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Molecular Prevalence of Entamoeba Species among Diarrheal Patients in Eastern Kenya

Caroline Makena Lepore, Lucy Kamau, Esther Kanduma

ABSTRACT

Data on the epidemiology of *Entamoeba* infections in Eastern part of Kenya is scanty. Diagnostic tests in use have limited capacity to differentiate common infecting species. The performance of Polymerase Chain Reaction (PCR) to differentiate between pathogenic *Entamoeba histolytica* and two nonpathogenic species *E. dispar* and *E. moshkovskii* is largely undetermined. Therefore, this study sought to determine the prevalence and associated factors for *Entamoeba* infections and evaluate the performance of PCR to differentiate *Entamoeba* complex species among diarrheal patient in Eastern region of Kenya. Stool samples were obtained from 400 patients attending Meru Teaching and Referral Hospital (MTRH) presenting with diarrhea. Samples were processed by direct wet mount using normal saline and iodine stain for microscopic examination. Entamoeba species differentiation was done using PCR targeting the 16S rRNA gene. A total of 33 (8.3%) samples had *Entamoeba* cysts/trophozoites by microscopy while 29 (7.3%) were identified as *E. histolytica* by PCR. *Entamoeba* infections was most common among adults 23 (5.8%) and in females 20(5%). The sensitivity of microscopy was 29/29 (100%; 95% CI 88.1% - 100%) with a specificity 367/371 (98.9%; 95% CI 97.3 % - 99.7%). In multivariate analysis, factors that independently influenced Entamoeba infection included sources of drinking water, use of toilet with water, regular use of soap or sanitizer, having diarrhea that persists for two weeks and stool consistency. *Entamoeba* infection was found to be responsible for most diarrhea condition especially among children. Patients hygienic and sanitation characteristics contributes significantly to *Entamoeba* infection. The performance of microscopy to detect *Entamoeba* infection is comparable to those of PCR except for the lack of species differentiation. Molecular species differentiation will improve disease diagnosis, control and management. Continuous monitoring of patient presenting with diarrhea for *Entamoeba* infection would improve treatment outcomes.

Keywords: Diarrheal diseases, Eastern Kenyan, Entamoeba species, *Entamoeba dispar*, *Entamoeba moshkovskii,* epidemiology, molecular diagnostics.

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I. Introduction

Worldwide about 1 million people die each year from diarrhea majorly due to unsafe drinking-water, poor sanitation and hygiene (Center for Disease Control and Prevention, 2016). Diarrhea is a life-threatening condition associated with a substantial mortality especially for children under five years (Center for Disease Control and Prevention, 2016). Sadly, in Africa, two out of five people do not have a proper source of drinking water (World Health Organization, 2014). Kenya is among the countries confronted with underprivileged sanitation and unsafe drinking water circumstances especially in the slums and rural areas. Poor sanitation predisposes individuals to diarrhea, cholera, dysentery, typhoid, and polio (World Health Organization, 2019). One of the causes of diarrhea is parasitic infections such as amoebiasis caused by Entamoeba histolytica (Development Initiative organization, 2018). Diarrhea resulting from amoeba infection is common in sub–Saharan African countries, an example is Kenya (Centre for Disease Control and Prevention, 2015).

The genus Entamoeba comprises of six species colonizing human intestinal lumen include *E. histolytica, E. dispar, E. moshkovskii, E. poleki, E. coli* and E. hartmanni (Begum *et al.,* 2015). Morphologically *E. histolytica, E. dispar* and *E. moshkovskii* are alike but differ biochemically and genetically (Zebardast *et al.,* 2014). The *E. histolytica* infection is associated with significant morbidity and mortality early detection and differentiation is therefore imperative. The existence of nonpathogenic species causes confusion in the diagnosis of intestinal amoebiasis and *E. histolytica* is frequently inaccurately reported (López-López *et al.,* 2017). Molecular identification of pathogenic E. histolytica and nonpathogenic Entamoeba spp (Entamoeba *dispar/Entamoeba moshkovskii/Entamoeba bangladeshi*) has important role in clinical management of patients with amoebiasis (Nowak, 2015). Molecular epidemiology

therefore crucial in understanding species diversity for accurate treatment and avoidance of emergence of antimicrobial resistance among the nonpathogenic strains (Nowak, 2015). This study therefore sought to determine molecular epidemiology of Entamoeba infection and evaluated the test performance of microscopy using PCR as gold standard to detect Entamoeba infection among diarrheal patients in Meru County, Kenya.

