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Molecular Epidemiology of Amebiasis

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Abstract

Entamoeba histolytica, the causative agent of human amebiasis, remains a significant cause of morbidity and mortality in developing countries and is responsible for up to 100,000 deaths worldwide each year. *Entamoeba dispar*, morphologically indistinguishable from *E. histolytica*, is more common in humans in many parts of the world. Similarly *Entamoeba moshkovskii*, which was long considered to be a free-living ameba, is also morphologically identical to *E. histolytica* and *E. dispar*, and is highly prevalent in some *E. histolytica* endemic countries. However, the only species to cause disease in humans is *E. histolytica*. Most old epidemiological data on *E. histolytica* are unusable as the techniques employed do not differentiate between the above three *Entamoeba* species. Molecular tools are now available not only to diagnose these species accurately but also to study intra-species genetic diversity. Recent studies suggest that only a minority of all *E. histolytica* infections progress to development of clinical symptoms in the host and there exist population level differences between the *E. histolytica* strains isolated from the asymptomatic and symptomatic individuals. Nevertheless the underlying factors responsible for variable clinical outcome of infection by *E. histolytica* remain largely unknown. We anticipate that the recently completed *E. histolytica* genome sequence and new molecular techniques will rapidly advance our understanding of the epidemiology and pathogenicity of amebiasis.

Keywords

Molecular epidemiology; amebiasis; diagnosis; genetic diversity

Introduction

Entamoeba histolytica is an intestinal protozoan parasite and the causative agent of invasive amebiasis. Traditionally, about one-tenth of the world population is stated to be infected with *E. histolytica* (Walsh, 1986), resulting in up to 100,000 deaths worldwide each year (Anonymous, 1997, Haque et al., 2003a, Petri et al., 2000). However, although all the deaths could be due to invasive *E. histolytica* infections, the value for the prevalence of *E. histolytica* is an overestimate since it dates from before the separation of the pathogen *E. histolytica* from the non-pathogen *E. dispar* (Diamond & Clark, 1993). In addition, recent studies suggest that infection with *E. moshkovskii*, a morphologically identical species, is also

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common in some areas of *E. histolytica* endemicity (Ali et al., 2003, Beck et al., 2008, Fotadar et al., 2008, Khairnar et al., 2007, Parija & Khairnar, 2005) and could be contributing to the prevalence figures. Obtaining accurate species prevalence data remains a priority as there are gaps in our knowledge for many geographic regions.

It has long been known that not all *E. histolytica* infections lead to disease in the host (Walker and Sellards, 1913) and it is now clear that at most one in 4 *E. histolytica* infections progress to development of clinical symptoms (Gathiram & Jackson, 1987, Blessmann et al., 2003, Haque et al., 2006). Nevertheless, *E. histolytica* remains a significant cause of morbidity and mortality in developing countries (Haque et al., 2003a, Stanley, 2003). For example, the annual incidence of amebic dysentery in preschool children is 2.2% compared to 5.3% for *Shigella* dysentery in Bangladesh, a country in which one in every 30 children dies of diarrhea or dysentery before reaching his or her fifth birthday (Haque et al., 2003b). Similarly, the annual incidence of amebic liver abscess averaged 21 cases per 100,000 inhabitants in Hue City, Vietnam (Blessmann et al., 2002b).

Although only a minority of *E. histolytica* infections progress to development of intestinal diseases, such as diarrhea or dysentery, or extra-intestinal diseases like amebic liver abscess (ALA), the basis for this difference in clinical outcome remains mostly unsolved. A recent report suggests that the parasite genotype plays a role in determining outcome of infection by *E. histolytica* (Ali et al., 2007). Therefore, one of the current priorities in a postgenomic era is understanding the genetic factors determining the outcome of an *E. histolytica* infection.

Three morphologically identical species of *Entamoeba* that commonly infect humans: *E. histolytica*, *E. dispar* and *E. moshkovskii*

It is only *E. histolytica* that causes invasive disease in humans. This organism is the fourth leading cause of mortality due to a protozoan infection after malaria, African trypanosomiasis, and leishmaniasis and the third cause of morbidity among protozoa after malaria and trichomoniasis (Anonymous, 1998). *E. dispar* has never been documented to cause disease in humans, although a recent report suggests that a few strains may be able to produce liver abscesses in hamsters (Shibayama et al., 2007). It is now accepted that *E. dispar* infection is, in general, much more common than *E. histolytica* world-wide (Al-Hindi et al., 2005, Gatti et al., 2002, Hooshyar et al., 2004, Leiva et al., 2006, Nesbitt et al., 2004, Ramos et al., 2005a,b), although local prevalence may vary significantly.

The worldwide prevalence of *E. histolytica* and *E. dispar* as separate species is not well studied and that of *E. moshkovskii* is almost unknown. Amebic infection is prevalent in the Indian subcontinent, Africa, the Far East, and areas of South and Central America. In developing countries it depends largely on cultural habits, age, level of sanitation, crowding and socio-economic status. In developed countries, the infection is mostly due to *E. dispar* and is largely confined to certain groups: immigrants from or travelers to areas of endemicity, homosexual males, patients infected with human immunodeficiency virus, and institutionalized populations (Petri, 1996). In Japan, however, carriage of *E. histolytica* is more common, especially among male homosexuals (Takeuchi et al., 1990, Ohnishi et al., 2004). Case studies of patients with amebic colitis in Kwa-Zulu Natal, South Africa, showed that there was a peak incidence of infection among children <14 years of age and a second increase in infection in adults >40 years old (Gathiram & Jackson, 1985). Acuna-Soto et al. (2000) after reviewing all the published reports from 1929 to 1997 found that the male to female ratios for invasive intestinal amebiasis and asymptomatic carriage were 3.2:1 and 1:1, respectively. Most of the latter infections will be *E. dispar*.

