The diagnostic implications of the separation of *Entamoeba histolytica* and *Entamoeba dispar*

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For much of the last hundred years most cases of amoebiasis have been diagnosed by light microscopy. Only relatively recently have we become aware that this technique is usually incapable of distinguishing between two species – *Entamoeba histolytica* and *E. dispar* – only the first of which is a pathogen. The implications of this for patient management and, even more, for the validity of epidemiological surveys, are only slowly being addressed. What is clear is that methods are urgently required to distinguish between infections with these two species and this review attempts to summarise some of those, which have been developed to meet this need.

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1. Introduction

In 1875 Fedor Lösch (Kean *et al* 1978) described the case of a young farmer who had been admitted to his clinic in St Petersburg, Russia, two years earlier. The man had been suffering from chronic dysentery and Lösch found such large numbers of amoeba in his faeces that he became convinced that they were responsible for the dysentery. He named the organism *Amoeba coli* and showed that it produced colonic ulceration and dysentery in dogs. Subsequently Schaudinn renamed the organism *Entamoeba histolytica*.

2. Invasive amoebiasis

This patient of Lösch's was the first person known to have died from amoebiasis. Clinical disease results from the ability of *E. histolytica* to penetrate the wall of the large bowel and to spread extraintestinally. In brief, penetration of the gut wall may lead to ulceration which, if extensive enough, produces the classical signs and symptoms of amoebic dysentery. Extraintestinal spread most frequently involves the liver, causing hepatic amoebiasis or amoebic liver abscess; spread to other distant organs such as the brain is known but is very rare. This is not a clinical article and current texts should be consulted for advice on patient management (Ravdin and Petri 1994; Petri and Singh 1999; Hughes and Petri 2000).

The accurate diagnosis of hepatic amoebiasis (amoebic liver abscess) has always been easier, even with limited resources, than that of amoebic dysentery – a consequence of a characteristic clinical picture, significantly raised antibodies detectable with simple whole-antigen tests and lesions easily detected by widely-available ultrasonography (Petri and Singh 1999). Invasive intestinal amoebiasis, on the other hand, has always presented more difficult challenges.

3. The asymptomatic cyst-passer

By the start of the 20th century it had become very clear that many persons shedding cysts of "*E. histolytica*" had no symptoms of disease and in 1925 Emile Brumpt (Brumpt 1925) suggested that there were two separate but indistinguishable types of amoeba. However, because Walker and Sellards (1913) had already demonstrated that cysts isolated from asymptomatic patients could, on occasion, cause disease when fed to volunteers, and

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because there was no way of distinguishing between the cysts of the pathogenic and non-pathogenic species, Brumpt's suggestion was largely ignored. Indeed, in 1969 WHO (WHO 1969) defined amoebiasis as "infection with *Entamoeba histolytica*, with or without clinical manifestations", implying that all strains were potentially pathogenic.

For many years it was far from clear whether the outcome of infection was due to differences in host or parasite, but since the pioneering observation of Martínz-Palomo and colleagues on lectin-mediated agglutination (Martínez-Palomo *et al* 1973), 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. Subsequently a very large amount of isoenzyme work by Sargeaunt and Williams demonstrated consistent differences between pathogenic and non-pathogenic isolates (Sargeaunt 1988). In particular "fast" hexokinase bands became and remain the simplest marker of pathogenicity, while many of the original distinct zymodemes are now known to result from variation in culture conditions (Blanc and Sargeaunt 1991).

An increasing number of biochemical, immunological and genomic differences between the two species are now recognized and this information finally led to the formal separation of the two species (Diamond and Clark 1993) with the name *Entamoeba histolytica* being retained for the pathogenic species and Brumpt's name *E. dispar* being revived for the non-pathogen.

4. Biological characteristics that distinguish *E. histolytica* from *E. dispar*

- Isoenzyme patterns, particularly hexokinase.
- Specific epitopes, recognized by reaction with several monoclonal antibodies.
- Sequence differences in the rDNA episome.
- Significant (2–18%) sequence differences between homologous genes.
- A small number of genes, for example *ariel* (Willhoeft *et al* 1999a) and *cp5* (Bruchhaus *et al* 1996; Willhoeft *et al* 1999b) appear so far to be unique to *E. histolytica*.
- 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).
- Scanning electron microscopic examination of axenic cultures of both species show significant differences – particularly in the appearance of the surface (Clark *et al* 2000). This may be linked to the apparent lack of surface lipophosphoglycan (LPG) from *E. dispar* (Bhattacharya *et al* 2000).