II. Methods

A. *Study Setting, Design and Data Collection*

1) *Ethical Considerations*

The research protocol was presented for scientific and ethical approvals by the Scientific Steering Committee and the Ethical Review Committee of Kenyatta University (KU/ERC/APPROVAL/VOL.1(51)). Permission to conduct this research was given by both NACOSTI (NACOSTI/P/17/33314/18190) and Meru County government. A written informed consent was obtained from all the patients.

This cross-sectional study enrolled consenting patients presenting with diarrhea attending Meru Teaching and Referral Hospital assuming 50% of the diarrhea patients were due to Entamoeba infection. Using the formula for estimating the population proportion with specified relative precision described by Lemeshow *et al.,* (1990) setting the α at 0.05, a total of 400 patients were recruited to achieve 0.95 power. The study consecutively enrolled consenting patients who provided a stool sample. Structured interviews were used to gather socio-demographic, hygienic, sanitary, and clinical presentations. Face to face interviews using structured questionnaires were conducted to gather information regarding the social demographic, clinical presentations, hygiene, and sanitation related attributes of the patients.

B. *Laboratory Analysis*

1) *Sample Collections*

All the recruited patients were issued with a sterile pre-labeled polypots and advised on how to collect at least 10 grams of stool without contaminating with urine or any foreign material. Each stool sample was then divided into two portions; approximately 5 g of fecal sample was used for formal ether concentration technique and the remaining stool sample frozen at -200 °C.

2) *Direct Microscopy*

A direct wet preparation of the fecal sample was prepared and examined under a light microscope. This was done within one hour of collection, in order to identify the trophozoites/cysts of Entamoeba species as described by Cheesbroug, (2012).

3) *Formal Ether Stool Concentration*

The formal ether concentration technique was used to concentrate the faecal parasite cysts of Entamoeba spp as described by Cheesbroug, (2012). Briefly, 10 ml of 10% formal saline was put in a clean mortar and 3 grams of stool was added. The large stool particles were broken down using a pestle. The mixture was then sieved through two layers of gauze into a centrifuge tube. Three (3) ml of ether was added into the centrifuge tube, capped and shaken vigorously then centrifuged at 2000 rpm for 2 minutes. The mixture separated into four layers, using an applicator stick the thick plug of debris and fatty material was loosened and the supernatant discarded leaving the sediment. The sediments were picked using Pasteur pipette and transferred to a clean slide, covered with coverslip then examined microscopically using x10 and x40 objectives with condenser iris closed to give good contrast. If Entamoeba cysts were seen, a few drops of lugols iodine was added to the remaining sediment in centrifuge tube and transferred to clean slide for microscopic examination. Those found to contain Entamoeba cysts were identified and preserved at -200C for DNA extraction.

4) *Polymerase Chain Reaction Characterization*

a) *DNA Extraction*

Patients' stool samples were used to extract DNA using the QIAamp DNA mini-kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The quality of DNA was measured using UV spectrophotometer ND- 1000 (NanoDrop Technologies, Wilmington, DE, USA).

b) *PCR Amplification*

The Entamoeba spp was subtyped using multiplex PCR assay targeting the 16S ribosomal RNA gene, modified from method described by Zerbardast *et al.,* (2014). In the final volume of 20 µl, the master mix contained 1 µl forward and reverse primers, 10 µl Maq taq enzyme, 2 µl nuclease free water and 10 ng of extracted DNA (2 µl). The primers sequences used in this reaction were: Entamoeba forward primer 5'-ATGCACGAGAGCGAAAGCAT-3'; Entamoeba moshkovskii reverse primer 5'-TGACCGGAGCCAGAGACAT-3'; Entamoeba dispar reverse primer 5'-CACCACTTACTATCCCT-ACC-3' and Entamoeba histolytica reverse primer 5'-GATCTAGAAACAATGCTTCTCT-3'.The PCR amplifications were done in a GeneAmp PCR system 9700 (Applied biosystem) with initial denaturation at 95 °C for 10 minutes, followed by 35 cycles at 95 °C for 30 seconds denaturation, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 5 minutes. The PCR products were visualized using a 5% cyber green stained agarose gel. The positive controls included known DNA extracts of E. histolytica-HM1: IMSS, *E. dispar*-SAW 760 and *E. moshkovskii*-Laredo strain while nuclease free water was used as negative control.