Until recently *E. moshkovskii*, which is identical in both cyst and trophozoite form to *E. histolytica* and *E. dispar*, was considered to be mainly a free-living ameba, and therefore not a diagnostic concern. However, reports of high prevalence of human infections with this species have emerged from Bangladesh, India, Australia and Tanzania (Ali et al., 2003, Beck et al., 2008, Fotedar et al., 2007, 2008, Parija & Khairnar, 2005). Although there are several subtypes of *E. moshkovskii*, all the human isolates that have been characterized so far belong to only one group ('ribodeme 2'; Clark & Diamond, 1997, Haque et al., 1998b). Environmental isolates of *E. moshkovskii* originally came from sewage, and have subsequently also been found in fresh, brackish and salt water sediments (Clark & Diamond, 1997). There are a few physiological characteristics that distinguish *E. moshkovskii* from *E. histolytica* and *E. dispar*: (i) it is osmotolerant, (ii) it can grow at room temperature, and (iii) it is resistant to emetine (Clark & Diamond, 1997, Dreyer, 1961, Entner & Most, 1965, Richards et al., 1966). However, all of these attributes require cultures in order to be examined.

Human infections with *E. moshkovskii* have been reported to-date from North America, Italy, South Africa, Tanzania, Bangladesh, India, Iran, Turkey and Australia, and in general they are not associated with disease (Beck et al., 2008, Clark & Diamond, 1997, Haque et al., 1998b, Parija & Khairnar, 2005, Solaymani-Mohammadi et al., 2006, Tanyuksel et al., 2007), although one report suggests that it may be a potential pathogen in humans (Fotedar et al., 2008). Nevertheless, it is important to differentiate all three species. In the clinical setting, for example, an *E. dispar* or *E. moshkovskii* infected patient could be diagnosed as infected with *E. histolytica* and be treated unnecessarily with anti amebic chemotherapy. Most studies that have investigated the prevalence of *E. histolytica* and *E. dispar* have not considered the possible presence of *E. moshkovskii*. This was partly due to a lack of tools to detect *E. moshkovskii*. However tools are now available to diagnose these infections specifically using PCR (Ali et al., 2003).

There are many described species in the genus *Entamoeba* and several others infect humans in addition to *E. histolytica*, *E. dispar* and *E. moshkovskii*. These include *E. coli*, *E. hartmanni*, and *E. gingivalis*. There are also reports of *E. polecki* and *E. chattoni* infections in humans (Chacin-Bonilla, 1992, Kuroki et al., 1989, Sargeant et al., 1992, Verweij et al., 2001, Clark et al., 2006b) though they are normally associated with pigs and non-human primates, respectively.

Although *E. hartmanni* also produces cysts with four nuclei, their size is generally much smaller than those of *E. histolytica*/*E. dispar*/*E. moshkovskii*. Both *E. polecki* and *E. chattoni* have uninucleate cysts and they are now considered to be different variants of same species (Clark et al., 2006b), while *E. coli* produces cysts with 8 nuclei. *E. gingivalis*, a parasite of human oral cavity, is not known to encyst. As a result, as far as microscopic diagnosis of amebiasis is concerned none of these species is a problem, unless uninucleate cysts are misinterpreted as immature tetranucleate cysts.

Diagnosis of intestinal infection

From the diagnostic point of view specific detection of *E. histolytica* is necessary since *E. dispar* and *E. moshkovskii* are not proven to cause disease. Most of the epidemiological data for intestinal amebiasis are based on the identification of the species in stool specimens by light microscopy. However, microscopy is unable to differentiate the three species, is at best 60% sensitive, and can be confounded by false-positive results due to misidentification of macrophages and other species of *Entamoeba* (Pillai et al., 1999). Culture has been reported to be more sensitive than microscopy (Gonzalez-Ruiz et al., 1994), and isoenzyme analysis of cultured amebae enables the differentiation of *E. histolytica* from *E. dispar* and *E. moshkovskii* (Sargeant et al., 1980). However, culture and isoenzyme analysis requires a week

to complete and is negative in many microscopy-positive samples due to delays in sample processing or due to the initiation of antiamebic therapy prior to stool collection (Gonzalez-Ruiz et al., 1994, Haque et al., 1997, Strachan et al., 1988). Mixed infections could also be missed. Serological tests, particularly in areas of endemicity, can provide little information, as they are only 50% sensitive for diagnosis of intestinal infection and they cannot distinguish between current and past infections (Haque et al., 2007).