So, in summary, we now believe that four, not three species of *Entamoeba* (*E. histolytica*, *E. dispar*, *E. coli*, *E. hartmanni*) may regularly be found in the human large bowel, only one of which is a pathogen. There are also a few rare species: "atypical," "low temperature" or "Laredo" strains of *E. histolytica*, now known to be the normally free-living species *E. moshkovski* (Clark and Diamond 1991), *E. polecki*, *E. chattoni* and *E. gingivalis*, which will not be considered further here. Of the principal four, the important point is that the cysts of *E. coli* and *E. hartmanni* may be distinguished by light microscopy applying well-understood criteria from those of *E. histolytica* and *E. dispar* but that the latter two are indistinguishable from each other.

5. The Mexico meeting

In 1997 WHO convened a meeting in Mexico City (WHO 1997) to consider the implications of the separation of the two species. The definition of amoebiasis as "infection with *Entamoeba histolytica*, with or without clinical manifestations" was reaffirmed but the name *Entamoeba histolytica* is now to be used only for the pathogenic species, clearly separated from *E. dispar*. Amongst the conclusions of the meeting were:

- The criteria (such as size) used to form the classical taxonomic description of *E. histolytica* cannot distinguish between *E. histolytica* and *E. dispar* and, of particular importance in diagnostic microscopy, cysts of the two species are identical.
- When diagnosis is made by light microscopy, since the cysts of the two species are indistinguishable, they should be reported as *E. histolytica/E. dispar*.
- In asymptomatic individuals treatment is not appropriate when *E. histolytica/E. dispar* has been specifically detected
- Optimally, *E. histolytica* should be specifically identified and infections, if present, treated.

What this means in practice is that while it would be ideal if every infected patient had a specific diagnosis of either *E. histolytica* or *E. dispar* made, the diagnostic procedure most frequently employed in disease-endemic areas (light microscopy) is unable to do this.

6. Species-specific diagnosis of *E. histolytica* and *E. dispar*

Before considering the various methods available for distinguishing between the two species in faeces, it is worth noting that there are two situations (both acknowledged at the Mexico meeting) where a presumptive diagnosis of infection with *E. histolytica* can be made without actually identifying the organism present.

The first is where amoebic trophozoites containing ingested red blood cells ("haematophagous trophozoites") can be identified in the faecal specimen (González-Ruíz *et al* 1994). For this to be even possible the specimen must either be examined as soon as possible after being passed (within 30 min at most), meanwhile being kept warm if necessary or it must immediately be fixed and subsequently stained, usually with Trichrome. Once fixed, specimens may be stored for many months without deterioration. Detailed protocols may be found in standard reference books (Garcia and Bruckner 1997) or obtained from the Centers for Disease Control (http://www.dpd.cdc.gov/dpdx/HTML/DiagnosticProcedures.htm). Although *E. dispar* is perfectly capable of ingesting red blood cells *in vitro* this does not appear to occur in the colon even if blood is present as a result of a second infection such as shigellosis. It is also worth noting that for this reason the presence of trophozoites without ingested red blood cells, even in a specimen of bloody diarrhoea, does not enable the diagnosis of amoebic dysentery to be made.

A related but much more invasive technique is the examination of fixed and stained biopsy specimens. The identification of trophozoites deep within the tissues and containing ingested red blood cells enables a definitive diagnosis of invasive amoebiasis due to *E. histolytica* to be made; unfortunately normal haematoxylin and eosin staining is not ideal for these parasites. Periodic acid-Schiff (PAS) or direct fluorescent antibody staining is much superior (Gilman *et al* 1980).

