

REVIEW ARTICLE

Problem in Amoebiasis Diagnosis in Clinical Setting: A Review from Conventional Microscopy to Advanced Molecular Based Diagnosis

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Abstract

It is now well established that *Entamoeba moshkovskii* and *Entamoeba dispar* are two distinct nonpathogenic species, but microscopically indistinguishable from the pathogenic species *E. histolytica*, the causative organism of amoebiasis. Traditionally diagnosis of *E. histolytica* infection has relied upon microscopic examination of cyst or trophozoite stage in fresh or fixed stool specimens which has several limitations. New approaches to the diagnosis of amoebiasis are based on detection of *E. histolytica* specific antigen or DNA in stool and other clinical samples using ELISA, PCR and microarray based technique while none are cheap and it is unlikely that they will be in routine use in resource poor disease-endemic areas in the near future. In the era of post genomic, development of faster, sensitive, specific, affordable and reproducible diagnostic tools and their application in resource poor setting with high endemic rates lead to a better understanding of the disease epidemiology in terms of prevalence, morbidity and measures to control the disease.

Keywords: Misdiagnosis, *E. histolytica*, microscopy, amoebiasis, ELISA, PCR, DNA based technique.

Introduction

There is a general feeling that misdiagnosis is quite prevalent with many people giving untrustworthy accounts from their own experiences, it is sometimes arduous to get exact data. Whereas, there are many studies of adverse drug events and there is a relative lack of misdiagnosis studies. Amoebiasis also called Traveller's diarrhea is defined by the World Health Organization (WHO) and Pan American Health Organization (PAHO) as infection with *Entamoeba histolytica*, regardless of symptomatology. This parasite is also of interest because it is an early branching amitochondriate eukaryote infecting 50 million people resulting in 10,000 to 40,000 deaths per annum and on a global scale, amoebiasis is the third among parasitic causes of death (WHO, 1997).

Major problems with a wide array of imperfect tests for diagnosis of amoebiasis severely limit the understanding of its magnitude and epidemiology. A greater hindrance is the varied, inconsistent application of existing methods in different areas of the world (Walsh, 1986). Traditionally diagnosis of amoebiasis has relied upon microscopic examination of fresh or fixed stool specimens (Fotedar *et al.*, 2007). However, microscopy has several limitations, most importantly, the inability to distinguish the pathogenic *E. histolytica* from the morphologically indistinguishable nonpathogenic *E. dispar* and *E. moshkovskii* (Ali *et al.*, 2003; Fotedar *et al.*, 2007).

World Health Organization recommended that *E. histolytica* should be specifically identified and if present, treated; if only *E. dispar* or *E. moshkovskii* is identified, treatment is unnecessary (WHO, 1997).

There is a need for simpler and affordable tool suitable for identification of these amoebae in clinical specimens, not only for diagnostic purposes and patient care management, where nonpathogenic parasite infected patients could be treated unnecessarily with anti-amoebic chemotherapy but also for a better understanding of the epidemiology of these parasites in the human population. Improved molecular diagnostics capable of distinguishing *E. histolytica* from morphologically identical nonpathogenic two were subsequently developed which minimized conventional screening and over-representation and have led to a reevaluation of the epidemiology of amoebiasis in areas with high endemic rates.

Against these backdrops, the main objective of this review is to highlight the current status of amoebiasis diagnosis and the possibility of using advanced, sophisticated molecular based diagnostic methods for routine testing of this parasite in clinical setting of developing countries particularly resource poor disease-endemic areas in the near future to overcome over-presentation and thus, misdiagnosis.

Biological characteristics that differentiate *E. histolytica* from its morphologically indistinguishable spp.

- Isoenzyme patterns in electrophoresis, particularly hexokinase (Strachan *et al.*, 1988).
- Specific epitopes like heavy subunit of the galactose/N-acetyl-galactosamine inhibitable lectin, recognized by reaction with several monoclonal antibodies (Petri *et al.*, 1990a, b).
- Sequence differences in rDNA episome particularly ITS2, basis for designing specific primer and probe (Paul *et al.*, 2002).
- It has proved much easier to adapt *E. histolytica* to axenic growth. Axenic culture of *E. dispar* proved extremely difficult and has so far been achieved for only one strain (Clark, 1995).
- Species and strain-specific probes derived from repetitive DNA, UEE1 and retrotransposon EhSINE1 (Srivastava *et al.*, 2005).

Detection techniques

Microscopy: Historically the light microscopy has been the method of choice to diagnose amoebiasis which relies on identifying cyst or trophozoite stage of *E. histolytica*. The presence of amoebic trophozoite containing ingested RBC or charcot-Leyden crystal strongly support *E. histolytica* infection, however such a finding is rarely seen. In the absence of haematophagous trophozoite the sensitivity of microscope is limited because of its inability to distinguish between *E. histolytica* from its morphologically identical species and misidentification of macrophages (Haque *et al.*, 1998; Ali *et al.*, 2003). In a report from Australia, half of microscopy positive stool samples were PCR-positive for nonpathogenic *E. moshkovskii* (Fotedar *et al.*, 2008).

