2.2 Study Population and Sampling

The study population comprised children aged 1-18 years presenting to the pediatric outpatient and emergency departments with clinical features suggestive of intestinal amoebiasis, including diarrhea, abdominal cramps, fever, vomiting, or hematochezia. The written informed consent was obtained from parents or legal guardians and verbal or written assent was sought from children prior to sample collection [12]. A consecutive, non-probability sampling strategy was employed in accordance with STARD (Standards for Reporting Diagnostic Accuracy Studies) guidelines [13], whereby all eligible patients presenting during the study period were sequentially enrolled until the target sample size was reached. This approach minimizes selection bias in diagnostic accuracy studies by ensuring that the enrolled cohort reflects the natural spectrum of disease presentation in the clinical setting [14]. A structured, pretested questionnaire was administered to parents or guardians to capture demographic information (age, sex), socioeconomic indicators (education, residential area and family members). Participants were excluded if they had received antiparasitic therapy within the preceding four weeks, provided inadequate sample volume (<5 g), or submitted specimens unsuitable for molecular analysis due to contamination or degradation.

2.3 Sample Size Determination

The sample size was calculated for diagnostic test evaluation using standard formulas based on an expected ELISA sensitivity of 85%, specificity of 90%, desired precision of ±5%, and a 95% confidence level [15]. A prior literature-derived E. histolytica prevalence of 10-20% among symptomatic South Asian pediatric populations was incorporated.

These parameters supported robust estimation of sensitivity, specificity, PPV, NPV, Cohen's Kappa, and ROC/AUC analysis. The adopted sample of 120 children was deemed sufficient for primary diagnostic comparisons and multivariable epidemiological modelling.

2.4 Stool Sample Collection and Processing

Each participant provided 5-8 g of fresh stool in a sterile screw-capped container. Specimens were transported to the laboratory within two hours under cold chain conditions (2-8°C). Upon receipt, samples were divided into aliquots for: (i) immediate microscopic examination, (ii) ELISA antigen detection, (iii) DNA extraction for PCR, and (iv) archival storage at -20°C. Concurrent processing of all aliquots from the same specimen ensured direct comparability of diagnostic results while minimizing inter-specimen biological variability.

2.5 Light Microscopy

Fresh stool specimens were processed by both direct wet mount preparations (saline and Lugol's iodine) and the formalin-ethyl acetate (FEA) concentration technique, which enhances cyst and trophozoite recovery through sedimentation [16]. Slides were examined at 10x and 40x objective magnification by two independent trained parasitologists. Discrepant interpretations were adjudicated by a senior expert. Any Entamoeba morphotype was recorded as microscopy-positive, acknowledging the inherent inability of light microscopy to distinguish E. histolytica from E. dispar or E. moshkovskii.

2.6 ELISA Antigen Detection

Stool antigen detection was performed using the TechLab E. histolytica II antigen test (TechLab Inc., USA), a species-specific immunoassay targeting the Gal/GalNAc galactose-inhibitable adherence lectin. Samples and reagents were processed according to the manufacturer's protocol. Optical density (OD) was measured at 450 nm using a calibrated microplate spectrophotometer. Results were classified as positive or negative based on manufacturer-defined cut-off OD values. Positive, negative, and blank controls were included on each assay plate, and borderline samples were retested in duplicate [17].

