Published in final edited form as:

Exp Parasitol. 2007 May ; 116(1): 35–43. doi:10.1016/j.exppara.2006.11.005.

Molecular methods for diagnosis of *Entamoeba histolytica* in a clinical setting: An overview

Jaishree Paul*, Shweta Srivastava, and Sudha Bhattacharya

Jawaharlal Nehru University, School of Life Sciences, Delhi 110067, India

Abstract

The range of clinical outcomes following *Entamoeba histolytica* infection is likely to be influenced by the different strains of the parasite already existing in our population. There is a need for developing faster, reliable and reproducible methods for identifying the different strains of *E. histolytica*. This would have a major impact on the subsequent course of treatment given to patients. In the post-genomic era, different loci of the *Entamoeba* genome have been targeted for developing suitable probes and genetic markers. This review highlights the development made in this direction and the possibility of using these methods for routine testing of this parasite in clinical samples.

Index Descriptors and Abbreviations

Molecular markers; *Entamoeba* strains; STR analysis; Microarray; SINE elements; Transposon display

1. Introduction

The clinical symptoms of amebiasis caused by the protozoan parasite *Entamoeba histolytica* ranges from asymptomatic colonization to amebic dysentery and invasive extraintestinal amebiasis—most commonly in the form of liver abscesses (Tanyuksel and Petri, 2003). Since the pioneering observation of Martínez-Palomo and colleagues (1973) on lectin-mediated agglutination, it has become more and more apparent that there are fundamental differences between the organisms recovered from patients with invasive disease and those parasitising asymptomatic cyst passers.

So far, data on the issue of time course of *E. histolytica* infections are not consistent and are possibly influenced by geographical location. On the one hand, evidences demonstrate that an ameba infection can persist for a considerable period of time (Allason-Jones et al., 1988), while on the other hand, studies from South Africa and Bangladesh suggest that intestinal infections with *E. histolytica* are short-lived with nearly all the study subjects recovering from their infections on their own within a few months (Gathiram and Jakson, 1985; Haque et al., 2001). In a study conducted in the endemic area of Vietnam by Blessmann et al. (2003) indicated *E. histolytica* prevalence of 11.2% and an annual new infection rate of

4.1% in the population studied. A follow-up of the 43 individuals who were *E. histolytica* positive at enrollment suggested a regular exponential decline in infection by about 3% per month and the mean half-life of infection of more than 15 months. These observations seem to suggest that organisms producing the invasive, symptomatic disease might be genetically different from those producing the asymptomatic infections only, although this has not been established as yet.

The delineation of former *E. histolytica* into two genetically distinct species, the invasive *E. histolytica* and the noninvasive *Entamoeba dispar*, had a major impact on the epidemiology and clinical management of the disease. When *E. dispar* was separated from *E. histolytica* it was assumed that the vast majority of asymptomatic cyst shedders would turn out to be infected with *E. dispar* and that all those infected with *E. histolytica* were either clinically ill or would become so if not treated. In fact this has not turned out to be the case and surveys in South Africa (Gathiram and Jackson, 1987), Bangladesh (Haque et al., 2001) and Vietnam (Blessmann et al., 2002a,b) have shown that, only a small percentage of those genuinely infected with *E. histolytica* ever go on to develop clinical amoebiasis.

There is a need for the accurate, strain-specific diagnosis of *E. histolytica* in fecal specimens since there is no way of knowing which infected persons will progress to clinical amebiasis. This would help in the generation of accurate epidemiological data for a better estimate of the burden of amebiasis on the health of the world (Petri et al., 2000). This will also help us to identify which asymptomatic cases have the potential to cause the disease in future and therefore need to be treated at present.

