

Original Research Article

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Detection of *Entamoeba* Species: A Comparative Analysis of Nested-Multiplex PCR and Recombinase Polymerase Amplification

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ABSTRACT

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Amoebiasis, caused by *Entamoeba histolytica*, remains a serious public health issue worldwide. For decades, microscopy remains preferred method for detection of the pathogenic species. The recent advent of molecular biology has helped in developing appropriate diagnostic technologies, of which isothermal amplification is considered to be robust and cost-effective compared to PCR that requires expensive instruments and highly skilled personnel. The aim of the present study was to compare the diagnostic ability of nested-multiplex PCR and Recombinase polymerase amplification for differential detection of *E. histolytica* and *E. dispar*. The results of this study showed good agreement between the two tests. This suggests that RPA can be used as clinical and epidemiological tool for diagnosis of amoebiasis in resource-limited setups and in endemic regions.

Introduction

Entamoeba histolytica causes amoebiasis which is globally considered as a leading parasitic cause of human morbidity and mortality (Haque *et al.*, 2003; Haque and Petri, 2006). Clinically, *E. histolytica* manifestation ranges from asymptomatic colonisation to amoebic dysentery and invasive extraintestinal amebiasis, which is presented most commonly in the form of liver abscesses. Reports suggest that approximately 50 million people suffer from invasive

amoebiasis, resulting in 100,000 deaths per year (WHO, 1997). *E. histolytica* is distributed worldwide; however, high prevalence rates have been reported from various developing and under-developed countries (Fotedar, 2007).

Entamoeba histolytica, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba hartmanni*, and *Entamoeba polecki* are the six species among the Genus *Entamoeba* that are found to reside in the human intestinal lumen. Recent studies have

isolated *E. dispar* and *E. moshkovskii* from individuals with gastrointestinal symptoms. The casual link between these parasites that are considered non-pathogenic and the disease is still not evident (Clark and Diamond, 1991). Until obvious evidence is established, the detection and differentiation of the different *Entamoeba* sp. is essentially important. Appropriate methodologies for specific diagnosis of amoebiasis in endemic areas are highly recommended because of the morphologically indistinguishable characteristics of *E. histolytica*, *E. dispar* and *E. moshkovskii*.

For many years, diagnosis of *E. histolytica* was primarily based on microscopic examination of the protozoan morphology. However, its sensitivity reaches up to only 60% and there can be issues with misidentification of non-pathogenic *Entamoeba* species. Unless the trophozoites contain ingested RBCs, the ability to differentiate *E. histolytica* from other look-alike species becomes technically tricky (Gonzalez-Ruiz *et al.*, 1994; Manochitra and Parija, 2017).

Recently molecular biology has revolutionized the field of pathogen identification and differentiation based on the difference in the genetic make-up. The sensitivity and the chances of specific identification have been improved by application of the molecular methods like polymerase chain reaction (PCR) assays. Nested multiplex PCR (NM-PCR) performed from faecal and liver abscess aspirate specimens, targeting the 18S SSU rRNA has been evaluated for diagnosing amoebiasis (Khairnar and Parija, 2007). However, it is time-consuming and requires expensive equipment for thermal cycling thus limiting their use in resource-poor setups. Isothermal DNA amplification platforms, such as the Recombinase polymerase amplification (RPA) used in this study, alleviate the need for

thermal cycling equipment and have the potential to broaden access to more sensitive diagnostics. RPA doesn't require thermocyclers to provide robust signal amplification. In RPA, the target sequence is scanned in the DNA template based on the interaction of the oligonucleotide primer with a nucleoprotein complex formed by a recombinase and its co-factor. The beginning of the strand invasion is marked by the recognition of a specific homologous sequence followed by extension of the opposing nucleotides by isothermal strand displacement amplification (Daher *et al.*, 2016). Based on the benefits of RPA over PCR, it has gained importance in the specific diagnosis of various diseases. In the present study, we have compared the diagnostic ability of NM-PCR and RPA for accurate detection and differentiation of *Entamoeba* sp.

Materials and Methods

This study was primarily hospital-based and was carried out over a period of one year (2016-2017) in the Department of Microbiology, JIPMER, India. The stool samples (n=180) from patients presenting with clinical symptoms of amoebic colitis like diarrhoea/dysentery, the presence of abdominal pain with or without the onset of fever and other gastrointestinal disturbances were randomly included in the study after obtaining clearance from JIPMER Institute Ethics Committee. The samples were stored at -20°C without the addition of any preservatives or fixatives until used.