New approaches used to detect *E. histolytica* and *E. dispar* are based on antigen detection in stool specimens (Haque et al., 1997, Haque et al., 1993, Haque et al., 2000, Haque et al., 1995) and species-specific PCR amplification of *E. histolytica*, *E. dispar* and *E. moshkovskii* DNA (Ali et al., 2003, Britten et al., 1997, El Hamshary & Arafa, 2004, Katzwinkel-Wladarsch et al., 1994, Newton-Sanchez et al., 1997, Romero et al., 1992, Sharma et al., 2003, Tannich & Burchard, 1991, among others), including DNA from formalin-fixed stool samples (Paglia & Visca, 2004). Recently Blessmann et al. (2002a) and Roy et al. (2005) described two very fast and highly sensitive Real-Time PCR tests for the detection and differentiation of *E. histolytica* and *E. dispar* in fecal samples. The comparative efficacy of different species-specific diagnostic methods has been the topic of several papers (Furrows et al. 2004, Haque et al., 1998a, Mirelman et al., 1997, Stark et al., 2008). In general, the authors concluded that antigen detection and PCR (standard PCR, PCR-SHELA and Real-Time PCR) all performed adequately and choice of method should depend on the budget and timeframe of the study. Some examples of the prevalence of *E. histolytica* and *E. dispar* infection determined using different diagnostic methods are provided in Table 1.

In conclusion, the accurate differential diagnosis of intestinal infection with *E. histolytica*, *E. dispar* and *E. moshkovskii* is important for two reasons: first, to understand the worldwide distribution of the three species individually, and second, to prevent unnecessary chemotherapy in patients infected with *E. dispar* or *E. moshkovskii*.

Diagnosis of amebic liver abscess

The most common extraintestinal manifestation of *E. histolytica* infection is amebic liver abscess (ALA), which is found predominantly in adult males. This used to be a progressive disease with high mortality a century ago, but since the introduction of effective medical treatment and rapid diagnosis, mortality rates have fallen to 1–3% (Abuabara et al., 1982, Adams & MacLeod, 1977, Barnes et al., 1987, Boonyapisit et al., 1993, Katzenstein et al., 1982, Shandera et al., 1998, Thompson et al., 1985). ALA arises from hematogenous spread through the portal circulation of *E. histolytica* trophozoites that have crossed the colonic mucosa barrier and can occur many months after infection with *E. histolytica*.

The diagnosis of amebic liver abscess is sometimes difficult since its clinical manifestations are highly variable. Signs and symptoms of ALA include upper right quadrant pain, low grade fever, enlarged and tender liver, and weight loss, but not jaundice. A response to anti-amebic treatment can often help the diagnosis of an ALA. In non-endemic countries, ALA patients often show a history of travel to *E. histolytica* endemic countries prior to the development of ALA. Imaging techniques such as ultrasound, computed tomography, and magnetic resonance have excellent sensitivity for the detection of a liver abscess arising from any cause but cannot distinguish amebic abscesses from pyogenic (bacterial) abscesses or necrotic tumors.

Studies have reported that amebic liver abscess patients usually do not have concomitant amebic colitis and often have no bowel symptoms. Stool microscopy may not be helpful for diagnosis: reports of amebae in stool range from less than 10% to as high as 70% of ALA patients (Irusen et al., 1992, Katzenstein et al., 1982). Serological tests demonstrate the presence of antiamebic antibodies in serum but are negative in one quarter to one half of patients (Haque et al., 2000). Several groups have reported the detection of amebic antigen in the serum

of ALA patients (Abd-Alla et al., 1993, Haque et al., 2000, Karki & Parija, 1999). For example, Abd-Alla and colleagues detected the Gal/GalNAc lectin, a major surface antigenic molecule in *E. histolytica*, in the sera of 75% of South African ALA patients. In Bangladesh, the TechLab *E. histolytica* II test detected Gal/GalNAc lectin in the sera of 96% of ALA patients tested prior to treatment with the antiamebic drug metronidazole. However, after a few days of treatment with metronidazole, sensitivity of this method was only 33% (32/98) and 41% (11/27) for detection of lectin antigen in serum and liver abscess pus, respectively (Haque et al., 2000).

Although no precise data are available, it appears that the prevalence and epidemiology of ALA varies among *E. histolytica* endemic countries (Table 2). Hue City in central Vietnam has been identified as having a remarkably high incidence of ALA (Linh Van et al., 1996). ALA predominantly occurred in adults (95% cases), with a peak incidence in the 30–49-year old age group. The male to female ratio of ALA in this age group was about 7:1 (Blessmann et al., 2002b). In Sonora, Mexico, a similar age and gender distribution was observed, although the male to female ratio was about 4:1 (Valenzuela et al., 2007). In contrast, in Bangkok, Thailand, only 53% of cases were male (Wiwanitkit, 2002).

Prevalence of *E. histolytica* infection in HIV/AIDS patients

The association of amebiasis with HIV-positive individuals is not clear-cut. Stool microscopic investigation of HIV/AIDS patients often finds high percentages to be positive for *E. histolytica* / *E. dispar* / *E. moshkovskii* cysts. However, enteric infections in general are more common in HIV-infected than in HIV-negative individuals, so that this does not indicate a specific association with amebiasis.