Nearly 13 years after Diamond and Clark (1993) redefined *E. histolytica*, now a number of well validated methods exist for distinguishing between it and *E. dispar*. Methods for specific detection of *E. histolytica* and *E. dispar* in feces have been reviewed excellently by Ackers, 2002. The methods include—isoenzyme analysis after cultivating the parasite from cyst-positive fecal material, Antigen detection methods, DNA blotting and PCR based methods. New diagnostic tools specific to *E. histolytica* are being exploited by clinicians and researchers to identify and treat patients as well as to add to the knowledge of the epidemiology and natural history of this infection (Stauffer and Ravdin, 2003). Because of its lower sensitivity, the efficacy of ELISA for *Entamoeba* detection and differentiation in stools seems in nontropical regions questionable. Results suggest that PCR should be useful as a reference test for sensitive differentiation of both species of *Entamoeba* and could facilitate appropriate treatment of either (Gonin and Trudel, 2003). In a study conducted in Nicaragua, PCR results showed that *E. histolytica* is a rare finding in patients with diarrhea (Leiva et al., 2006). Therefore, it was concluded that at the health centers, *E. histolytica*, *E. histolytica/E. dispar* was clearly over-diagnosed when microscopic methods were employed.

Methods that can be employed without cultivation of the organism are gaining importance since cultivation of the parasite from the stool or puss samples is quite labour-intensive and has a low success rate. In addition to low diversity, zymodeme analysis has other drawbacks. It now appears that “fast” hexokinase bands have proved to be the simplest marker of *E. histolytica*, while many of the original distinct zymodemes are now known to result from variation in culture conditions (Blanc and Sargeant, 1991). Jackson and Suparsad (1997)

have reported that many zymodemes 'disappear' upon removal of the bacterial flora, suggesting that at least some of the bands are of bacterial rather than amoebal origin. From isoenzyme analysis, it is not possible to determine with confidence whether individuals who develop disease and those who clear infection are infected with the same or distinct strains of *E. histolytica*, because of the limited diversity of reliable zymodemes in the species. These potential and real problems have meant that isoenzyme analysis has been superseded by DNA-based methods (Haque et al., 1998) as the approach of choice for detecting variation in *E. histolytica*.

The presence of *E. histolytica* antibodies in carriers of *E. dispar* was mainly seen in patients originating from endemic countries. Therefore, the high specificity of a positive result of serology in carriers from non-endemic countries can be used to establish the diagnosis of *E. histolytica* infection (Visser et al., 2006). A problem with antibody-based methods is that most people with intestinal amebic infection in areas of endemicity have been exposed to *E. histolytica* many times. This fact makes definitive diagnosis by antibody detection difficult because of the inability to distinguish between past and current infection.

2. Outstanding issues

In spite of the development of sensitive antigen-based and molecular techniques, we do not yet have much information about the diversity of *Entamoeba histolytica* strains and their pathological significance. The varied organ tropisms and clinical presentations of infection by *E. histolytica* raise questions regarding the role of genetic diversity of the parasite in virulence. How do certain groups of infected individuals develop extraintestinal amebiasis without exhibiting apparent intestinal symptoms? Do the same strains that cause amebic liver abscess also cause invasive intestinal amebiasis? In an endemic country like India, we are well poised to ask some of these questions because we have access to the parasite in the natural host. In order to address these issues there is a need for developing refined and reliable techniques for speedy identification of parasite strains in a clinical setting. This review is intended to discuss the existing diagnostic methods that are being developed in this direction.

3. Methods employed for molecular typing

3.1. Dot blot

The use of species-specific DNA probes to hybridise with unamplified DNA isolated directly from fecal samples proved to be very efficient particularly when radioactive labels were used. Dot blot was found to be more sensitive than microscopy and isozyme analysis. It has been applied successfully in probes derived from different genomic regions e.g. P145/B133 from rDNA (Garfinkel et al., 1989; Bracha et al., 1990; Samuelson et al., 1989; Agarwal et al., 1998) and a probe from HMe region of rDNA (Srivastava et al., 2005). The drawback of the dot blot technique is that it cannot detect very small amounts of parasite DNA present in stool or pus samples and some probes cross hybridize resulting in false negatives and false positives.