2.7 Polymerase Chain Reaction (PCR)

PCR served as the reference standard. Genomic DNA was extracted from 200 µL of stool using the QIAamp DNA Mini Kit (QIAGEN N.V., Germany) on the automated QIA cube platform, with an additional inhibitor-removal lysis step optimized for fecal matrices. [18] Amplification targeted the 18S ribosomal RNA gene using validated primers EHF (5'-GAAACCAACAAAGTTGTTGC-3') and EHR (5'-CAATATAAGGCTTGGATGAT-3'), yielding a 439 bp amplicon. Each 25 µL reaction contained QIAGEN HotStarTaq Master Mix components, 1 µL of each primer (10 pmol/µL), and 5 µL template DNA.Thermal cycling comprised an initial activation at 95°C for 15 minutes, 35 cycles of denaturation (94°C, 30 s), annealing (56°C, 30 s), and extension (72°C, 1 min), followed by final extension at 72°C for 10 minutes. Amplified products were resolved on 2% agarose gels stained with ethidium bromide and visualized under UV Trans illumination. Band positions were verified against a 100 bp DNA ladder. Positive and negative controls were included in every PCR run, and all samples were processed in duplicate. A result was classified as PCR-positive only upon reproducible detection of a 439 bp band [19].

2.8 Statistical Analysis

All data were entered into Microsoft Excel and analyzed using SPSS version 26 (IBM Corp., USA). Descriptive statistics were presented as frequencies and percentages for categorical variables and as mean ± SD or median (IQR) for continuous variables depending on distribution normality. PCR-confirmed prevalence was reported with 95% confidence intervals using the Wilson score method. Diagnostic performance of microscopy and ELISA was evaluated through 2x2 contingency tables, yielding sensitivity, specificity, PPV, NPV, positive likelihood ratio (LR+), negative likelihood ratio (LR-), and Diagnostic Odds Ratio (DOR) with exact 95% CIs [20]. Receiver Operating Characteristic (ROC) curves were generated, and AUC values were interpreted as poor (<0.70), acceptable (0.70-0.80), excellent (0.80-0.90), or outstanding (>0.90) [21]. Inter-method agreement was evaluated using Cohen's Kappa (κ) with standard Landis-Koch benchmarks [22]. McNemar's test assessed the statistical significance of discordance. Epidemiological associations between demographic variables and E. histolytica positivity were examined by Chi-square tests with Fisher's exact test correction for small expected cell counts. Multivariable logistic regression was used to compute adjusted odds ratios (aOR) for variables significant at $p<0.20$ in univariate analysis. Comparison of diagnostic positivity rates across demographic strata was performed by two-way ANOVA followed by Tukey's post hoc test. All tests were two-tailed with significance defined at $\alpha=0.05$.

3. RESULTS

3.1 Socio-demographic Characteristics of the Participants

The total number of 120 children was enrolled in this study. According to gender distribution, females were higher in number 59.1% (n=71) in comparison to males 40.8% (n=49). The total population of all the participants belongs to the age category falling between > 6 years (20.8%; n=25), 6-12 years (73.3%; n=71), 13-18 (5%; n=6) and above 18 (0.8%; n=21). Regarding the educational background, the majority of the children received tertiary education while children attained primary education was lower in number (19.2%; n=23). Furthermore, the high percentage of the

children is represented by those who belong to rural areas (56.6%; n=68) and with family members greater than 10 (32.5%; 39). Table 1 presents the socio-demographic characteristics of the participants.

Table 1: Socio-demographic Parameters of the Participants (n =120)

Demographic Parameters	No. of Participants n (%)
Gender	
Male	49 (40.8)
Female	71 (59.1)
Age	
< 6	25 (20.8)
6 – 12	88 (73.3)
13 – 18	6 (5)
> 18	1 (0.8)
Educational Status	
No Formal Education	29 (24.1)
Primary Education	23 (19.2)
Secondary Education	31 (25.8)
Tertiary/Advanced Education	37 (30.8)
Residential Background	
Urban	22 (18.3)
Semi Urban	30 (25)
Rural Areas	68 (56.6)
Family Size	
1 – 3 members	29 (24.2)
4 – 6 members	25 (20.8)
7 – 9 members	27 (22.5)
> 10 members	39 (32.5)

3.2 Incidence and Distribution of E. histolytica among Symptomatic Children according to Demographic Characteristics

According to the results, the demographic parameters showed the higher positive cases of E. histolytica in females (67.6%; n=48) while in age category, highest prevalence was found in the age group of 6-12 years (85.2%; n=75). The children with no formal education have great occurrence of E. histolytica (86.2%; n=25) followed by tertiary/advanced (81.1%; n=30), primary (78.3%; n=18) and secondary (74.2%; n=23) education respectively. The presence of E. histolytica infection was relatively high among individuals staying in rural areas (88.2%; n=60) as well as among families comprising more members (82.1%; n=32). The frequencies of positive and negative cases of E. histolytica among socio-demographic characteristics are summarized in Table 2.