Species-specific diagnostic methods help clarify the situation somewhat. For example, in Mexico the prevalence of *E. histolytica* in HIV/AIDS patients (*E. histolytica* mono-infections plus *E. histolytica* / *E. dispar* mixed infections) was 25.3% compared to 18.4% in a control HIV- group. The prevalence of mixed infections in particular, as determined by PCR, was very high in HIV/AIDS patients (13.3%) compared to a control group (0.7%) (Moran et al., 2005). In the Mexican study, none of the *E. histolytica*-infected individuals, whether HIV-infected or not, developed clinical symptoms within 12 months. A comprehensive analysis of risk factors in Taiwan indicated that HIV-positive male homosexuals are at a significantly higher risk of acquiring an *E. histolytica* infection than other HIV-positive individuals (Hung et al., 2008), but this appears to be true regardless of their HIV status. Although HIV-positive individuals are at higher risk of *E. histolytica* / *E. dispar* / *E. moshkovskii* infection compared to control groups, at least in certain countries, there is little evidence to suggest that they are more likely to develop symptomatic infections or more severe disease.

Intra-species genetic variation in *E. histolytica* and *E. dispar*

Genetic variation among *E. histolytica* isolates has been investigated extensively, as it may provide important clues as to why most infections are asymptomatic whereas some cause invasive disease.

Isoenzyme analysis

In the earliest extensive studies, predating the separation of *E. histolytica* and *E. dispar*, Sargeant and colleagues (Sargeant & Williams, 1978, Sargeant et al, 1978) used isoenzyme electrophoresis to study variation in *Entamoeba* species of humans. After studying approximately 10,000 intestinal amebic isolates using four enzymes (Sargeant, 1987), they identified more than 20 different isoenzyme patterns (known as 'zymodemes'): 9 proved to be from what is now called *E. histolytica* (formerly pathogenic zymodemes, PZ) while the rest were from *E. dispar* (formerly non-pathogenic zymodemes, NPZ). However, subsequent

studies suggest that there are only 4 zymodemes that are reliable for isolate typing (those found in axenic cultures; Jackson & Suparsad, 1997). The remaining zymodemes appear to be due to interference from bacterial enzymes (present in the xenic cultures used by Sargeant et al.). Even though additional enzyme systems have been studied by Blanc (1992), which revealed even more variation, the number of detectable variants remains small. In addition, isoenzyme analysis is time consuming, laborious, and it requires organisms to be established in cultures, which does not have a very high success rate in most laboratories. As a result this method is no longer widely used, although it played a very important role in the recognition of *E. dispar* as a separate species.

Polymorphism in protein coding genes

In studies involving strain typing the most widely used gene is the one encoding the serine-rich *E. histolytica* protein (SREHP), an immunodominant surface antigen containing tandem repeats of related dodeca- and octa-peptides (Köhler & Tannich, 1993, Stanley et al., 1990). Variation in repeat number and sequence are detected by either restriction enzyme digestion (Aye-Kumi et al., 2001, Clark & Diamond, 1993) or sequencing (Ghosh et al., 2000, Haghighi et al., 2002, 2003) of PCR-amplified genes. The degree of polymorphism is very high in both the *E. histolytica* gene (Table 3) and in its homolog from *E. dispar* (E. Paez, 1997, unpublished MSc thesis). One of the drawbacks of SREHP is that PCR often produces multiple bands from single strain because of allelic variation. As a result, it would be very difficult, if not impossible, to detect a mixed infection of two strains in the same patient, and it can make sequence analysis difficult.

Polymorphism has also been investigated, although less widely, in the repeat-containing chitinase gene of both *E. histolytica* and *E. dispar* (Table 3), which is expressed only during encystation of the ameba (de la Vega et al., 1997a, de la Vega et al., 1997b). Polymorphism in other genes has been studied very rarely. The Gal/GalNAc lectin, a major amebic virulence protein, shows only very limited diversity among strains of *E. histolytica* (Beck et al., 2002).

Polymorphism in non-coding DNA

A few PCR-based DNA typing methods have been reported that use repetitive elements contained within non-coding DNAs. In addition to its chromosomal genome *E. histolytica* also contains highly repetitive extrachromosomal DNA circles of about 25 kb in size and 200 copies per genome that encode the ribosomal RNAs (Bhattacharya et al., 1988, Bhattacharya et al., 1989, Huber et al., 1989). The 'strain specific gene' (SSG) (Burch et al., 1991) or 'Tr' (Sehgal et al., 1994) also forms part of this episome. It produces a non-coding transcript and has been localized to a region upstream of the rRNA cistrons. SSG contains tandem 26bp repeats and PCR amplification has revealed variations in the number of repeats among *E. histolytica* strains (Clark & Diamond, 1993). However, in some *E. histolytica* strains this region of the episome is completely absent and *E. dispar* strains also do not possess this gene, which limits its utility.

Polymorphism has been investigated using 'random amplification of polymorphic DNA' (RAPD). Gomes et al. (2000) have reported RAPD analysis using axenic strains of *E. histolytica*. Using 11 reference strains of *E. histolytica* originally isolated from 4 asymptomatic and 7 symptomatic individuals, mainly from Brazil and Mexico, the authors found that only about 70% of the RAPD bands were shared. However, no relationship between the RAPD profiles and the clinical manifestations of infected individuals or the zymodeme of the strains was detected. Analysis of a few north Indian isolates showed a high degree of polymorphism (Prakash et al., 2002), but each isolate invariably produced multiple PCR products, which is not ideal as analysis of results is difficult. A major problem with this study is that they did not use DNA from axenic strains so it is not clear whether the bands are derived from ameba DNA.

The need for axenic cultures makes RAPD an unlikely contender for future use as an epidemiologic tool.