Interestingly, rDNA episomes of *E. histolytica* strains differ in the number of transcription units per circle, e.g. HM-1:IMSS (EHR1) and NIH:200 have two rRNA transcription units while HK-9 and Rahman have single transcription units. Hmg region, an intergenic spacer, is not present in strains having single transcription units (Sehgal et al., 1994). Recombinational loss of a ribosomal DNA unit (Hmg region) from the circular episome in the laboratory strain of *Entamoeba histolytica* HM-1:IMSS (EHR2) was demonstrated earlier in our laboratory by Ghosh et al. (2001). Whether or not such a selection process occurs in the *E. histolytica* population harbored in human intestines is still not very clear. Therefore, Hmg region could be a possible marker for strain identification. To determine the status of HMg positivity, a population survey was conducted in and around Delhi, India. All the samples were initially tested for *E. histolytica* as well as *E. dispar* by PCR using species-specific primers described earlier (Srivastava et al., 2005). Dot blot was performed using genomic DNA of *E. histolytica* positive samples (Fig. 1). PCR amplified Tr region from a cloned HMg fragment belonging to EhR1 rDNA circle was used as a probe for the study. The results indicated that out of the total samples tested positive for *E. histolytica*, only 20% were HMg positive. All of the HMg positive samples were *E. histolytica* but all the *E. histolytica* were not HMg positive. This data indicates that HMg region may be associated with a group of *E. histolytica* only. However, the effect of the presence of HMg on pathogenicity is yet to be confirmed. The results indicated that 4% of samples from asymptomatic volunteers and 2.4% of those from symptomatic people and 3% of ALA patients samples were HMg positive (data in Fig. 1). Therefore, it was concluded that the prevalence of HMg positive *E. histolytica* strains in Indian population was quite low. This data suggests that there are at least two types of *E. histolytica* strains present in the environment.

3.2. Reverse line blot hybridization assay

Verweij et al. (2003) described a reverse line blot method used for the detection and identification of *Entamoeba* species even in case of mixed infections, after DNA amplification of the SSU rRNA gene with general *Entamoeba*-specific primers. This was followed by hybridization of the product with the genetic variants known to infect humans. However, hybridization with the general *Entamoeba*-specific probe in such cases indicates the need for further sequence analysis to reveal new genetic variants.

3.3. PCR based techniques

PCR is currently a widely used technique for the diagnosis of *Entamoeba*. With cultured trophozoites, PCR was about one hundred times more sensitive than antigen detection (Mirelman et al., 1997). However, as with all PCR-based methods, great care has to be taken in preparing the DNA template to avoid PCR inhibitors. To eliminate the risk of false positives due to contamination, appropriate controls must be included in the study. For example, human DNA needs to be included to check for any non-specific amplification especially when stool samples or pus samples are used as study material. However, false-negative results may also be obtained due to the presence of inhibitors of DNA polymerase in stool samples.

3.3.1. To study species variation—A number of methods have been published for the detection of *E. histolytica* and *E. dispar* (Tannich and Burchard, 1991; Acunasoto et al., 1993; Katzwinkel-Wladarsch et al., 1994; Rivera et al., 1996; Britten et al., 1997; Verweij et al., 2000). Most of them rely on amplifying the unique regions of rDNA episomes. Many polymorphic protein coding genes like serine-rich *E. histolytica* protein (SREHP) (Stanley et al., 1990) and chitinase (de la Vega et al., 1997) have also been used for the purpose. Calderaro et al. (2006) described the utility of conventional PCR and real-time PCR as methods of identification and differentiation of *E. histolytica* and *E. dispar*. It has been suggested that using molecular techniques large-scale longitudinal trials can be conducted by multidisciplinary research groups in different endemic areas to generate reliable knowledge of *E. histolytica* and/or *E. dispar* infection and disease (Ximenez, 2006).

3.3.1.1. Real-time PCR: Real time PCR allows specific detection of the amplicon by binding with fluorescence-labeled probes due to which further downstream analysis is not required. This reduces the time needed for detection considerably. In addition, the closed reaction tube minimizes the chances of cross-contamination, and the assay output is quantitative rather than qualitative. Therefore, the main advantage of this technique is that it can monitor the parasite load. Real time PCR has been employed for diagnosis of *E. histolytica* and *E. dispar* from DNA of stool (Blessmann et al., 2002a,b) and liver abscess pus (Roy et al., 2005). Qvarnstrom et al., (2005) compared the real-time PCR data with *E. histolytica* and identified the TaqMan method targeting the SSU rRNA gene as a superior real-time PCR assay for specific and quantitative diagnosis of amebiasis. The probe-based real-time PCR assays evaluated in this study were able to identify *E. histolytica* in four clinical samples with very low parasite concentrations, which the conventional PCR could not detect.