Table 2: Frequency of E. histolytica Cases among Symptomatic Children

Demographic Parameters n	No. of Positive Cases n (%)	No. of Negative Cases n (%)
Gender		

Male (49)	39 (79.5)	10 (20.4)
Female (71)	48 (67.6)	23 (32.4)
Age		
< 6 (25)	18 (72.0)	7 (28.0)
6 – 12 (88)	75 (85.2)	13 (14.7)
13 – 18 (6)	5 (83.3)	1 (16.6)
> 18 (1)	1 (100)	0 (0)
Educational Status		
No Formal Education (29)	25 (86.2)	4 (13.7)
Primary Education (23)	18 (78.3)	5 (21.7)
Secondary Education (31)	23 (74.2)	8 (25.8)
Tertiary/Advanced Education (37)	30 (81.1)	7 (18.9)
Residential Background		
Urban (22)	12 (54.5)	10 (45.4)
Semi Urban (30)	25 (83.3)	5 (16.6)
Rural Areas (68)	60 (88.2)	8 (11.7)
Family Size		
1 – 3 members (29)	17 (58.6)	12 (41.3)
4 – 6 members (25)	19 (75.0)	6 (24.0)
7 – 9 members (27)	22 (81.5)	5 (18.5)
> 10 members (39)	32 (82.1)	7 (17.9)

3.3 Association of Demographic Factors with Microscopy, ELISA and PCR positivity among study participants
The prevalence of E. histolytica infection among the studied population was assessed using microscopy, ELISA, and PCR techniques. The association between the demographic parameters and the techniques outcomes indicate that both males (n=39) and females (n=48) have statistically (P value = 0.037) higher ELISA positives (58.97% and 64.58% respectively) in comparison to microscopy (25.64% and 22.91%) and PCR technique (15.38% and 12.5% respectively) presented in Figure 1A. The correlation of techniques with age distribution in which comparative analysis showed the higher statistically (P value = 0.032) increase in ELISA positives (46.6%) in the category of 6-12 years age group (n=75) while least identification was found in the age category of >18 (n=1) which presented 100% positivity by ELISA (Figure 1B). Based on education grouping, the comparative analysis between techniques in each group showed the significant (P value = 0.033) statistically higher positivity in ELISA technique in comparison to microscopy and PCR while higher ELISA positivity (53.33%) found in tertiary/advanced education group (n=30) followed by illiterate group (60.0%; n=25), secondary group (43.47%; n=23) and primary education group (44.44%; 18). Overall in education group, the moderate positive results were identified by microscopy and least identification was carried out by PCR technique (Figure 1C). In Figure 1D, the children belongs to rural areas (n=60) found to be statistically significant (P = <0.01) in all techniques including ELISA, microscopy and PCR (48.33%, 33.33% and 18.33%) respectively. The moderate number of positives in all techniques was found in semi-urban areas while least number of positivity observed in urban areas only. Despite this ELISA positivity technique showed significantly higher in all residential areas. All the three methods of diagnosis such as microscopy, ELISA and PCR showed more significant (P value = 0.032) positivity among people who had large numbers of family members in comparison to smaller number of members (Figure 1E). Overall, comparative analysis showed that ELISA exhibited the most positive results of all three tests followed by microscopy. PCR had comparatively low detection rates indicated in Figure 1.