Polymorphism in short tandem repeat loci linked to tRNA genes

In 2005, the genome sequence of the *Entamoeba histolytica* strain most widely used in research laboratories, HM-1:IMSS, was completed (Loftus et al., 2005). The genome consists of about 24 megabases of DNA distributed over an unknown number of chromosomes (at least 14; Willhoeft & Tannich, 1999). One of the striking findings was the abundance and unique organization of the tRNA genes (Clark et al., 2006a). Over 10% of all the sequence reads contained tRNA genes and almost all were organized in tandem arrays. Intergenic regions are rich in A+T (about 80%) and contain non-coding short tandem repeats (STRs).

Some of the tRNA array STRs were originally identified independently by other groups (Huang et al., 1997, Lohia et al., 1990, Michel et al., 1992, Rosales-Encina and Eichinger (GenBank accession number AF265348)) but their potential as polymorphic markers was investigated first by Zaki and Clark (2001). Diversity in these loci among *E. histolytica* strains is mainly due to varying numbers of STRs (Zaki & Clark, 2001). When a comparison is made between *E. histolytica* and *E. dispar* these loci vary not only in the number but also in the sequence and arrangement of STRs (Zaki et al., 2002, Tawari et al., 2008).

The diversity at some of these loci is almost as high as in the SREHP gene, even in geographically restricted regions, such as Kwa-Zulu Natal, South Africa (Zaki, 2002, Zaki et al., 2003a), Recife and Macaparana, Northeast Brazil (Pinheiro et al., 2005), Japan (Haghighi et al., 2002, 2003). Ali et al. (2005) have described a system for typing *E. histolytica* strains based on 6 of the tRNA gene-linked STRs. Using this method, a recent study of *E. histolytica* samples collected from asymptomatic, diarrhea/dysenteric and amebic liver abscess patients in Bangladesh revealed that the parasite genotype prevalences identified in the 3 groups were significantly different from each other, suggesting that the parasite genotype plays a role in the outcome of infection in humans (Ali et al., 2007).

Choice of method

For epidemiology studies, the choice of polymorphic marker is crucial. The marker needs to have the apparently conflicting characteristics of polymorphism and stability. The technique for detecting the markers must be reliable, employ methodology that is transferable among laboratories, and ideally should be suitable for use with large numbers of samples. The degree of diversity among *E. histolytica* strains varies among target loci and method of detection used (Table 3).

In the past few years the tRNA-linked STR loci appear to have become the target of choice. Different studies have employed variable numbers of loci but always with good results, indicating that the methodology is transferable. Polymorphism is clearly high, and although diversity is not quite as high as for the SREHP gene these loci do have other advantages.

Among the advantages of this method compared are that i) it shows very high discriminatory power in detecting variation among strains of *E. histolytica*, ii) a single PCR amplicon is generated for each STR (in the 6-locus system), iii) no restriction endonuclease digestion is necessary, iv) cross-species and species-specific amplification are both available, v) cultures are not needed as PCR amplification from stool DNA is possible, vi) because the targets are multi-copy in the genome, PCR requires a very small amount of target DNA, and vii) the use of multiple loci reduces the likelihood of chance similarity among strains.

Three STR-based studies in 3 countries have used multiple samples from the same infected individuals (Ali et al., 2007, Blessmann et al., 2003, Zaki et al., 2003a). In the South African study (Zaki et al., 2003a), family members were usually infected with the same genotypes of *E. histolytica* and *E. dispar*. Similar epidemiological linkage was observed using SREHP in the Philippines (Rivera et al., 2006) where individuals living in close proximity also shared the same genotypes. This indicates a common source of infection, and also suggests that transmission of the parasite from one member of a family/group to other does not lead to the change in parasite genotype. In Vietnam, PCR patterns remained the same after 1 year for 23 out of 28 individuals infected with *E. histolytica* (Blessmann et al., 2003). In Bangladesh, follow-up samples in 13 out of 23 children showed the same genotypes (Ali et al., 2007), while the evidence suggested that the other 10 children had acquired new infections.

Genotypes obtained using DNA prepared from cultures are identical to those seen with DNA extracted from the original stool sample, indicating that while culture is not necessary it is not misleading either (Zaki et al., 2003b). No changes in the patterns at any of the STR loci have been observed using DNAs extracted from *E. histolytica* HM-1:IMSS or *E. dispar* SAW760 at various times over several years or in different laboratories around the world (unpublished observations). Likewise, mouse passage of strains failed to result in any changes (unpublished observations).

The conclusion from these studies is that the markers appear to be sufficiently stable to use in molecular epidemiological studies. However, it is worth noting that recently our laboratory has identified two closely related populations in the long-term cultured strain *E. histolytica* HM-1:IMSS, after cloning using the soft agar method (Mueller & Petri, 1995). The two populations differ from each other by only one repeat in 1 of the 6 tRNA-linked polymorphic loci tested (unpublished data). In DNA extracted from uncloned cultures or stool only the predominant form of the locus is seen after amplification/sequencing and this needs to be remembered.

Infections with multiple genotypes

Several reports suggest that mixed infections of *E. histolytica* and *E. dispar* are not uncommon (Acuna-Soto et al., 1993, Beck et al., 2008, Haque et al., 1998a, Khairnar et al., 2007, Zaki et al., 2003a). Similarly, *E. moshkovskii* infections have often been found to be associated with *E. dispar* and/or *E. histolytica* infections (Ali et al., 2003, Beck et al., 2008, Fotedar et al., 2008, Khairnar et al., 2007, Parija & Khairnar, 2005). Use of species-specific PCR amplification can usually detect and differentiate multiple infections, at least where distinct primers are used for each species.