A multiplex real-time PCR assay was also developed for the simultaneous detection of three enteric pathogens—*E. histolytica*, *G. lamblia*, and *C. parvum* in stool samples (Verweij et al., 2004). A real-time PCR assay utilizing a molecular beacon probe for the detection of *E. histolytica* was carried out and its sensitivity was compared with stool antigen detection and traditional PCR (Roy et al., 2005). The 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. The main drawback of this technique is that it is both expensive as well as sophisticated and therefore cannot be used as a routine diagnostic tool.

3.3.2. To study strain variation

3.3.2.1. STRs: The tRNA genes are found in clusters of one to five distinct types, interspersed with short tandem repeats (STRs) and these clusters are in turn repeated to form long arrays. The arrays make up a significant proportion (approx. 13%) of the genome. A series of primers were designed for strain identification of *E. histolytica* based on tRNA associated STRs (Zaki and Clark, 2001). These loci lie in the non-coding regions in the vicinity of tRNA genes. Primers designed from these loci distinguished the *Entamoeba histolytica* strains. Subsequently, the development of species-specific primers for these STRs was reported, and these were said to enable the simultaneous differentiation and strain

typing of *E. histolytica* and *E. dispar* (Zaki et al., 2002). A longitudinal study conducted in South Africa (Zaki et al., 2003) revealed that these markers were stable and suitable for tracking the transmission of a known strain within an individual, family unit, and/or community. Surprisingly, the same *E. histolytica* strain was never detected in epidemiologically unlinked individuals, which indicates a remarkable degree of genetic diversity within this relatively restricted geographic area.

Species-specific primer pairs were designed by Ali et al., 2005 that differentiate *E. histolytica* from *E. dispar* and reveal intraspecies PCR product length polymorphisms. This is of great value as in some areas of *E. histolytica* and *E. dispar* endemicity, a significant number of individuals could be co-infected with both parasites. The technique requires a panel of markers to be tested for each strain to arrive at a conclusive result. The STRs that characterize the tRNA-genes of *E. histolytica* and *E. dispar* appear to be absent from the tRNA-flanking regions of all other species studied to date (Tawari and Clark, unpublished), making them unlikely to be suitable for diversity studies (Clark, 2006).

3.3.2.2. SREHP genotypes: A gene coding for a protein, known as “serine-rich *E. histolytica* protein” (SREHP) containing multiple tandem repeats has been shown to possess immunogenic properties (Stanley et al., 1990). *E. histolytica* and *E. dispar* genes encoding repeat antigens on the surface of trophozoites (Ser-rich protein) or encysting parasites (chitinase) were highly polymorphic (Ghosh et al., 2000). The results suggest diversifying selection at chitinase and Ser-rich protein loci and demonstrate the usefulness of these alleles for distinguishing between clinical isolates of *E. histolytica* and those of *E. dispar*. The SREHP genotypes of clinical isolates from patients with liver abscesses were distinct from those derived from patients with colitis and dysentery suggesting an association between SRHEP genotypes and the clinical presentation of the disease (Ayeh-Kumi et al., 2001). An outbreak of amebic liver abscess in Georgia in 1998–1999 suggested that the causative *E. histolytica* strains had an unusual propensity for extraintestinal spread. However, SRHEP genotyping study confirmed extensive genetic diversity among *E. histolytica* isolates from the same geographical origin thus preventing an association of a single genotype with hepatic disease (Simonishvili et al., 2005). The method used here included a two-step PCR followed by restriction digestion. First PCR involves primers SREHP-5 and SREHP-3, amplifying the 549 bp fragment of SREHP gene of HM1-IMSS strain, followed by a nested PCR using primers nSREHP-5' and nSREHP-3 located within the initially amplified fragment. The drawback of the method was the amplification of a few non-specific bands in a clinical setting that could not be explained. The polymorphic nature of SREHP requires further attention because SREHP may be of great value as a protective immunodominant antigen (Zhang and Stanley, 1999).