Secondly, in symptomatic individuals the presence of high titres of specific antibody is strongly correlated with the presence of invasive amoebiasis. In patients normally resident in non-endemic areas with low or undetectable levels of pre-existing antibody, serological testing using simple techniques and crude antigens (such as ELISA or indirect haemagglutination) is an effective diagnostic technique for intestinal as well as hepatic invasive amoebiasis (Pillai *et al* 1999). Results are much less clear-cut in disease-endemic areas; while virtually everyone infected with *E. histolytica* will be seropositive whether or not they are symptomatic (Jackson *et al* 1985), these antibodies will persist for months or years following spontaneous or drug-induced loss of the infection. This, plus the fact that titres tend to be lower than in cases of hepatic amoebiasis, makes simple serology of limited diagnostic value where infection is common (Hossain *et al* 1983; Shetty *et al* 1988).

The use of recombinant antigens and defined epitopes reduces but does not completely eliminate the problem of persisting seropositivity (Lotter *et al* 1995; Stanley *et al* 1998; Haque *et al* 1999). The specific measurement of IgM rather than IgG may also help to distinguish current from past infections (Abd-Alla *et al* 1998).

7. Asymptomatic carriage of *E. histolytica*

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 probably 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 (E Tannich, personal communication) have shown that, surprisingly, only a few percent of those genuinely infected with *E. histolytica* ever go on to develop clinical amoebiasis. Why this should be is not clear and there are many possible explanations but it does not reduce the need for the accurate, species-specific diagnosis of *E. histolytica* in faecal specimens. This is needed firstly because, since there is no way of knowing which infected persons will progress to clinical amoebiasis, all should be treated; secondly because they are (as Walker and Sellards showed all those years ago) excreting cysts which can cause clinical amoebiasis in others and thirdly, because only in this way can we obtain accurate epidemiological data from which to make a better estimate of the burden of amoebiasis on the health of the world (Petri *et al* 2000).

8. Specific detection of *E. histolytica* and *E. dispar* in faeces

8.1 *Isoenzyme analysis*

This technique, which was amongst the earliest to suggest that "pathogenic" and "non-pathogenic" *E. histolytica* were in fact two separate species, had until recently been applied to more specimens than any other and thus deserved its reputation as the "gold standard" against which newer methods need to be validated (Sargeaunt 1988). In practice, however, it has several disadvantages: culture of microscopically cyst-positive faeces is by no means always successful (Sehgal *et al* 1995); it may take seven to fourteen days to grow enough trophozoites to prepare the lysates for analysis and the process itself is somewhat cumbersome. Even though the original procedure involving starch-gel electrophoresis of four enzymes is often simplified to merely examining hexokinase mobility in agarose mini-gels (Strachan *et al* 1988), isoenzyme electrophoresis is now rarely used in clinical diagnosis.

8.2 *Antigen detection*

Although only a very few genes specific to *E. histolytica* and absent from *E. dispar* have been discovered as yet, almost all homologous proteins contain amino-acid

substitutions which result in the expression of species-specific epitopes. Detection of these epitopes with monoclonal antibodies is the basis of a number of quick and convenient diagnostic methods. The target molecule which has been most intensively studied is the heavy subunit of the galactose/N-acetyl-galactosamine inhibitable lectin; of six monoclonal antibodies raised against this molecule (Petri *et al* 1990b), only two reacted with *E. histolytica* and *E. dispar* while the other four reacted only with *E. histolytica* (Petri *et al* 1990a). These antibodies are the basis of two kits manufactured by TechLab Inc. (www.Techlab.com) one of which (based on one of the non-specific monoclonal antibodies) identifies *E. histolytica*/*E. dispar* while the other, based on a specific one can identify *E. histolytica*. Sequential application of these two kits can specifically identify both species although they cannot distinguish mixed infection with *E. histolytica* and *E. dispar* from infection with *E. histolytica* alone. Evaluation in Bangladesh shows clearly that these kits are more sensitive and specific than either wet-film microscopy or culture (Haque *et al* 1998, 2000). Because the gold-standard test requires cultivation of the organism which is known not to be 100% sensitive it is difficult to assess whether the kits produce false-positive results, but PCR (see below) suggests that most culture or microscopy negative but antigen positive samples are true positives (Haque *et al* 1998). The speed and convenience of these kits is also a strong point in their favour (Evangelopoulos *et al* 2001). Other workers have used similar monoclonal antibodies with equal success for species specific diagnosis in Egypt (Abd-Alla *et al* 2000b; Abd-Alla and Ravdin 2002).