Infection with multiple strains of *E. histolytica* has only been reported in two cases, both of which suggest transient superinfection with a second strain: one in South Africa (Zaki et al., 2003a) and one in Bangladesh (Ali et al., 2007). This is possibly due to limitations of the method used for genotyping. There are several factors that can mask a multi-genotype infections, for example, if one strain is present in significantly higher number compared to the other, or if the patterns are too similar to be differentiated unambiguously by the genotyping method used. Experimental mixing of two *E. histolytica* strains showed that it is possible to detect both products even when the minor population represents only 4% of the cells (unpublished data). However, differentiating between faint bands that are PCR artefacts and those that indicate infection with multiple strains of *E. histolytica* would be very difficult with 'real' samples.

Sequencing v. product size for genotyping

The majority of studies mentioned above have employed PCR product size to differentiate genotypes rather than DNA sequencing. This is largely a matter of cost and as sequencing becomes more affordable the method of choice will likely shift away from PCR product size to PCR product sequence. Several studies employing DNA sequences have indeed already been published.

Haghighi et al. (2002, 2003) sequenced PCR products from 4 loci – chitinase, SREHP, and two tRNA-linked STRs - for 79 *E. histolytica* strains isolated from asymptomatic and symptomatic individuals in at least 8 different countries. The chitinase gene showed limited PCR product size variation but somewhat greater sequence diversity, with a total of 9 sequence types and an equal number of predicted peptide sequences. SREHP showed the most polymorphism with a total of 37 sequence types and 31 different peptide sequences. The two tRNA-linked STRs showed intermediate polymorphism with 13 to 15 sequence types. No single nucleotide polymorphisms (SNPs) were reported by these authors in any of the 4 loci outside of the repeat regions, and no clinical correlation with sequence types was observed. Tawari et al. (2008) have reported sequence diversity at 3 additional STR loci of *E. histolytica* and in a large number of samples. The number of sequence types ranged from a low of 12 in 138 samples to a high of 18 in 54 samples, depending on the locus examined. Importantly, in both these studies it was noted that PCR products of the same size do not necessarily have the same sequence, clearly showing the advantage of sequence over the PCR product-size in detecting diversity.

While there have been several DNA-based studies of inter- and intra-species variation in *E. histolytica* and *E. dispar*, only a very few studies of intra-species variation in *E. moshkovskii* have been reported (Ali et al., 2003, Clark & Diamond, 1991, 1997). Sargeant et al. (1980) reported *E. moshkovskii* variants using isoenzyme analysis long before Clark and Diamond (1991, 1997) reported 6 groups among 5 human and 20 environmental isolates, using restriction enzyme digestion of PCR amplified small subunit rRNA genes. Ali et al. (2003) have described intraspecific size and sequence polymorphism based on PCR amplification of a tRNA locus but diversity data are very limited at present. In contrast to *E. histolytica* and *E. dispar*, *E. moshkovskii* does not have STRs linked to its tRNA genes (Tawari et al., 2008) but diversity in the intergenic spacer regions exists nevertheless.

Insights gained from genotyping of *E. histolytica* strains

Although the studies of Haghighi et al. (2002, 2003) did not detect a link between genotype and symptoms, their samples were from geographically diverse sources and acquired over a number of years. The study of Ali et al. (2007) used a geographically and temporally restricted sample set, and in contrast did find a distinction among sample groups. Although PCR product size rather than sequence was used in genotyping the samples, the resulting loss of discrimination among distinct products of the same size would be expected to decrease the associations and so the degree of difference between populations is probably underestimated. The need for samples associated in space and time in order to detect the associations is probably linked to the rate of change at STR loci. Although the genotypes appear to be stable by the criteria we can measure, they must be mutating to generate the diversity we see in populations. As a result, over time any associations would be lost as new genotypes arise in the population.

A recent study using size and sequencing of tRNA-linked STRs has given insights into links between diversity and virulence. Paired stool and liver abscess pus samples from 18 ALA patients from Bangladesh, Italy and USA surprisingly revealed that the genotypes of the intestinal amebae were different from those in the corresponding pus samples of the same patient (Ali et al, 2008). The explanation for this finding is as yet unclear, but could indicate

either that the intestinal population contained multiple genotypes and only a subpopulation migrated to the liver, or that DNA reorganization or recombination events are taking place prior to or during migration of the amebae from the intestine to the liver.

Alternative approaches to genotyping

Comparative genomic hybridization with microarrays has been used to investigate the genotypic differences among 4 reference strains of *E. histolytica* and 2 of *E. dispar* (Shah et al., 2005), and a number of genes restricted to *E. histolytica* were identified. One of the drawbacks of this approach is that it requires a large amount of genomic DNA (5 to 10 µg), which has so far restricted its use to laboratory strains only. It is possible that with further methodological refinements this approach to detecting genome-wide variation could have wider application in the future.