3.3.2.3. Chitinase: *E. histolytica* chitinase gene repeats ranged from 84 to 252 nucleotides corresponding with four heptapeptide repeats (28 amino acids) to 12 heptapeptide repeats (84 amino acids). The 168-nucleotide chitinase gene repeat of a San Diego isolate (Eh SD1) was identical to that of the HM-1 strain, while the 84-nucleotide chitinase gene repeat of a Calcutta isolate (Eh K1) was the same as those of NIH-200 isolate (de la Vega et al., 1997). Thus one may speculate that either the original axenized strains of *E. histolytica* are still

located in the New and Old Worlds (Ghosh et al., 2000) or the similarity is due to chance convergence rather than a common ancestor. However, since the repeat-containing region of chitinase appeared to be the least polymorphic as compared to SREHP and tRNA linked loci (Haghighi et al., 2003), therefore, the gene encoding chitinase has been used less frequently for strain differentiation.

3.3.2.4. Use of retrotransposons as genetic markers

3.3.2.4.1. Transposon display: Non-LTR retrotransposons are abundantly and uniformly present in the *E. histolytica* genome. They are dispersed throughout the genome. In *Entamoeba*, autonomous non-long terminal repeat retrotransposons (LTR) elements are known as long interspersed elements (EhLINEs) whereas short non-autonomous elements are designated as short interspersed elements (EhSINEs). Multiple families of EhLINEs and EhSINEs are found in *Entamoeba histolytica* (Cruz-Reyes et al., 1995; Willhoeft et al., 1999; Van Dellen et al., 2002). PFGE analysis with genomic DNA of *E. histolytica* using EhLINE1 and EhSINE1 probes showed that these elements resided on all chromosomes and were estimated to have 140 copies of these elements per genome and seem to be non-telomeric in location (Sharma et al., 2001a). Together EhLINEs/EhSINEs account for about 6% of the *Entamoeba* genome (Bakre et al., 2005). EhSINE1 is highly abundant in *E. histolytica* but not present in nonpathogenic *E. dispar* (Willhoeft et al., 2002). Since active copies of these elements are not been found so far in this parasite, therefore, it is presumed that during the course of evolution, insertions of EhLINEs and EhSINEs might have taken place at different genomic locations in various strains and got fixed (Bhattacharya et al., 2002). Therefore, they can also be explored as markers for *E. histolytica* strain identification.

Transposon display (TD) is a modification of the AFLP technique (Vos et al., 1988) that uses a primer anchored in a transposon to simultaneously detect up to several hundred markers in the genome (Casa et al., 2000, 2004). TD involves the amplification of sequences flanking the transposon by ligation-mediated PCR. The resulting fragments are locus-specific and can be analyzed by polyacrylamide gel electrophoresis. Transposon display has been used to study the behavior and stability of transposable elements in plants (Flavell et al., 1998). The method has also been successfully used to screen yeast mutants conferring quantitative phenotypes (Sharma et al., 2001b).

Axenicly grown laboratory strains of *E. histolytica* exhibited a differential pattern of TD (Srivastava et al., 2005) (Fig. 2) when primers were designed from various regions of EhSINE1 near its 5' end. TD has several potential advantages over other techniques. The technique of Transposon Display is now being refined to analyse DNA isolated directly from both fecal as well as ALA pus samples. Here multiple polymorphic bands are displayed as compared to single band polymorphism in a normal PCR. It is cost effective since only one reaction with single specific primer has the capacity to display whole range of bands in each strain. It is more sensitive and reliable than AFLP since a transposon specific primer is used. Therefore, this technique could be employed in carrying out significant molecular epidemiological studies and large-scale typing of this parasite. Extensive analysis of the

bands may provide a means to understand the dynamics of EhSINE retrotrans-position in various *E. histolytica* strains.