C. *Statistical Analysis*

Descriptive statistics: frequency (%), mean, standard deviation, median Interquartile range was used to describe the patients' characteristics and laboratory parameter. The test performance (sensitivity, specificity, predictive values, and kappa statistics) was analyzed as follows: Sensitivity = number of true positive (TP)/sum of the number of TP and number of false negative (FN). Specificity = Number of true negatives (TN)/sum of TN and the number of false positives (FP). The positive predictive value (PPV) = TP/sum TP + FP; while the negative predictive value (NPV) = TN/TN + FN. The 95% confidence interval for the test performance were also calculated. Cohen's kappa coefficient (k) analyses was used to test the agreement of tests against a reference standard. Factors associated with Entamoeba infection was achieved using logistics regression analysis. The prevalence of Entamoeba infection was determined for all patients. In

bivariate analyses, odds ratios (OR) and 95% confidence intervals (CI) for the association between Entamoeba infection and socio-demographic, hygienic and sanitation, clinical presentation factors were calculated using Poisson regression. In multivariate analyses, a manual backward elimination approach was used to reach the most parsimonious model including factors that were associated with Entamoeba infection among study patients at the significance level of $P \leq 0.05$. All statistical analyses were performed using STATA v 13 (StataCorp LP, College Station, TX, USA).

III. Results

A. Demographic Characteristics of Study Patients

All the 400 patients recruited in this study provided stool sample and demographic information and were included in analysis (100% response rate). Table I summarizes the baseline characteristics of study patients. There were near equal number of females 201 (50.3%) and 199 (49.7%) male patients. The mean age of the patients was 27.9 (Standard Deviation - SD 18.6) years with median age of 24 years (interquartile range (IQR) = 13 – 39.5 years). Among the study patients there were 43 (10.8%) children under five years and 62 (15.5%; 95% CI 12.3% – 19.4%) elderly patients aged 51 years and above. The majority 177 (44.3%) of the patients had secondary level of education.

The majority 231 (57.7%) of the patients sourced drinking water from pipped water source, 175 (47.8%) stored drinking water in containers with lids, 169 (42.3%) treated drinking water by boiling while 236 (59%) disposed their sluice water into the environment. Further, only 75 (18.7%) had access to toilet or latrines with covers while 163 (40.8%) of the patients washed their hands with soap or used hand sanitizers.

The majority 375 (93.8%) of patients had fever, 250 (62.5%) had diarrhea for two weeks, while 43 (10.8%) had diarrhea lasting longer than 14 days. Further, majority 199 (49.8%) study patients had mild dehydration while only 6 (1.5%) of the patients were severely malnourished and 67 (16.88%) being categorized as malnourished. Majority of the patients 176 (44%) had watery stools.

TABLE I: DESCRIPTIVE CHARACTERISTICS OF STUDY PATIENTS

Variables	Units	Frequency	Percent	95% CI	
Gender	Female	201	50.3	45.3	55.2
	Male	199	49.7	44.8	54.7
Age (Years)	Median (IQR)	24 (13-39.5)			
	1 to 10	62	15.5	12.3	19.4
	11 to 20	118	29.5	25.2	34.2
	21 to 30	62	15.5	12.3	19.4
	31 to 40	61	15.3	12	19.1
	41 to 50	35	8.8	6.3	12
	≥ 51	62	15.5	12.3	19.4
Education Level	Informal	46	11.5	8.7	15
	Primary	80	20	16.4	24.2
	Secondary	177	44.3	39.4	49.2
	Tertiary	97	24.3	20.3	28.7
Main Source of Drinking Water	Borehole and Open well	111	27.9	23.4	32.4
	Pipped	211	52.8	47.7	57.7
	Rivers/Springs	57	14.3	10.9	18.1
	Water Vendors	21	5	3.3	7.9
Water Storage	Container with Lid	175	43.8	38.9	48.7
	Container without Lid	143	35.8	31.2	40.6
	Jerrycans	82	20.5	16.8	24.8
Water treatment	Boiling	169	42.3	37.5	47.2
	Chemicals	140	35	30.5	39.8
	Stand to Settle	91	22.8	18.9	27.1
Availability of Toilet/Latrine with Cover	Yes	75	18.7	15.2	22.9
	No	325	81.3	77.1	84.8
Disposal of Sluice Water	Environment	236	59	54.1	63.7
	Septic Tank	121	30.3	25.9	35
	Sewage	43	10.8	8.1	14.2
Hand Washing	After using Toilet	117	29.3	25	33.9
	Before Food Preparation	144	36	31.4	40.8
	Before Meals	139	34.8	30.2	39.6
Presence of Fever	Yes	375	93.8	90.9	95.7
	No	25	6.3	4.3	9.1
Did This Diarrhea Last Longer than 14 Days?	Yes	43	10.8	8.1	14.2
	No	357	89.2	85.8	91.9
Presenting Symptoms	>3 Loose/Watery Stools/Day	248	62	57.1	66.6
	Blood in Stools	8	2	1	4
	Increased Thirst	90	22.5	18.7	26.9
	Sunken eyes (Dehydration)	54	13.5	10.5	17.2
Dehydration	Mild	199	49.8	44.8	54.7
	Moderate	106	26.5	22.4	31.1
	Normal	54	13.5	10.5	17.2
	Severe	41	10.3	7.6	13.6
Nutritional Status	Malnutrition	67	16.8	13.4	20.8
	Mild Malnutrition	210	52.5	47.6	57.4
	Nourished	117	29.3	25	33.9
	Severe Malnutrition	6	1.5	0.7	3.3
Stool Consistency	Bloody	5	1.3	0.5	3
	Mucoid	173	43.3	38.5	48.2
	Watery	176	44	39.2	48.9
	Watery and Bloody	46	11.5	8.7	15