Isoenzyme analysis: This technique, which was amongst the earliest to suggest that "pathogenic" and "non-pathogenic" *E. histolytica* were in fact two separate species (Sargeant, 1988). Even though the original procedure involving starch-gel electrophoresis of four enzymes (malic enzyme, hexokinase, glucose phosphate isomerase and phosphoglucomutase isozyme) is often simplified to merely examining hexokinase mobility in agarose mini-gels (Strachan *et al.*, 1988). However, an isoenzyme analysis takes usually one to several weeks before the results are reported as it relies on establishing the amoebae in culture and also requires special laboratory facilities, making it impractical for use in the routine diagnosis of intestinal amoebiasis (Khairnar *et al.*, 2007). Furthermore, the amoebic cultures and therefore isoenzyme analyses are negative for many microscopy positive stool samples (Fotedar *et al.*, 2007).

Culture methods: Robinson medium (Robinson, 1968) and TYSGM-9 medium (Diamond, 1982) are more often used for axenic cultivation of *E. histolytica*. Axenic cultivation involves cultivation in the absence any other metabolizing cell in the monophasic media like TP-S-1, TY I-S-33 and YI-S (Diamond *et al.*, 1995).

It has proved much easier to adapt *E. histolytica* to axenic growth. Axenic culture of *E. dispar* proved extremely difficult and has so far been achieved for only a few strains (Clark, 1995). However, culture methods are time-consuming, laborious and often unrewarding, with a sensitivity of only about 50% and further testing is required for speciation (Hal *et al.*, 2007). As culture of *E. histolytica* from clinical samples such as feces or liver abscesses has a significant false-negative rate and is technically difficult, it is not undertaken in a routine clinical laboratory (Fotedar *et al.*, 2007).

Antibody tests: Being varying sensitivity, depending on the presence or absence of invasive disease and the type of invasive disease, it is nowadays not a method of choice. The sensitivity of serology is about 95% for amoebic liver abscess and 84% for invasive intestinal disease. Antibody testing to diagnose carriage of *E. histolytica* is unhelpful, as the sensitivity is only 8% (WHO, 1997). Recently, heavy subunits of *E. histolytica* lectin (152 KDa) and pyruvate phosphate dikinase (110 KDa) were used for serodiagnosis of amoebic liver abscess (ALA), which showed sensitivities above 80% and undetectable cross-reactivity in the western blot analysis and this molecule merits further evaluation on its diagnostic value using a larger panel of serum samples (Wong *et al.*, 2011).

In a report from Malaysia, two in-house indirect ELISAs were found to be efficacious for diagnosis of ALA and EEA is easier to prepare than the commonly used CSA (Tan *et al.*, 2013). The main drawback with antibody based diagnosis is that even after successful therapy; the serum antibody test often gives false positive, as antibody titres can remain high for years after initial infection and successful treatment. This fact makes definitive diagnosis by antibody detection difficult because of the inability to distinguish between past and current infection (Paul *et al.*, 2006).

Antigen detection: Diagnosis based on detection of specific epitope with monoclonal antibodies is often considered as quick and convenient one. Of six monoclonal antibodies raised against heavy subunit of the galactose/N-acetyl-galactosamine inhibitable lectin, only two reacted with *E. histolytica* and *E. dispar* while the other four reacted only with *E. histolytica* (Petri *et al.*, 1990a, b) and are the basis of two kits manufactured by TechLab, Inc. (Blacksburg, VA), one of which identifies *E. histolytica/E. dispar* while the other identify *E. histolytica*. Comparative study carried out in different parts of the world showed the high sensitivities of TechLab antigen kit between 95 and 100% (Sharma *et al.*, 2003; Mohammadi *et al.*, 2006; Al-Harathi and Jamjoom, 2007). However, a study conducted in a region of northern Ecuador found that the TechLab *E. histolytica* II test performed poorly, with a sensitivity of 14.3% and a specificity of 98.4% compared to isoenzyme analysis (Gatti *et al.*, 2002).

According to a study conducted in Australia the TechLab ELISA (enzyme-linked immunosorbent assay) kit did not prove to be useful in detecting *E. histolytica*, as it failed to identify any of the *E. histolytica* samples which were positive by PCR. With the TechLab kit, cross-reactivity was observed for three specimens, one of which was positive for both *E. dispar* and *E. moshkovskii* while the other two samples contained *E. moshkovskii* (Stark *et al.*, 2008). Moreover, the TechLab *E. histolytica* II ELISA was not sensitive in detecting amoebic antigen in samples from ALA patients (Zeehaida *et al.*, 2008). Other ELISA kits for antigen detection include the *Entamoeba* CELISA PATH kit (Cellabs, Brookvale, Australia), which uses a monoclonal antibody specific for lectin of *E. histolytica*, and the ProSpecT EIA (Remel Inc.; previously manufactured by Alexon-Trend, Inc., Sunnyvale, CA), which detects *E. histolytica* specific antigen in fecal specimens (Fotedar *et al.*, 2007).