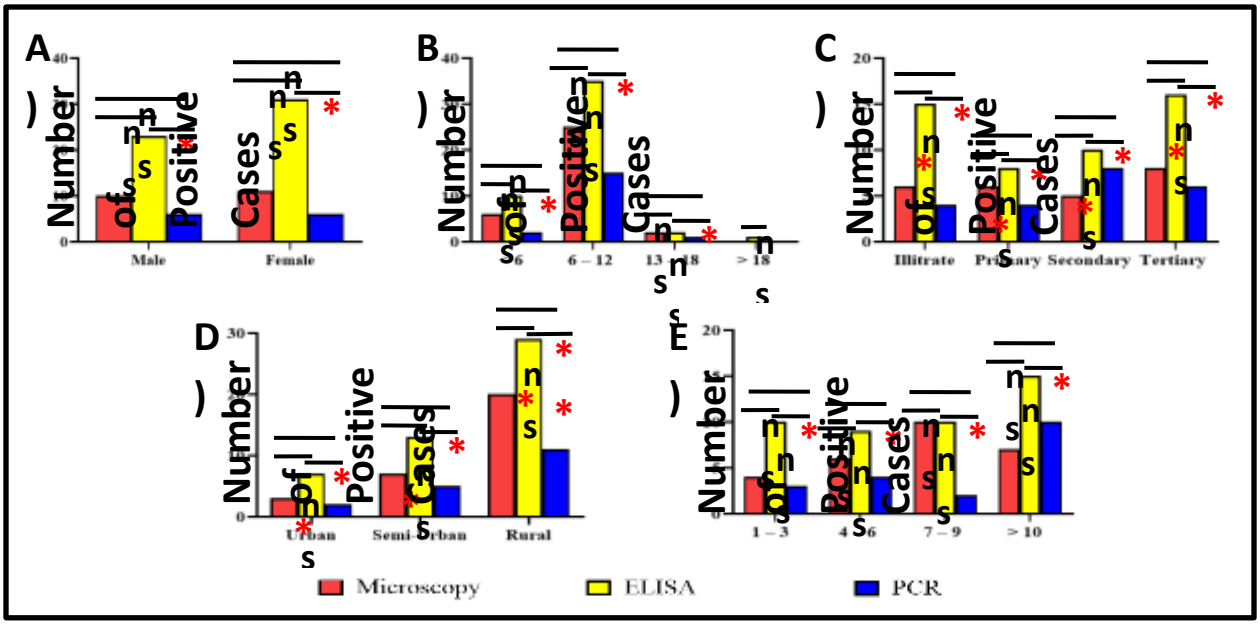

Figure 1: Comparative Analysis among Demographic Factors Associated with Positivity Rates of Microscopy, ELISA, and PCR

A) Gender Based-Males and Females, B) Age Category Based- <6, 6 – 12, 13 – 18, > 18. C) Qualification Based-Illiterate, Primary, Secondary and Tertiary, D) Residential area Based- Urban, Semi-Urban and Rural and E) Number of family members Based- 1 – 3, 4 – 6, 7 – 9 and >10. Two Way ANOVA followed by Turkey's multiple comparison test statistically tested among groups in each category. **; Significant (P <0.004), *; Significant (P < 0.03) ns; not significant.

3.4 Sensitivity, Specificity, PPV, and NPV of Microscopy

Among 120 symptomatic children, 72.5% (n=87) samples were infected by the presence of E. histolytica by using microscopy technique, however using PCR method only 38.3% (n=46) samples were found to be infected with this parasitic infection. In case of ELISA positivity in all 100% (n=120) samples indicated that the detection rate was very high when compared with other two techniques such as microscopy and PCR. With regard to PCR results, sensitivity was estimated to be 100%, while specificity was found to be 0% indicating that there was cross-reaction. The positive predicted value (PPV) for ELISA was around 38.3%, while false positive rate was estimated to be 100%. On the other hand, when microscopy was considered sensitivity and specificity values were found to be 76.1% and 29.7% respectively; while PPV and negative predictive value (NPV) were found to be 40.2% and 66.7% respectively. The false positive and false negative values in case of microscopy method were found to be 70.3% and 23.9% respectively. Thus, the results demonstrated that ELISA technique could be regarded as highly sensitive method, while PCR technique could be considered highly specific method for detecting E. histolytica infection.