CI-Confidence Interval

B. Prevalence of Entamoeba Infections among Study Patients

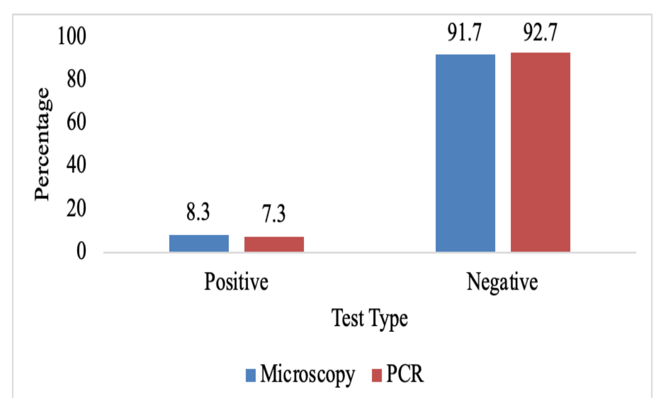

Fig. 1. Prevalence of Entamoeba infection among study patients using microscopy and PCR.

Prevalence of Entamoeba infection varied depending on the test used: Using microscopy 33/400 (8.3%; 95% CI 5.6% – 10.9%) had Entamoeba infection while 29/400 (7.3%; 95% CI 4.9% – 10.3%) were positive by PCR (Fig. 1).

C. Sensitivity and Specificity of Microscopy using PCR as Gold Standard

Table II summarizes the test performance of microscopy using PCR as gold standard. The microscopy results were concordant with those of PCR 396/400 (99%; 95% CI 97.5% – 99.7%). A kappa value of 0.9641 which measures the level of agreement between tests showed almost perfect agreement between microscopy and PCR to detect Entamoeba infection. The sensitivity of microscopy was 29/29 (100%; 95% CI 88.1% - 100%) with a specificity of 367/371 (98.9%; 95% CI 97.3 % - 99.7%). The positive predictive value (PPV) of microscopy was 29/33 (87.9%; 95% CI 71.8% - 96.6%) with a negative predictive value (NPV) of 367/367 (100%; 95% CI 99 % - 100%) (Table II).

TABLE II: PERFORMANCE OF MICROSCOPY AGAINST PCR RESULTS AS THE GOLD STANDARD TO DETECT ENTAMOEBA INFECTION

PCR (Gold Standard)								
Test	N	Concordant Results (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	NPV (%) 95% CI	PPV (%) 95% CI	Kappa	Agreement
Microscopy	400	99 (97.5-99.7)	100 (88.1-100)	98.9 (97.3-99.7)	100 (99-100)	87.9 (71.8-96.6)	0.9641	Almost perfect

N-Number; %-Percentage; CI-Confidence Interval; NPV; Negative Predictive Value; PPV; Positive Predictive Value

