Limited genetic diversity among genotypes of *Enterocytozoon bieneusi* strains isolated from HIV-infected patients from Sydney, Australia

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Microsporidia are intracellular parasites, with over 1200 species belonging to 143 genera described to date. They are opportunistic pathogens in humans and can cause chronic diarrhoea in immunosuppressed patients. Both *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* cause intestinal disease, with *Enterocytozoon bieneusi* more commonly identified in patients with human immunodeficiency virus (HIV) infection. In this study, intestinal microsporidial clinical isolates from patients in Sydney, Australia, were genotyped. All specimens were from HIV-infected men with low CD4⁺ T-cell counts (<100 cells mm⁻³). Genotyping of the internal transcribed spacer regions of the rRNA gene showed the presence of only one genotype, the anthroponotic *Enterocytozoon bieneusi* genotype B strain. This study thus highlighted the limited genetic diversity among Australian *Enterocytozoon bieneusi* isolates, and it is hypothesized that, due to the reduced incidence of microsporidia and the subsequent reduction in the human reservoir of the anthroponotic genotype B, locally acquired intestinal microsporidiosis will rarely be seen in HIV-infected persons undergoing highly active antiretroviral therapy in the future in Australia.

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INTRODUCTION

Microsporidia are ubiquitous, obligatory intracellular eukaryotic parasites. To date, over 1200 species belonging to 143 genera have been described, infecting a wide range of vertebrate and invertebrate hosts (Didier & Weiss, 2006). Microsporidia are recognized as opportunistic infectious agents worldwide in both developed and developing countries and as a major cause of chronic diarrhoea in immunocompromised patients, particularly in human immunodeficiency virus (HIV)-infected patients with CD4⁺ T-cell counts below 100 cells mm⁻³ (Didier & Weiss, 2006; van Hal *et al.*, 2007).

Of the 15 species identified as human pathogens, two species of microsporidia can cause gastrointestinal disease, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, with *Enterocytozoon bieneusi* being the more commonly identified microsporidium in patients with HIV infection (Didier & Weiss, 2006).

The prevalence of intestinal microsporidiosis in HIV-infected patients varies widely from <1% to 50%

depending on the population being studied (Navin *et al.*, 1999). Since the advent of highly active antiretroviral therapy (HAART) in developed countries, the frequency of all opportunistic infections, including microsporidia, has decreased (Dworkin *et al.*, 2007; van Hal *et al.*, 2007). However, in developing countries, where there is a rapidly increasing HIV infection rate coupled with limited access to HAART, the incidence of microsporidiosis remains high (Breton *et al.*, 2007; Espern *et al.*, 2007).

Considerable genetic diversity within *Enterocytozoon bieneusi* isolates has been found, with over 65 distinct genotypes based on nucleotide sequence polymorphism of the internal transcribed spacer (ITS) of the rRNA gene (Breton *et al.*, 2007). To our knowledge, no studies have been undertaken on the geographical distribution of human-derived genotypes from Australia. Our aim was to compare the genotypes of microsporidial samples over a period of time to determine the genotypes present in HIV-infected patients in Sydney, Australia.

METHODS

Faecal samples. The study was performed retrospectively on stored faecal samples known to contain microsporidia by microscopy from patients who presented at St Vincent's Hospital, Sydney, Australia. All stool samples were originally analysed for microsporidial spores using UV light microscopy and the fluorescent dye Uvitex 2B (van Hal *et*

Abbreviations: HAART, highly active antiretroviral therapy; ITS, internal transcribed spacer; SSU, small subunit.

The GenBank/EMBL/DDBJ accession number for the internal transcribed spacer region of the small-subunit rRNA gene of *Enterocytozoon bieneusi* is FJ174697.

al., 2007). The specimens were stored at -80°C or 4°C mixed in equal volumes of 70% ethanol. Samples had been stored over time dating back to 1995. A total of 54 individual patient samples were included in the study. All patients were HIV-infected males presenting with symptomatic infection (diarrhoea), with CD4^{+} T-cell counts below 200 cells mm^{-3} (median CD4^{+} T-cell count 80 cells mm^{-3}).

DNA extraction and PCR. DNA extraction was performed as described previously (Verweij *et al.*, 2007). To exclude inhibition from faecal inhibitors, all specimens were spiked with an equal volume of genomic DNA from controls and run in parallel with unspiked specimens. Confirmation of microsporidia was by conventional PCR targeting the small-subunit (SSU) rRNA gene of microsporidia as described previously (Fedorko *et al.*, 1995). Both *Enterocytozoon bienewsi* and *Encephalitozoon intestinalis* DNA were used as controls.

Sequencing. The amplicons were purified using a QIAquick PCR purification kit (Qiagen) following the manufacturer's recommendations and sequenced in both directions using an ABI PRISM 3700 DNA analyser. The SSU rRNA gene sequences obtained were then compared with those available in GenBank using the BLASTN program run on the National Center for Biotechnology Information Server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Genotyping. *Enterocytozoon bienewsi* genotyping was undertaken by nucleotide sequencing of the ITS region of the rRNA gene as described previously (Breton *et al.*, 2007). The ITS sequences obtained were compared with 66 previously published sequences retrieved from GenBank. Multiple alignments were performed using the CLUSTAL_W program using default parameters. Only complete ITS sequences were included.