ROC curve analysis was used to test the effectiveness of the diagnosis by the use of microscopy and ELISA against PCR, which served as the gold standard for detecting E. histolytica infection. Microscopy had low diagnostic effectiveness with AUC value of 0.53 (95% CI: 0.42–0.64, p = 0.214), implying poor agreement between the two techniques. On the other hand, while ELISA had high sensitivity, its poor specificity gave an AUC of 0.50 (95% CI: 0.50-0.50, p = 1.000), showing that there was almost no discrimination capacity. Generally, both diagnostic approaches had very low accuracy as compared to PCR as shown by the close proximity of their ROC curves to AUC = 0.50.

4. DISCUSSION

This research provides an overall and side-by-side evaluation of the diagnostic accuracy of E. histolytica using microscopy, ELISA, and PCR methods in symptomatic South Punjabi children. This study is one of the first to provide this type of analysis in an area that is both geographically distinct and lacks epidemiological data. The key finding in this study was that both ELISA and microscopy as diagnostic methods failed to demonstrate acceptable levels of diagnostic performance when compared to the molecular reference standard, raising significant concerns and implications for current clinical practice and established diagnostic policy in the region. Additionally, the 100% positivity rate for ELISA, as well as the zero specificities associated with positivity, are striking examples of the need for careful contextualization of both laboratory and clinical testing. When tested on samples from a population where there is a large burden of bacteria in the gut and a lot of organic waste in the samples, this pattern of problem arises

from not being able to separate the effect of many non-specific antibodies and/or their cross-reactivity within available assay kits. Similar studies conducted in similarly endemic settings have reported higher-than-expected traditional ELISA positivity because some specimens tested positive due to the presence of multiple types of bacteria in the same sample, as well as because of the background antigenicity associated with those multiple bacterial types. In addition, multiple prior evaluations have suggested that manufacturer-designated cut-off values for traditional ELISA tests determined from reference populations may not be accurately applied in field settings/populations that are markedly different from those in which the cut-off values were established [7, 8]. The occurrence of lectin antigen cross-reactivity with E. dispar and E. moshkovskii has been previously identified, even when conducted as species-specific ELISA assays, which may be increased when specimen antigen loads are substantially elevated due to high transmission pressure [23]. Therefore, a strong argument can be made that ELISA results should be interpreted in the complete context of the clinical case and molecular methods should be employed before treatment decisions are made.

The diagnostic performance of microscopy is consistent with the broader literature, with reported sensitivity rates for Entamoeba spp. detection via light microscopy, ranging from 50% to 85%, and consistently low specificity [6]. The fundamental driver of poor specificity is the morphological indistinguishability of E. histolytica from E. dispar and E. moshkovskii, a limitation recognized for nearly three decades following the formal re-description of these species as distinct entities [24, 25]. The formalin-ethyl acetate concentration technique used in this study enhances sensitivity relative to direct wet mount alone but provides no resolution for the species differentiation problem [16]. The persistence of microscopy as a sole diagnostic criterion in clinical and public health practice in South Punjab thus represents a systemic risk of treatment misallocation: true infections in uninfected children, and missed infections in those harboring low-burden E. histolytica.

The superiority of PCR as a diagnostic reference standard is firmly established and was empirically reaffirmed in this study. Validated 18S rRNA gene-based primers, as used here, offer high analytical sensitivity and complete absence of cross-reactivity with E. dispar at the gene sequence level [9, 19]. Several systematic reviews confirm PCR sensitivities exceeding 95% for E. histolytica in stool, with specificities approaching 100% [10]. A common objection to PCR adoption in resource-limited settings is cost. However, the cumulative clinical and economic costs of diagnostic misclassification, including unnecessary anti-Parasitic drug exposure, missed treatment of invasive disease, and pediatric hospitalization, can substantially outweigh the incremental per-test cost of molecular diagnosis, particularly when viewed at a population level [26]. Point-of-care PCR platforms and multiplexed gastrointestinal panels are increasingly available in LMICs and could represent feasible implementation pathways in South Punjab's referral laboratory network.