D. Linear Regression Model

In the final linear regression model (Table III), factors significantly associated with Entamoeba infection in the multivariate analysis were; patients who obtained drinking water from water boreholes/open wells (adjusted odd ration aOR = 2.8, 95% CI = 1.2 – 6.6; p = 0.022), from rivers and springs (aOR = 3.3, 95% CI = 1.2 – 8.9; p < 0.018), from water vendors (aOR = 5.1, 95% CI = 1.5 – 17.7; p = 0.009), patients who used toilets without cover (aOR = 294.8, 95% CI = 16.6 – 539; p = 0.0001), patients whose diarrhea lasted longer than 14 days (aOR = 11.9, 95% CI = 4.1 – 33.7; p < 0.0001) and patients who had bloody stool (aOR = 6.4, 95% CI = 1.2 – 32.9; p = 0.026) and watery and bloody diarrhea (aOR = 5.7, 95% CI = 1.5 – 21.8; p = 0.01) were independently associated with high rates of Entamoeba infection. On the other hand, patients who disposed their sluice water into sewage systems (aOR = 0.2, 95% CI = 0.03 – 0.9; p = 0.036), those who cleaned their hands using soap and water or sanitizers (aOR = 0.08, 95% CI = 0.024 – 0.0; p = 0.0001) were independently associated with reduced rates of Entamoeba infection.

TABLE III: UNIVARIATE AND MULTIVARIATE REGRESSION ANALYSIS OF FACTORS ASSOCIATED WITH ENTAMOEBA INFECTION AMONG STUDY PATIENTS

Variables	Infected			Bivariate		Multivariate	
	N	Freq	%	uOR (95% CI)	P Value	aOR (95% CI)	p Value
Gender							
Male	201	20	9.9	0.7 (0.3-1.3)	0.238	0.6 (0.3-1.3)	0.246
Female	199	13	6.5	Referent	Referent	Referent	Referent
Age (Years)							
1 to 10	62	4	6.5	0.6 (0.2-1.9)	0.372	0.6 (0.2-1.9)	0.384
11 to 20	118	7	5.9	0.5 (0.2-1.5)	0.229	5.3 (0.4-67.8)	0.202
21 to 30	62	4	6.5	0.6 (0.2-1.9)	0.372	0.7 (0.2-2.3)	0.529
31 to 40	61	10	16.4	1.5 (0.6-3.8)	0.449	1.5 (0.6-3.9)	0.409
41 to 50	35	1	2.9	0.3 (0.03-2.1)	0.199	0.3 (0.03-2.1)	0.199
≥ 51	62	7	11.3	Referent	Referent	Referent	Referent
Main Source of Drinking Water							
Borehole and Open well	111	12	10.8	2.5 (1.1-6)	0.035	2.8 (1.2-6.6)	0.022
Rivers/Springs	57	8	14.1	3.2 (1.3-8.5)	0.014	3.3 (1.2-8.9)	0.018
Water Vendors	21	4	19	4.4 (1.4-14.5)	0.013	5.1 (1.5-17.7)	0.009
Pipped	211	9	4.3	Referent	Referent	Referent	Referent
Water Storage							
Jerrycans	82	10	12.2	0.9 (0.01-0.7)	0.023	0.1 (0.02-0.9)	0.047
Container without Lid	143	10	6.7	0.6 (0.3-1.3)	0.203	1.3 (0.6-2.8)	0.468
Container with Lid	175	13	74.0	Referent	Referent	Referent	Referent
Use Toilet with Cover							
Yes	75	10	13.3	Referent	Referent	Referent	Referent
No	325	23	7.1	39.2 (4.4-351)	0.0001	294.8 (16.6-539)	0.0001
Disposal of Sluice Water							
Sewage	43	3	6.9	0.9 (0.3-3.1)	0.886	0.2 (0.03-0.9)	0.036
Septic Tank	121	12	9.9	1.3 (0.6-2.7)	0.481	0.6 (0.2-1.7)	0.31
Environment	236	18	7.6	Referent	Referent	Referent	Referent
Hand washing							
After Using Toilet	117	9	7.7	0.8 (0.4-1.9)	0.886	0.2 (0.3-0.9)	0.036
Before Food Preparation	144	11	7.7	0.8 (0.4-1.8)	0.481	0.6 (0.2-1.7)	0.31
Before Meals	139	13	9.4	Referent	Referent	Referent	Referent
Use Soap or Hand Sanitizers							
Yes	163	4	1.8	0.2 (0.01-0.6)	0.004	0.08 (0.024-0.3)	0.0001