DNA extraction and nested multiplex PCR

DNA was extracted from the stored stool specimens according to the manufacturer's instructions with the QIAamp Stool DNA Mini Kit. The NM-PCR with the primer targeting the 18S SSU rRNA gene was carried out as previously described (Khairnar and

Parija, 2007) in Sure Cyclor 8800 (Agilent Technologies). The amplified product was visualised on 1.8% agarose in a gel documentation unit (GelDoc XR, BioRad).

Recombinase polymerase amplification

The RPA assays were performed using the TWISTAMP[®] basic kit (TwistDx, Cambridge, United Kingdom) following the manufacturer's instructions. Amplifications were performed based on the previously described protocol with some minor modifications (Nair *et al.*, 2015). A set of forward primers specific for *E. histolytica* (EHF 5'GTACAAAATGGCCAATTCAT TCAAT3') and *E. dispar* (EDF 5' AAGTATAAAGACCAAGTAGGATGAAA3 ') and common reverse primer (EHEDR 5'ACTACCAACTGATTGATAGATCAG3') targeting the 18S SSU rRNA gene were used. Each primer set could amplify 132 bp product for *E. histolytica* (EHF/EHEDR) and 234 bp product for *E. dispar* (EDF/EHEDR). The amplified product was purified, and 5 µL of each RPA reaction was analyzed by electrophoresis in 1% agarose gel followed by ethidium bromide staining for visualization under UV trans-illuminator.

Results and Discussion

A total of 180 stool samples were subjected to NM-PCR and RPA. The species-specific NM-PCR product size for *E. histolytica* and *E. dispar* was 439 and 174 bp respectively (Figure 1). Of the total 180 samples, 13 were positive by NM-PCR, of which 11 were found to be *E. histolytica*, and only 2 were *E. dispar* (Table 1).

After amplification by RPA which yielded products of 132 bp size for *E. histolytica* and 234 bp size for *E. dispar* were visualized on 1% agarose gel (Figure 2). Amplified products could be visualised in a total of 11 samples, of

which 9 were positive for *E. histolytica* and 2 for *E. dispar* (Table 1).

Based on the results, the sensitivity and specificity of RPA were statistically calculated, which was found to be 84.62% and 100% respectively; the positive predictive values and negative predictive values for RPA were 100% and 98.83% respectively when compared with NM-PCR (Table 2). The previous report shows that the sensitivity and specificity of NM-PCR to be 94% and 100% (Khairnar and Parija, 2007). On the other hand the Kappa agreements between the two tests were found to be very good.

Recently in the developed countries, PCR has been the method of choice for clinical and epidemiological studies and has been strongly recommended to be used as a diagnostic tool by the WHO (Haque, 2006; Khairnar and Parija, 2007; Tanyuksel and Petri, 2003; Calderaro *et al.*, 2006; Hamzah *et al.*, 2006; Zaki *et al.*, 2002).

A variety of clinical specimens (faeces, tissues, saliva, liver abscess aspirate) have been tested for identification of *E. histolytica* (Khairnar and Parija, 2008) various diagnostic methods are being developed for rapid, specific and differential detection of the *Entamoeba* sp., (Parija *et al.*, 2014; Korpe *et al.*, 2012) which may aid in formulating appropriate treatment strategies. Isothermal amplification methods are robust and are currently in the developmental stage for the diagnosis of various pathogens. RPA has been tested for parasites such as *Plasmodium*, *Cryptosporidium*, *Schistosoma haematobium* and few other bacteria and viruses (Jauset-Rubio *et al.*, 2016). There have been very few reports about utilization of RPA for diagnosis of amoebiasis and they are mainly used in research laboratories (Nair *et al.*, 2015; Crannell *et al.*, 2016).

Table.1 Comparison of NM PCR and RPA

Samples N=180	Positive by NM PCR		Positive by RPA	
	EH	ED	EH	ED
	11	2	9	2
Total	13		11	