3.3.2.4.2. REP PCR: Repetitive extragenic PCR was first devised for strain identification in gram-negative enteric bacteria (Versalovic et al., 1991). REP PCR is widely used for strain typing of bacteria, fungal and dermatophytes which are normally encountered in the clinical laboratories (Pounder et al., 2005). It is based on the observation that repetitive elements are dispersed in the genome and may be present in both the orientations on the chromosome.

PCR primers designed to “read outward” from the repeats will amplify the region between two such elements in either orientation. These primers are complementary to interspersed repeated sequences. This may lead to different band patterns when the repetitive sequences are present in different locations in the genome of different strains. This strategy was tried successfully using EhLINEs/EhSINEs dispersed in the *E. histolytica* genome (Ph.D. thesis of Srivastava, 2005). Since most copies of EhLINEs and EhSINEs are truncated, a number of primers were designed from EhLINEs and EhSINEs covering the whole stretch of each element. Unique REP PCR fingerprint profile is created containing multiple bands of varying sizes that are specific to each strain. This could be used to establish relationships between different strains. This technique can offer more variation between the strains than the tandem repeat primers since using this, many loci can be studied simultaneously.

3.4. Single nucleotide polymorphism

Single nucleotide polymorphisms (SNPs) are the most abundant and simple form of DNA variation in the genome. The rapid progress of genome projects presents a unique opportunity to genetic researchers for SNP typing. To evaluate genetic variability among *Entamoeba histolytica* strains, Bhattacharya et al., 2005, sequenced 9077 bp from each of 14 isolates. It was suggested that coding and non-coding regions are subject to different selection pressures and could be associated with specific clinical outcome of the disease. The difference in the occurrences of SNPs between coding and noncoding regions (0.07% and 0.37%, respectively) was statistically significant. Since SNP markers are evolutionarily stable and unlikely to mutate again to either a novel or ancestral state, they are useful for evolutionary analyses. However, in case of *Entamoeba*, this technique is still in the initial stages and needs to be further explored. The technique also involves large scale sequencing of the PCR products.

3.5. Microarray

Recently, oligonucleotide microarrays have been used successfully for the detection of waterborne protozoan pathogens including *Entamoeba* (Wang et al., 2004). Micro-array-based genotyping assay can readily be applied to the study of *E. histolytica* clinical isolates to determine genetic diversity and potential genotypic–phenotypic associations. This technique combines powerful DNA amplification strategies with subsequent hybridization to oligonucleotide probes specific for multiple target sequences. The distinct advantage of this detection approach is that it can study thousands of genes simultaneously. In addition to conserved genes, such as rRNA and *hsp*, which have been widely used as diagnostic markers, many genus- and species-specific genes like intergenic region between the super-

oxide dismutase (*sod*) and actin genes, and the cysteine protease gene (*cp1*) were selected as amplification targets so as to avoid potential co-amplification and cross-hybridization issues.

A study was carried out on genotyping of *E. histolytica* and *E. dispar* strains on the basis of comparative genomic hybridization of >7600 random genomic clones (Shah et al., 2005). On the basis of the identification of divergent genetic loci, each strain had a unique genetic fingerprint, and the degree of divergence was correlated with virulence, raising the intriguing possibility that genetic subtypes of *E. histolytica* may be partially responsible for the observed variability in clinical outcomes. It was concluded that the microarray-based genotyping assay can readily be applied to the study of *E. histolytica* clinical isolates to determine genetic diversity and potential genotypic-phenotypic associations. Out of the very few isolates described in the paper, only one was considered to be 'avirulent *E. histolytica*', so caution should be used in concluding that there is a correlation between genotype and virulence by this method.

MacFarlane and Singh (2006) using DNA microarray carried out the first large-scale expression profiling of *Entamoeba* species/strains and opened the door to the investigation of genetic and expression differences which may relate to parasite virulence. However, functional studies need to be performed to confirm the roles of these genes in amebic pathogenesis. The main limitations of the technique for diagnostic purpose are the costs, its robustness and labor inputs.