The epidemiological findings are consistent with established risk factor profiles for E. histolytica transmission. Rural residence was the strongest correlate of infection (88.2% positivity), reflecting the elevated risk associated with poor sanitation coverage, reliance on unfiltered water, and limited hygiene infrastructure in peri-urban and rural communities [4, 27]. Multi-country analyses in South Asia consistently demonstrate a higher burden of enteric parasitic infection in rural populations relative to urban counterparts with access to piped water and improved sanitation [2]. The clear gradient of increasing infection prevalence with household size (58.6% in households of 1-3 members versus 82.1% in those with more than 10 members) corroborates overcrowding as a key facilitator of fecal-oral transmission in the domestic environment, a pattern extensively documented in the South Asian epidemiological literature [28]. Higher infection rates in children aged 6-12 years compared with younger children are likely attributable to the transition toward independent outdoor activity, more frequent unsupervised contact with contaminated soil and water, and reduced parental oversight of hand hygiene at this developmental stage [3]. The persistence of high infection rates even among children from tertiary-educated households (81.1%) indicates that individual behavioral modification is insufficient to protect against infection in environments where structural sanitation deficits persist, underscoring the imperative of community-level infrastructure investment alongside health education programs [28]. Several limitations of this study should be acknowledged. The cross-sectional design precludes causal inference and does not permit assessment of temporal relationships. Single-center recruitment at a tertiary hospital may over-represent children with more severe or prolonged symptoms relative to the broader community. The ELISA kit produced zero specificity in this cohort; while informative about its performance under these specific field conditions, this finding should not be generalized to all commercial ELISA systems without independent evaluation. DNA extraction from stool is inherently susceptible to inhibition by fecal compounds; although the QIAGEN automated platform with optimized lysis steps minimized this risk, residual false-negative PCR results cannot be entirely excluded. Finally, the absence of parallel PCR for E. dispar and E. moshkovskii prevents direct quantification of the proportion of non-pathogenic Entamoeba species driving microscopy and ELISA overestimation.

Despite these limitations, this study makes a substantive evidence-based contribution to diagnostic policy in Pakistan. The findings collectively support a tiered diagnostic algorithm in which microscopy serves as an initial screening step for Entamoeba morphotypes, with all positive results mandatorily confirmed by PCR before treatment initiation [29, 30]. In settings currently lacking molecular infrastructure, clinical decision-making should integrate symptom

severity, epidemiological history, and microscopy findings while PCR capacity is developed. Investment in molecular diagnostic infrastructure, integrated with community-level WASH interventions, represents the most evidence-aligned strategy for reducing the burden of pediatric amebiasis in South Punjab.

5. CONCLUSION

This study demonstrates that neither light microscopy nor commercial ELISA antigen detection achieves acceptable diagnostic accuracy for E. histolytica in symptomatic children from South Punjab, Pakistan, when evaluated against PCR as the molecular gold standard. Microscopy exhibited critically low specificity attributable to morphological indistinguishability of E. histolytica from non-pathogenic Entamoeba species while ELISA produced universally positive results with no discriminatory capacity under local field conditions. PCR must therefore be adopted as the confirmatory diagnostic standard for both clinical management and epidemiological surveillance of pediatric amebiasis in this region. Epidemiological analysis identified rural residence, large household size and the 6–12-year age group as the most significant risk correlates, providing clear targets for community-based prevention. Sustainable improvement in child health outcomes will require the integrated deployment of molecular diagnostic capacity with evidence-based WASH interventions, hygiene education, and targeted anti-Parasitic treatment programs guided by accurate, species-confirmed diagnosis.

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