Other kits are available from Cellabs in Australia (http://www.cellabs.com.au/) (detects *E. histolytica* and *E. dispar*); R-Biopharm in Germany (http://www.r-biopharm.com/Human/HumanFrame.html) (*E. histolytica*/*E. dispar* only; Schunk *et al* 2001) and Remel in the USA (http://www.remel.com/products/clinical/level2/MicrowellFormat.cfm) (*E. histolytica* only; Ong *et al* 1996). A dipstick test for *E. histolytica*/*E. dispar*, *G. intestinalis* and *C. parvum* is also available commercially (http://www.biosite.com/products/micro.asp).

8.3 *DNA blotting*

The use of species-specific DNA probes to hybridise with unamplified DNA isolated from faecal samples has the great attraction of simplicity, particularly if non-radioactive labels are used. The method has been applied successfully (Samuelson *et al* 1989; Agarwal *et al* 1998) but not widely taken up, probably because of doubts about its sensitivity. The polymerase chain reaction (PCR) method, however, has been as widely tested for amoebiasis as for most other diagnostic problems and forms the subject of much of the remainder of this review.

8.4 *PCR based methods*

The ability of the PCR to specifically amplify minute amounts of pathogen DNA has revolutionised the diagnosis of many infectious diseases, and the numerous sequence differences between homologous genes in *E. histolytica* and *E. dispar* make it a natural candidate for identifying these two species. A number of methods have been published (Tannich and Burchard 1991; Acuna-Soto *et al* 1993; Katzwinkel-Wladarsch *et al* 1994; Rivera *et al* 1996; Britten *et al* 1997; Troll *et al* 1997; Evangelopoulos *et al* 2000; Verweij *et al* 2000). Most, but not all, rely on amplifying unique regions of the SSUrRNA episome, its high copy number providing increased sensitivity. Although in the original procedures the product was often detected by gel electrophoresis followed by ethidium bromide staining, colorimetric detection using specific probes is now frequently employed. This provides the advantage of an easy-to read and familiar microtitre plate format. Light Cycler PCR has now been applied to the diagnosis of amoebiasis (E Tannich, personal communication; see also http://www.artus-biotech2.de/). PCR has also proved capable of detecting *E. histolytica* DNA in liver abscess contents (Tachibana *et al* 1992; Britten *et al* 1997; Zaman *et al* 2000).

In *in vitro* testing with cultured trophozoites PCR was about one hundred times more sensitive than antigen detection (Mirelman *et al* 1997); it also has the advantage that it can be developed to provide strain (as well as species) identification. However, it is important to be aware of the disadvantages of the method. Firstly, three separate steps are required – DNA extraction, amplification and product detection. While the necessary equipment is now widely available the process is not as quick or as simple as the use of an antigen detection kit. Secondly, as with all PCR-based methods, great care has to be taken to eliminate the risk of false positives due to contamination from product prepared earlier – although the Light Cycler technique greatly reduces this danger. Neither PCR nor antigen detection kits are fully sensitive if the faeces have been frozen or preserved and neither method is affordable for routine use in most disease-endemic countries. In practice, the theoretically higher sensitivity of PCR is balanced by the speed and convenience of a kit (Evangelopoulos *et al* 2001) and the method chosen will usually depend on local resources and preferences.

9. Specific detection of *E. histolytica* antigens in other samples

Some interesting results have been published suggesting that it may be possible to detect *E. histolytica* antigen in

the saliva (Abd-Alla *et al* 2000a) and circulating in the blood (Abd-Alla *et al* 1993) of infected patients. This is provided either of these specimens may be more acceptable to patients and these are interesting reports, although sensitivity might be a problem.

10. Conclusions

Nearly ten years after Diamond and Clark redefined *E. histolytica*, we now have a number of well-validated methods for distinguishing between it and *E. dispar*. While none are cheap and it is unlikely that they will be in routine use in disease-endemic areas in the near future, they do provide the tools to re-examine the important question raised by Julia Walsh (Walsh 1986) – just how common is *E. histolytica* in the world?

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