Dot-Blot Hybridization: The utilization of specific DNA probes for *E. histolytica* derived from different genomic regions like highly repeated sequences and ribosomal DNA (Samuelson *et al.*, 1989; Garfinkel *et al.*, 1989; Bracha *et al.*, 1990) and a 4.5 kb rDNA fragment from HMe region of EhR1 (Verma *et al.*, 2012) which include both *E. histolytica* and *E. dispar* to hybridize with DNA isolated directly from fecal samples proved to be very competent. The main drawback of the dot blot technique is that it cannot detect minuscular amounts of parasite DNA present in stool; however being more sensitive than microscopy, culture and isoenzyme analysis can be employed as a screening tool in resource poor setting where the disease is endemic, however the main drawback with this is further PCR amplification using specific primer is required for speciation.

DNA based methods: The ability of Polymerase Chain Reaction (PCR) to specifically amplify minute amounts of pathogen DNA has revolutionized the diagnosis of many infectious diseases including amoebiasis. A number of methods have been published, most, but not all, rely on amplifying unique regions of the SSUrRNA as its high copy number provide increased sensitivity. Conventional multiplex and nested multiplex PCR have been developed by many workers for simultaneous differential diagnosis of morphologically indistinguishable *E. histolytica/dispar/moshkovskii* complex (Que and Reed, 1991; Hamza *et al.*, 2006; Khainar *et al.*, 2007). A nested PCR-RFLP based technique was also developed for differential diagnosis of the three *Entamoeba* spp. and found that only 19% of the stool samples, resembling *E. histolytica* by microscopy, were actually *E. histolytica*, implying that 81% of suspected infections were misdiagnosed and would have been treated unnecessarily with anti-amoebic drugs (Parija and Khainar, 2005). In a comparative study among traditional PCR, stool antigen detection and real-time PCR assay utilizing a molecular beacon probe for the detection of *E. histolytica*, it was found that sensitivity of

traditional PCR was 72% and the specificity was 99% whereas the sensitivity and specificity achieved by the real-time PCR assay and the antigen detection test were 79% and 96%, respectively (Roy *et al.*, 2005). Several real-time PCR methods have been designed from different parts of the world (Blessmann *et al.*, 2002; Qvarnstrom *et al.*, 2005; Haque *et al.*, 2007; Hamza *et al.*, 2010). The main drawback of this technique is that it is expensive as well as sophisticated and thus cannot be used as a routine diagnostic tool, however as the assay output is a quantitative one; it is very useful to monitor the parasite load. Recently, DNA microarrays have been employed successfully for investigating *E. histolytica* pathogenesis (Singh and Shah, 2002). An oligonucleotide microarray was developed for simultaneous detection and genotyping of the three most frequently identified protozoan parasites causing waterborne disease outbreaks *E. histolytica*, *E. dispar*, *G. lamblia* and *C. parvum* (Wang *et al.*, 2004). The DNA microarray technique came into focus as it combines powerful DNA amplification strategies with subsequent hybridization to oligonucleotide probes specific for multiple target sequences. A novel one-step, closed-tube, loop-mediated isothermal amplification (LAMP) assay for detecting *E. histolytica* was developed showing levels of sensitivity and specificity similar to those of nested PCR implying its useful for clinical detection and active surveillance of *E. histolytica* parasites in countries where amoebiasis is endemic (Liang *et al.*, 2009).

Conclusion

Entamoeba histolytica/dispar/moshkovskii complex with possible differentiation has revolutionized our understanding of the epidemiology of amoebiasis and can have led to important treatment and diagnostic recommendations. Clinicians should follow the precise guidelines promulgated by the WHO/PAHO in 1997 including definitive differentiation of *E. histolytica* from morphologically identical nonpathogenic species to avoid unnecessary and possibly harmful chemotherapies. Antigen detection using fecal ELISA could be used in areas of endemicity, however recent studies conducted in different parts of the world showing the sensitivity of the fecal antigen test is about 100 times less than that of PCR and in addition, several studies have highlighted its low specificity because of cross-reaction with other *Entamoeba* spp. As none of the above are cheap and those are cheap showing cross-reactivity, continued development of specific and affordable diagnostic tools will be required for use in resource-poor settings and thus, appropriate technique for amoebiasis diagnosis till today remains a major public health priority for the developing world with high endemic rates.

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