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No	237	29	12.2	Referent	Referent	Referent	Referent
Fever							
Yes	375	32	8.5	1.9 (0.3-14.3)	0.51	1.1 (0.1-9.4)	0.956
No	25	2	8	Referent	Referent	Referent	Referent
Had Diarrhea in the Past 2 Weeks							
Yes	43	27	62.8	23.2 (9.6-56.2)	0.0001	11.9 (4.1-33.7)	0.0001
No	357	6	1.7	Referent	Referent	Referent	Referent
Presenting Symptoms							
>3 Loose/Watery Stools/Day	248	27	10.9	3.3 (1.0-10.8)	0.05	0.9 (0.2-4.6)	0.869
Blood in Stools	8	0	0	ND	0.995	ND	0.995
Increased Thirst	90	3	3.3	1.7 (0.3-8.2)	0.531	1.6 (0.1-17.5)	0.717
Sunken eyes (Dehydration)	54	3	5.6	Referent	Referent	Referent	Referent
Nutritional Status							
Malnutrition	67	9	13.4	3.1 (1.1-9.3)	0.04		0.989
Mild Malnutrition	210	19	9.1	2.1 (0.8-5.7)	0.647		0.989
Severe Malnutrition	6	0	0.0	ND	0.993	ND	0.990
Nourished	117	5	4	Referent	Referent	Referent	Referent
Stool Consistency							
Bloody	5	5	100	58.3 (13.9-244.1)	0.0001	6.4 (1.2-32.9)	0.026
Watery and Bloody	46	15	32.6	17.9 (5.2-61.7)	0.0001	2.5 (0.7-9.3)	0.174
Watery	176	10	5.7	3.4 (0.9-12.4)	0.062	5.7 (1.5-21.8)	0.01
Mucoid	173	3	1.7	Referent	Referent	Referent	Referent

N= total population; Freq- Pfrequency; %- Percentage; OR- Odds Ratio; CI-Confidence Interval; uOR and aOR-Un and Adjusted Odd Ratio respectively; P-Level of Significance; P≤0.05 indicates the relationship is significant

IV. Discussion

Amoebiasis caused by intestinal parasite Entamoeba histolytica, has an estimated worldwide prevalence of 500 million infected people and is responsible for 40,000 - 100,000 deaths each year (Mulinge *et al*., 2021). It is an important health problem, especially in developing countries (Sebastian et al., 2007). Microscopy, the main diagnostic test used in many hospitals is unable to distinguish between *E. histolytica and Entamoeba dispar* (Nowak, 2015; López-López *et al*., 2017). More sensitive and specific molecular techniques that are able to distinguish *E. histolytica from E. dispar and E. moshkovskii* have been developed (Nowak, 2015). Inevitably, prompt management of amoebiasis requires accurate diagnosis to distinguish between species of public health importance. It is on this background that this study was designed to determine the prevalence and associated factors of Entamoeba infections in diarrheal patients attending MTRH.

The prevalence of Entamoeba infection in this population was dependent on the test used: Using microscopy 8.3% of the study patients were found infected with Entamoeba while 29/400 (7.3%) patients were found infected with E. histolytica by PCR. No other Entamoeba strains were identified by PCR. Varied prevalence of Entamoeba has been reported globally. Infection with *E. histolytica*, the protozoan parasite that causes amoebic colitis and liver abscess, results in 34 million to 50 million symptomatic cases of amoebiasis (all illnesses caused by *E. histolytica*, including amoebic dysentery) worldwide each year, causing 40 thousand to 100 thousand deaths annually (Stauffer et al., 2006). In Africa, the burden of amoebiasis is high with an estimated E. histolytica infection median rate of 796 per 100,000 people (WHO, 2019). Studies conducted in Kenya indicated prevalence of 6–11% of *E. histolytica / E. dispar* in children at selected hospitals and 11–32% among adults (Sebastian et al., 2007; Kagira *et al*., 2011; Mbae *et al*., 2013). Moreover, the prevalence of E. histolytica by quantitative PCR was recorded at 15% in Bungoma County, Western Kenya (Easton *et al*., 2016), while a much lower prevalence of 0.4% was reported among children with vertically transmitted HIV infection (Matey *et al*., 2016). This data suggests that amoebiasis is a heavy burden among the Kenyan population which is also plagued by other diseases such as malaria, HIV-AIDS, tuberculosis and other non-communicable diseases. In other regions, amoebiasis is also a problem in the developed world in travelers, immigrants, and men who have sex with men (Haque *et al*., 2003). Entamoeba histolytica usually causes asymptomatic infection but in a minority of cases causes symptoms ranging from a few loose stools to profuse bloody diarrhea (Kosek *et al*., 2003). The difference in this prevalence can be explained largely due to epidemiological, environmental distribution difference, poor personal hygiene practices, environmental sanitation and ignorance of health-promotion practices.