SNP analysis may well be the main tool in the future as such markers will be more stable than the STRs and potentially could also be directly linked to the outcome of infection. To date, SNP analysis has been limited to only one report (Bhattacharya et al., 2005) where the authors detected 0.007% SNPs in coding regions among the *E. histolytica* strains studied, with non-coding region SNPs being 5 fold more frequent. However, with recent advances in high-throughput sequencing technology, it is likely that whole genome sequences will soon be available for several additional strains of *E. histolytica*. This will rapidly lead to the evaluation and implementation of SNP-based typing methodologies for molecular epidemiological studies.

Current challenges

Large gaps in our knowledge of species prevalence rates in different parts of the world remain. To address this, species-specific diagnosis of *E. histolytica*, *E. dispar* and *E. moshkovskii* needs to be implemented, especially in countries where *E. histolytica* and *E. dispar* are endemic but elsewhere also. Based on the limited information available to date it appears that *E. moshkovskii* is being detected mostly when PCR is used. This may indicate that the number of cysts generated in infections with this species is low compared to *E. histolytica* and *E. dispar* (Beck et al., 2008). Screening of microscopy negative or random samples would be needed to identify *E. moshkovskii* infections and obtain a clear picture of prevalence. Evaluation of the pathogenic potential of *E. moshkovskii* strains in humans should also be a priority, but until species identification is widespread this will not be possible.

Data on the persistence of infections are contradictory at present. The study of Blessmann et al. (2003) in Vietnam indicated a mean half-life of *E. histolytica* infection of over a year, whereas in Bangladesh the study of Haque et al. (2002) indicated less than two months. Further investigation will be necessary to see whether this discrepancy is due to the differences in study population (adults v. children), detection techniques (PCR v. antigen), parasites strains, or currently unknown factors.

The tRNA-linked STR genotyping system has provided evidence that the parasite genome does influence the outcome of infection. However, as these results were based exclusively on samples from Bangladesh, it is also important to know whether the observations can be replicated in other areas of *E. histolytica* endemicity such as Mexico, Vietnam or India. tRNA-linked STR genotyping was also behind the recent observation of differences between parasite genotypes in the intestine and the liver abscess of same patient. It will be very difficult to prove what is actually happening in vivo to give rise to this result but worth the effort.

Although most *E. histolytica* appear to have the ability to invade host tissues and to produce disease, we still do not know whether some strains of *E. histolytica* are intrinsically avirulent.

It is highly unlikely that tRNA-linked STR variation is directly linked to virulence in any way. Other approaches, such as SNP identification coupled with microarray based analysis of gene expression or proteomic comparisons among parasites will be needed to identify the actual genes responsible for these results and to help us to understand the mechanism of parasite virulence and pathogenesis. Additional sequencing of *E. histolytica* genomes from carefully selected strains will be required to identify the SNPs and this is currently ongoing.

These are exciting times for those interested in the epidemiology of amebiasis. There should be few limits to the scope for molecular and genetic investigations to be combined effectively into epidemiological and clinical studies. The currently available genome sequence is not only helping understanding the parasite better but is also bringing researchers in the amebiasis community together to share and work to achieve common goals in solving unsolved questions. The future availability of additional genome data will only enhance this cooperation. We can anticipate rapid development of this field in the next few years.

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Prevalence of intestinal amebiasis. Reported prevalence of infection with *E. histolytica*, *E. dispar* and *E. moshkovskii* are given where available. This list is not intended to be comprehensive but focuses on the most recent reports.