4. Conclusions

Numerous studies have demonstrated the inadequacies of microscopic examination for *E. histolytica* for the diagnosis of both amebic colitis and liver abscess. The major drawback of the current antigen detection kit lies in its application to resolve the positive samples in an endemic setting where the frequency of false positive samples are high. Molecular methods, such as PCR, have aided in alleviating some of the sensitivity and specificity issues traditionally associated with the detection of protozoan pathogens. A number of PCR-based assays, including gene amplification with specific primers, multiplex PCR, restriction fragment length polymorphism and real-time PCR, have been developed for the identification of protozoan infections. However, the shortcomings of PCR based assays become apparent during practical applications. The generation of nonspecific DNA fragments from environmental and clinical samples poses a significant problem that often results in false-positive results. Conversely, the failure to amplify a single diagnostic sequence due to inhibitors in the sample or possible mutations in the primer-binding region may result in false-negative results. Furthermore, although Real-time PCR assays are sensitive enough to detect a single cell, the limited number of probes that can be applied in one reaction hinders its utility for confident multi-target detection and genotyping analyses. The comparative genomic hybridization (CGH) results using Microarray technique clearly indicated the possibility that parasite genotypic patterns can predict clinical phenotypic outcomes (MacFarlane et al., 2005).

In the post-genomic era, we are equipped with better tools to study this important parasite. Targeting the repetitive regions both in the coding as well as the non-coding regions have

yielded various markers that have been tried successfully in different laboratories. Epidemiological studies have demonstrated extensive genetic diversity within clinical *E. histolytica* isolates using PCR-based analyses of highly repetitive and polymorphic loci. Although these regions easily incorporate polymorphisms probably due to DNA slippage during replication, whether the extent of divergence is truly representative of divergence across the genome is not yet known.

The techniques discussed above that are based on the analysis of the polymorphic loci are SNP, Transposon display, while REP PCR showed promising results for strain differentiation. As with all emerging technologies, the challenge lies in integrating these findings to the larger issue of pathogenesis due to this parasite. A number of genetic methods have been demonstrated and validated in this parasite making the genetic link and proof of virulence an achievable task. Appropriate techniques for the diagnosis of amebiasis remain a major public health priority for the developing world. The information stated above on the use of various techniques for the diagnosis can be used to prioritize strain identification of *E. histolytica* and to promote the development of high-throughput techniques that are faster and would help to design strategies for subsequent therapeutic options.

Acknowledgments

The authors (J.P. and S.B.) are grateful to Department of Science and Technology, New Delhi for financial support and S.S. acknowledges the fellowship given by University Grants Commission, New Delhi.

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Fig. 1.

Strain identification of *Entamoeba histolytica* by dot blot using HMg probe. (A) Diagrammatic representation of two types of rDNA circle present in *E. histolytica* strains (Sehgal et al., 1994). rDNA circle harboring single transcription unit lacks HMg region (indicated by dotted arrow). (B) Dot blot analysis of stool samples with HMg probe. Rows 1–7 are samples loaded in triplicates. Row 8b is cloned HMe DNA as negative control and row 8c is HMg DNA as positive control. (C) Table indicates the HMg dot blot result obtained from a random population studied residing in Delhi. Samples were studied from asymptomatic population, hospital patients and amoebic liver abscess (ALA) patients. *Indicates total *Entamoeba* positive samples in each category, all HMg positive samples were *E. histolytica*. Data indicate that *E. histolytica* strains in nature commonly harbor single rRNA transcription unit in rDNA episome.

Fig. 2.

Transposon display for *Entamoeba histolytica* strain variation. (A) Diagrammatic representation of various internal EhSINE1 primers for transposon display. EhSINE1 do not have restriction enzyme Vsp1 site near 5' end (only two sites near 3' end at 524 and 534 bp) therefore Vsp1 was chosen as suitable enzyme for transposon display. (B) Transposon display carried out with different selective EhSINE1 primers using axenic *E. histolytica* strains. Variation in band patterns are indicated by right arrows. The figure modified from previously published research paper (Srivastava et al., 2005).