Environmental, socio-economic, demographic and hygiene-related behavior is known to influence the transmission and distribution of intestinal parasitic infections (Norhayati *et al*., 2003). In this study hygienic and sanitary status such as obtaining household waters from unhygienic water sources including water vendors and lack of access to covered toilets or pit latrines were independently associated with higher risk of Entamoeba infections. This agrees with previous studies which have shown that the rate of infection by E. histolytica differs among countries, socio-economic and sanitary conditions and populations (Al-Harthi *et al*., 2007). The infection is highly endemic throughout poor and socio-economically deprived communities in the tropics and subtropics. A study in Brazil identified place of residence, age, ingestion of raw vegetables and drinking water quality as important risk factors (Norhayati *et al*., 2003).

Proper waste disposal such as the use of sewage system rather than environment and practicing recommended hygienic guidelines such as hand with soap or sanitizers were protective against Entamoeba infection. Reports show that E. histolytica in humans is transmitted directly following fecal-oral transmission routes. The risk pattern identified in our study follows this logic. In particular, the transmission routes via contaminated hands play a major role, documented in our study with a more than three- fold risk increase if hands are

not washed properly, in agreement with a study by Duc *et al*., (2011).

This study also found association of Entamoeba infection with stool consistency. Patients who had bloody or watery stool remained independently associated with higher Entamoeba infection. Even though presence of bloody stool remains a significant sign of intestinal parasite, indeed, studies have established the fecal oral route for the transmission of Entamoeba (Anuar *et al*., 2012). Watery stool or bloody stool have higher chance of spreading fecal droplets across wider surface compared to firm stool which could be a cause of infection and re-infection especially to those in contact with these patients. The presence of watery stool or bloody stool poses greater risk of further transmission within the family. A study by Al-Areeqi *et al*, (2017) showed that within the family, the presence of other infected family members significantly increased the odds of an individual being infected with *E. histolytica*. A previous study from Malaysia found a similar finding with *E. dispar* (Anuar *et al*., 2012). The infection could easily be passed on through contaminated food and drinks prepared by infected family members who have inadequate personal hygiene. The trophozoite of Entamoeba spp. survives for a short time but the cyst lives longer and withstands unfavorable conditions; it can be found on the hands and clothes of children as well as in containers used for washing clothes and in shower trays, which implies that contact with an infected person's belongings and water they have used also transmits the infection (Roberts *et al*., 2009).

Although age was not found to influence Entamoeba infection in this study, previous reports have shown an increasing prevalence of Entamoeba infection to reach its highest rate among patients aged 21–30 years then decreasing to its lowest among those aged more than 40 years (Al-Areeqi *et al*., 2017). By contrast, a previous study among Malaysian rural communities reported a significantly higher prevalence of *E. dispar* infection among patients younger than 15 years (Anuar *et al*., 2012). Gender in this study was not found to be associated with Entamoeba infection. However, gender has been cited as a key factor for Entamoeba infection with more infections occurring among males. This difference could be explained by a higher exposure of adult males to sources of infections as a result of their daily activities such as more frequent consumption of contaminated outdoor food, contaminated water sources and contact with infected individuals (Al-Areeqi *et al*, 2017).

Accurate laboratory diagnosis of any disease-causing pathogen is critical for proper management as false results can potentially have severe consequences such as serving as reservoirs for onward transmission and death (Elston, 2006). Additionally, presenting false results can significantly undermine both clinical confidence and credibility of laboratory results, consequently resulting in wrong prescription that may cause drug wastage (Footedar *et al*., 2007) and unnecessary costs. In the current study based on PCR test results as the gold standard, the sensitivity and specificity of microscopy was 100% and 98.9% respectively. The PPV and NPV of microscopy was 87.9% and 100% respectively. Lower sensitivity 64.2% and specificity 83.6% of microscopy were recorded in a previous study in western Kenya (Emisiko *et al*., 2020). On the other hand, sensitivity of microscopy in the diagnosis of E. histolytica seems to be varied with some studies reporting values ranging between 10% and 60% (Fotedar *et al*., 2007; Pereira *et al*., 2014). Diagnostic tests occasionally show varied sensitivities and specificities depending on the setting especially in endemic and non-endemic sites (Abu-Madi *et al*., 2017).