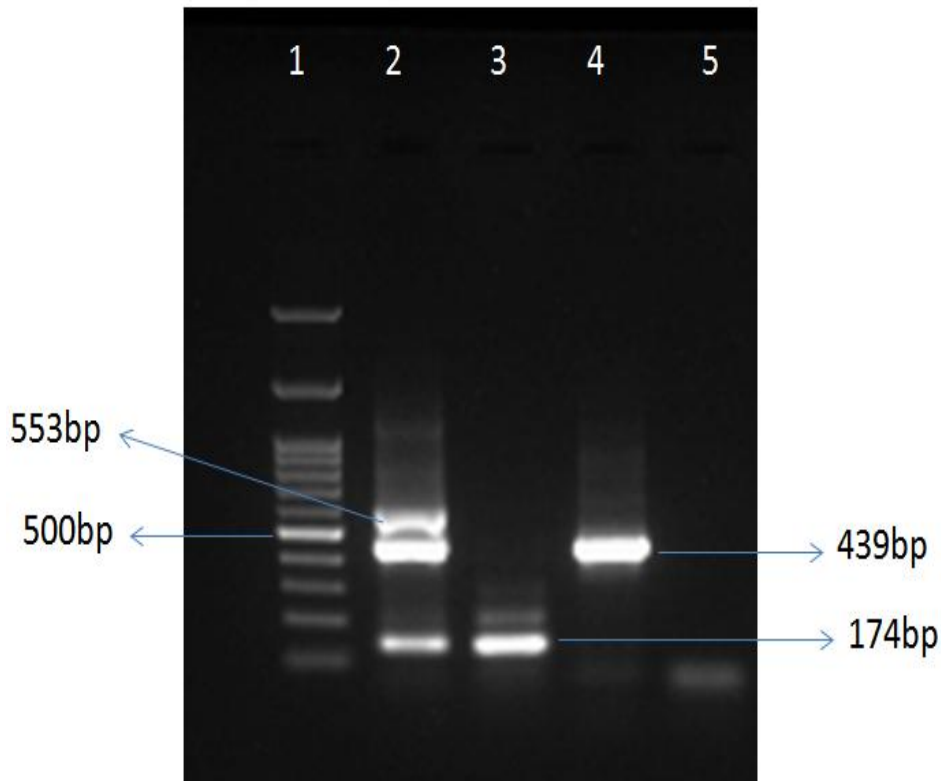
Table.2 Two-way contingency table for comparing RPA against NM PCR

	NM PCR Positive	NM PCR Negative	Total
RPA Positive	11	0	11
RPA Negative	2	167	169
Total	13	167	180

Sensitivity -84.62% specificity- 100% Positive Predictive value -100%
 Negative predictive value 98.83 Kappa- 0.911
 The strength of agreement is considered to be 'very good'

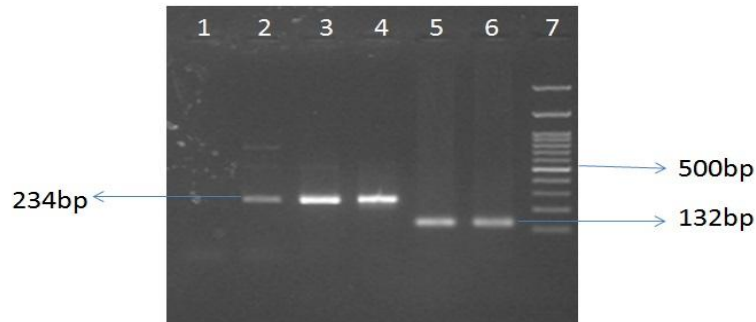
Sensitivity and Specificity was calculated by using Medcalc. Kappa agreement was calculated by graph pad.

Fig.1 PCR products of NM-PCR in 1.8% agarose gel



Lane 1- 100bp Marker, Lane 2- positive control (EH-439bp, ED-174bp, EM-553bp) Lane-3&4 sample, Lane 5- negative control

Fig.2 RPA products in 1% agarose gel



Lane 1- negative control, Lane 2&3 samples, Lane-4 –Positive control(ED), Lane-5 – sample, Lane-6-Positive control (EH), Lane-7-100bp Marker

The study by Nair *et al.*, (2015) has tested the ability of RPA for utilization in the detection of the *Entamoeba* sp., and they found that RPA showed 86% and 98% correlation with the positive and negative samples which were screened by PCR and ELISA. A study by Crannell *et al.*, (2016) has attempted to develop a multiplex assay using RPA for intestinal parasites like *Giardia*, *Entamoeba* and *Cryptosporidium*. In our study, we have directly tested clinical specimens and have successfully differentiated *E. dispar* from *E. histolytica*.

As RPA for *E. moshkovskii* has not been studied so far, we have used it only for detection of *E. histolytica* and *E. dispar*. Considering this, for comparative purpose the NM-PCR parameters were slightly modified, wherein the primers for *E. moshkovskii* were not included.

RPA eliminates the need for thermocyclers, thereby reducing the high cost spent on the equipment. This enhances its utility in developing and under-developed countries where the disease burden is high. RPA when combined with lateral flow (LF) techniques, makes it an efficient point-of-care test.

Future studies combining RPA with LF and testing it on larger samples may help in evaluating its ability as an epidemiological tool in endemic areas.

RPA is a simple, rapid and efficient diagnostic method that can be used in both clinical and epidemiological studies. The nominal cost and efficiency are the important factors that have to be considered in widely implementing this method. This study strongly suggests that RPA could be a valuable tool in resource poor settings if developed in-house so as to bring down the cost.

This study represents the first effort to evaluate RPA in comparison with NM PCR in an parasite endemic region like India.

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