Table 1

Method of diagnosis	Country	Population studied	Prevalence (%)	Reference
Microscopy	Korea	Handicapped people	1.80%	Lee et al., 2000
	Thailand	Institutionalized handicapped	7.10%	Sirivichayakul et al., 2003
	Lebanon	General population	2.30%	Saab et al., 2004
	Gaza Strip, Palestine	6–11 year old children	7.00%	Astal et al., 2004
	Cambodia	School children	0.80%	Part et al., 2004
	Ethiopia	Schoolchildren and prisoners	27.60%	Kebede et al., 2004
	Ethiopia	AIDS patients	10.30%	Halemariam et al., 2004
	Iran	General population	1.40%	Hooshyar et al., 2004
	Tanzania	Adults in Kilimanjaro region	8.70%	Nesbitt et al., 2004
	Mexico	Rural population	12.80%	Ramos et al., 2005a
	Brazil	Urban poor	5.80%	Pinheiro et al., 2005
	Egypt	Hospitalized patients	16.20%	El-Kadi et al., 2006
	Australia	Reference laboratory	2.90%	Fotedar et al., 2007
	Libya	Children and neonatus	36.60%	Kassem et al., 2007
	India	Hospital	11.70%	Khairnar et al., 2007
	Turkey	Hospitalized patients	5.30%	Koroglu et al., 2007
	India	HIV infected/uninfected	17.5%/3.0%	Ramakrishnan et al., 2007
	Brazil	Villagers	21.00%	Santos et al., 2007
	South Africa	Semi-rural	1.0% Eh and 9.0% Ed	Gathiram and Jackson, 1985
	UK	Male homosexual population	20% Ed, no Eh	Allason-Jones et al., 1986
Isoenzyme analysis	Seychelles	General population	2.6% Eh, 12.8% Ed	Sargeant, 1992
	Ecuador	School children	Ed is 3.7 times higher than Eh	Gatti et al., 2002
ELISA: Antibody detection	Mexico	In general Mexican population	8.4% Eh	Caballero-Salcedo et al., 1994
	Bangladesh	Pre-school children in Mirpur	76.0% Eh	Haque et al., 1999
ELISA: Antigen detection	Mexico	Puebla region	6.4% Eh	Sanchez-Guillen et al., 2000
	Tanzania	Adults in Kilimanjaro region	0.8% Eh and 7.4% Ed	Nesbitt et al., 2004
PCR	Bangladesh	Hospitalized urban children	4.2% Eh	Haque et al., 2006
		Asymptomatic children from urban slum	4.3% Eh	
		Asymptomatic children from rural village	1.0% Eh	
	South Africa	Hospitalized patients	18.8% Eh and 25.3% Ed	Samie et al., 2006
		School children	2.1% Eh and 8.5% Ed	
	Egypt	Hospitalized patients	9% Eh	El-Kadi et al., 2006
	Saudi Arabia	Hospitalized patients	2.7% Eh	Bamawi et al., 2007
	Tanzania	HIV infected	4% Eh	Beck et al., 2008
	Vietnam	Asymptomatic adults	11.2% Eh	Blessmann et al., 2003
	Ethiopia	School children, prisoners, and HIV patients	Only Ed	Kebede et al., 2003, 2004
	Iran	General population	Ed 11.7 times more common than Eh	Hooshyar et al., 2004
	Brazil	Urban poor	Only Ed	Pinheiro et al., 2005
	Gaza Strip, Palestine	Hospitalized patients	69.6% Eh, 22.8% Ed, 7.6% Eh+Ed **	Al-Hindi et al., 2005
	Mexico	Rural population	5.4% Eh, 48.6% Ed, 16.2% mixed **	Ramos et al., 2005a
	Nicaragua	Diarrhea patients	1.5% Eh and 7.5% Ed	Leiva et al., 2006
	Italy	Reference laboratory	5.6% Eh and 8.3% Ed	Calderaro et al., 2006
	Australia	Reference laboratory	5.6% Eh, 70.8% Ed and 61.8% Em **	Fotedar et al., 2007
	India	Hospital	3.5% Eh, 9.3% Ed and 1.9% Em	Khairnar et al., 2007
	Brazil	Villagers	0.8% Eh, 8.7% Ed	Santos et al., 2007
	Tanzania	HIV +ve	5% Ed, 13% Em	Beck et al., 2008
			** = % of cyst positive samples	

Method of diagnosis	Country	Population studied	Prevalence (%)	Reference

Table 2

Prevalence of ALA.

Region and/or country	Origin	Period	No. of ALA cases	No. of ALA cases per year	References
Hue City, Vietnam	Central Hospital records	1990–1998	2031	225.7	Blessmann et al., 2002b
San Francisco, USA	Medical histories	1979–1994	56	3.5	Seeto and Rockey, 1999
Mexico and Southeast Africa	Literature search	1979–1997	5642	81.8	Acuna-Soto et al., 2000
Bangkok, Thailand	King Chulalongkorn Memorial Hospital	1992–2001	62 (23 HIV +)	6.2	Wivanitkit, 2002
Bordeaux, France	Bordeaux University Hospital Center	1995–1999	20 (18 HIV +)	4	Djossou et al., 2003
Palma de Mallorca, Spain	Hospital Universitario Son Dureta	1991–2001	7 (2 HIV +)	0.6	Ruiz de Gopegui et al., 2004
Seoul, South Korea	Seoul National University Hospital	1990–2005	31 (10 HIV +)	1.9	Park et al., 2007
Sonora, Mexico	4 hospitals in Hermosillo	2000–2005	319	53.2	Valenzuela et al., 2007
Taipei, Taiwan	National Taiwan University Hospital	1994–2005	40 (40 HIV +)	3.3	Hung et al., 2008

Table 3

Genetic diversity studies

Genetic locus	Method	Origin of samples studied	Diversity (genotypes/sample number)	Reference
Serine Rich <i>E. histolytica</i> Protein (SREHP)	PCR-RFLP	Diverse laboratory strains	11/18	Clark & Diamond, 1993
		Bangladesh	34/54	Ayeh-Kumi et al., 2001
		Republic of Georgia	4/8	Simonishvili et al., 2005
Chitinase	Sequencing	Institution for the mentally retarded, Philippines	6/74	Rivera et al., 2006
		Cameroon, Zimbabwe, South Africa	33/61	Samie et al., 2008
		Japan, Thailand, Bangladesh	37/79	Haghighi et al., 2003
tRNA-linked STR loci (locus ID)	PCR	Japan, Thailand, Bangladesh	9/79	Haghighi et al., 2003
		Rural population, Mexico (<i>E. dispar</i>)	13/60	Ramos et al., 2005c
		South Africa	5/41 (D-A), 10/41 (I-W), 4/41 (E-Y), 5/41 (Y-E), 9/41 (V-F), 8/41 (F-V)	Zaki, 2002
	Sequencing	Brazil (<i>E. dispar</i>)	6/39 (D-A), 19/39 (I-W)	Pinheiro et al., 2005
		Bangladesh	10/111 (S-D), 6/111 (R-R), 10/111 (N-K2)	Ali et al., 2007
		Japan, Thailand, Bangladesh	10/111 (S-Q), 4/111 (D-A), 5/111 (A-L)	Haghighi et al., 2003
		Primarily Bangladesh	13/79 (D-A), 15/79 (I-W)	Tawari et al., 2008
			17/128 (S-D), 12/136 (R-R), 18/53 (N-K2)	