Entamoeba infections are traditionally diagnosed via microscopic examination of stool samples, fresh or fixed. Microscopic examination of stool specimens in saline wet mount is a less sensitive technique even when viewed by an expert microscopist (Parija *et al*., 2014). Moreover, this technique is often subjective and is prone to misdiagnosis and has other limitations. For instance, in microscopy one cannot distinguish between cysts and trophozoites within degenerated polymorphonuclear cells (Shirley *et al*., 2018). The Center for Disease Control recommends examination of adequate samples within 30 minutes of collection to improve sensitivity of microscopy. It is important to note that changes in sample pH as well as prior use of antibiotics before sample collection kills trophozoites decreasing sensitivity of microscopy (Shirley *et al*., 2018). Due to the limitations of microscopy, other techniques have been developed including serological based techniques like Rapid Diagnostic Tests (RDTs) and molecular based techniques such as polymerase chain reaction (PCR) (Aly *et al*., 2018). PCR has been adopted as the gold standard method for the diagnoses of amoebiasis (Shirley *et al*., 2018). However, utilization of PCR in routine diagnosis of E. histolytica in resource limited settings is impractical (Emisiko *et al*., 2020). As such, a combination of serologic tests with microscopy detection offers the best approach to diagnosis of E. histolytica [30]. However, in most resource limited settings, microscopy is the only diagnostic test used for detection of most enteric parasites (Emisiko *et al*., 2020). It is important that continuous monitoring and evaluation of the test is done to ensure reliability of microscopy. Moreover, laboratory personnel have different levels of training, experience, and skills.

One key objective of this study was to identify *E. histolytica, E. dispar* and *E. moshkovskii* using PCR assay. By microscopy all the *Entamoeba* isolates were identified as *E. histolytica*. However, using specie specific PCR, 29 (87.9%) out of 33 isolates identified by microscopy were found to be *E. histolytica* while the remaining 4 did not amplify and thus could not be typed by PCR. This is not unique to the current study only, Lau *et al*., (2013) found nine samples identified by microscopy as negative for real-time PCR even upon retesting. It is possible that the samples which were detected positive by microscopy but not by PCR may belong to other Entamoeba species such as *E. coli, E. polecki* and *E. hartmanni* (Parija *et al*., 2014). A study done by Petri and co-workers in Bangladesh showed the limitations of microscopy, whereby only 40% of children diagnosed by microscopy were proven to have E. histolytica infection by PCR test (Petri *et al*., 2000).

Studies have repeatedly shown that pathogenic amoeba, E. histolytica is indistinguishable in its cyst and trophozite stages from *E. dispar* and *E. moshkovskii*, the non-pathogenic species (Khhairnar and Parija, 2007). It has also been shown that the sensitivity and specificity of microscopy is less optimal in differentiating the various species of Entamoeba

(Tanyuksel *et al*., 2003). Given the discrepancies of microscopy, various approaches have been implemented. The epidemiology of Entamoeba can be further be studied by culturing trophozoites and determining isoenzyme patterns by gel electrophoresis (Lau *et al*., 2013). These techniques are costly, time-consuming, laborious and not practical for regular diagnosis (Lau *et al.,* 2013). An enzyme immunoassay kit (TechLab II antigen test) has been specifically designed for the detection of *E. histolytica*. However, this kit is marketed for examination of stool samples only though fixed stools samples have been found not suitable for enzyme-linked immunosorbent assay (ELISA) (Qvarnstrom *et al*., 2005). Due to the need to study the epidemiology of Entamoeba, the polymerase chain reaction (PCR) is now the method of choice, and this technique has been approved by the World Health Organization (WHO) (Hamzah *et al*., 2010). In a study conducted by Stark et al. (2008), it was demonstrated that PCR has improved sensitivity and specificity over ELISA-based kits. PCR also has the ability of specifically targeting and detecting *E. histolytica, E. dispar,* and *E. moshkovskii* infections (Parija *et al*., 2014).

V. Conclusion

The following conclusions can therefore be drawn from the findings of this study; that prevalence of Entamoeba infection still remains a significant problem among patients presenting with diarrhea diseases. The importance of hygiene and sanitary status as important avenue for Entamoeba infection cannot be overstated as observed in this study in agreement with many others. In the absence of molecular assays, microscopy is a reliable test in detecting Entamoeba though its limitation in differentiating different Entamoeba species is still a challenge.

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

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