RESULTS AND DISCUSSION

Of the 54 samples used, SSU rRNA gene PCR products were obtained from 46. Sequencing of the amplicons confirmed all isolates as *Enterocytozoon bienewsi* and genotyping of the ITS regions was performed on all specimens, but was successful only for 29. All isolates that were genotyped were identified as genotype B and the sequence was deposited in GenBank. All patients were male and HIV-infected, and had a median CD4^{+} T-cell count of 80 cells mm^{-3} . All patients had localized intestinal microsporidiosis and were symptomatic with diarrhoea. The number of microsporidial cases diagnosed decreased from 29 in 1995 to 0 cases from 2004 onwards (Table 1). In Australia, HAART therapy became available in late 1996; however, it was not until 1998 that effective HAART therapy became widespread. It should be noted that at this time there was a significant reduction in the number of cases of intestinal microsporidiosis seen at St Vincent's Hospital, with the incidence of microsporidiosis in HIV-infected patients decreasing from 12% in 1997 to 3% in 1999, and from 2004 onwards the incidence declined to 0%.

This is the first study of the molecular epidemiology of microsporidiosis among HIV-infected patients in Australia. All microsporidia identified by PCR were determined to be *Enterocytozoon bienewsi*. According to genotyping of the ITS region of the 29 isolates, all were

Table 1. Summary of cases of intestinal microsporidiosis during the period of study

Year	No. of cases	Positive result by:	
		PCR	Genotyping
1995	29	6	1
1996	28	4	2
1997	29	7	3
1998*	25	5	4
1999	17	9	7
2000	16	6	4
2001	6	3	3
2002	4	4	3
2003	5	2	2
2004	0	0	0
2005	0	0	0
2006	0	0	0
2007	0	0	0

*Denotes the start of widespread use of HAART in Australia.

genotype B. Genotypes A and B are considered to be host-specific, as to date they have been isolated exclusively from humans. These anthroponotic genotypes appear to be the dominant strains in many parts of the world. The type B genotype is the dominant strain in France (Liguory *et al.*, 2000), Germany (Rinder *et al.*, 1997), Switzerland (Breitenmoser *et al.*, 1999) and the UK (Sadler *et al.*, 2002). This is in contrast to many other microsporidial genotypes that are not host-specific and are found in a variety of mammals and as such have a zoonotic potential (Dengjel *et al.*, 2001).

Given the fact that genotype B *Enterocytozoon bienewsi* strains are anthroponotic and the only identified reservoir is humans, we hypothesize that, due to the reduced incidence of microsporidial infection and a reduction in the human reservoir, locally acquired intestinal microsporidiosis in HIV-infected patients undergoing HAART will rarely be seen in the future in Australia. This is supported by the fact that no patients have been diagnosed with intestinal microsporidiosis since 2004 in our institution. It should be noted, however, that other groups or individuals may be carriers of *Enterocytozoon bienewsi*. The organism has been reported in HIV-negative children (Mungthin *et al.*, 2005), implicated as a cause of travellers' diarrhoea (Wichro *et al.*, 2005) and has been seen in the elderly (Norhayati *et al.*, 2008) and others who are immunocompetent who may exhibit transient or asymptomatic infection (Abreu-Acosta *et al.*, 2005; Nkinin *et al.*, 2007). Clinically relevant cases have also been reported in transplant patients undergoing immunosuppression (Lanternier *et al.*, 2008). However, to date no studies have been undertaken in Australia to examine the exact extent of microsporidial infection or carriage in these particular groups.

Our findings that most cases of microsporidiosis were diagnosed in patients with low CD4⁺ T-cell counts (<100 cells mm⁻³) is consistent with previously published data. The effect of HAART on the incidence of microsporidiosis has been dramatic and is highlighted in this study. We found a decrease in the incidence of microsporidiosis from 12 to 0% in only 7 years. Several studies have reported the reduced incidence of intestinal microsporidiosis since the advent of HAART (Dworkin *et al.*, 2007; van Hal *et al.*, 2007). Effective HAART therapy results in suppression of HIV replication and CD4⁺ T-cell count restoration, therefore eradicating the risk of microsporidial infection. There is also evidence that the protease inhibitors used in HAART may have a direct antiparasitic effect on microsporidia (Pozio & Morales, 2005).

To our knowledge, this is the first study of the molecular epidemiology of microsporidiosis among HIV-infected patients in Australia, and this study highlights the limited genetic diversity among Australian *Enterocytozoon bienersi* isolates and the dramatic effect that HAART therapy has had on the incidence of intestinal microsporidiosis. We hypothesize that, due to the limited genetic diversity found in this study, a reduction in the human reservoir of the anthroponotic genotype coupled with the widespread use of HAART will effectively eradicate locally acquired microsporidiosis in HIV-infected persons in Australia.

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