Recurrent candidaemia in a neonate with Hirschsprung's disease: fluconazole resistance and genetic relatedness of eight *Candida tropicalis* isolates

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The incidence of candidaemia among immunocompromised patients in Malaysia is increasing at an alarming rate. Isolation of clinical strains that are resistant to fluconazole has also risen markedly. We report here the repeated isolation of *Candida tropicalis* from the blood of a neonatal patient with Hirschsprung's disease. *In vitro* fluconazole susceptibility tests of the eight isolates obtained at different time points showed that seven of the isolates were resistant and one isolate was scored as susceptible dose-dependent. Random amplification of polymorphic DNA fingerprinting of the isolates using three primers and subsequent phylogenetic analysis revealed that these isolates were highly similar strains having minor genetic divergence, with a mean pairwise similarity coefficient of 0.893 ± 0.041 . The source of the infectious agent was thought to be the central venous catheter, as culture of its tip produced fluconazole-resistant *C. tropicalis*. This study demonstrates the utility of applying molecular epidemiology techniques to complement traditional mycological culture and drug susceptibility tests for accurate and appropriate management of recurrent candidaemia and highlights the need for newer antifungals that can combat the emergence of fluconazole-resistant *C. tropicalis* strains.

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INTRODUCTION

Systemic *Candida* infections in patients with extensive surgery or burns or who are receiving intensive antibiotic therapy are always very serious and have a high mortality rate. *Candida albicans* is an opportunistic fungus that can cause a wide spectrum of infections from the superficial, cutaneous or subcutaneous to systemic candidiasis in immunocompromised hosts. *C. albicans* has been regarded as the major cause of invasive fungal infections (Wingard *et al.*, 1991). However, during the past decade, an increasing trend of systemic and fatal infections with non-*albicans* species such as *Candida tropicalis*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, *C. lipolytica*, *C. inconspicua* and *C. norvegensis* has been reported (Nguyen *et al.*, 1996; Weinberger *et al.*, 1997; Ng *et al.*, 1999).

Abbreviations: CVL, central venous line; ERIC, enterobacterial repetitive intergenic consensus primers; RAPD, random amplification of polymorphic DNA.

Scoring of polymorphic bands from RAPD-PCR analysis and a distance matrix resulting from the RAPD-PCR results are available as supplementary material in JMM Online.

In a study on candidaemia in a Middle Eastern hospital, *C. tropicalis* was found to be the second commonest *Candida* species in terms of prevalence after *C. albicans* (Ellis, 2001). A study on *Candida* species isolated from blood samples in the University Malaya Medical Centre in Malaysia (Ng *et al.*, 2001) revealed that *C. parapsilosis* and *C. tropicalis* surpassed *C. albicans* in terms of prevalence. In a prospective, population-based surveillance for candidaemia in two United States cities, *C. tropicalis* infection was most often seen in persons with cancer and diabetes mellitus, making up 46 and 24 %, respectively, of the cases due to *C. tropicalis* (Kao *et al.*, 1999).

Antifungal azoles such as fluconazole, ketoconazole and intraconazole are now widely used to treat infections caused by opportunistic *Candida* species. These azole compounds are advantageous over amphotericin B due to their favourable bioavailability and safety profiles. The emergence of azole-resistant *Candida* strains as the result of use and occasional overuse of fluconazole had gradually become prominent among immunocompromised patients (Boschman *et al.*, 1998). Moreover, antifungal azoles are fungistatic but

not fungicidal, and this also contributes to candidiasis treatment failure. Although recurrent oropharyngeal and vaginal candidiasis have been reported frequently and studied phylogenetically, very few cases of recurrent candidaemia have been described. Krcmery *et al.* (1998) defined the relapse of candidaemia as 'a positive blood culture up to 10 days after initial clinical improvement or cure'. However, Clancy *et al.* (2000) defined recurrent candidaemia as 'occurring at least 1 month after the apparent complete resolution of an infectious episode caused by the same *Candida* species'.

The repeated isolation of *Candida* species from a patient receiving either antifungal prophylaxis or a therapeutic regimen indicates that the drug is not capable of eliminating the fungus. This recurrent candidaemia could be due to reinfection by the same species or strain, or to infection by a completely new strain or a different species. Thus, molecular epidemiology can help to determine whether recurrent infections are caused by reinfection or strain replacement. Genotyping methods are also helpful in identifying the infectious source, such as the catheter, the hospital workers or endogenous body sites like the gastrointestinal tract.

We report here the isolation and molecular epidemiology of eight isolates of *C. tropicalis* from the blood of a single patient within a 3-month period. The strains obtained were subjected to *in vitro* susceptibility testing and genotyping by random amplification of polymorphic DNA (RAPD) analysis.

METHODS

Culture and maintenance. The *Candida* isolates were cultured from the blood sample of a neonatal patient at the University Malaya Medical Centre, a teaching hospital affiliated to University of Malaya, Malaysia. Eight yeasts were isolated from this patient between December 2002 and February 2003.

The BACTEC 9240 Fluorescent Blood Culture System was used to isolate the yeasts from blood specimens. *Candida* cultures were maintained in Sabouraud's dextrose agar or broth for species identification and DNA extraction purposes. For long-term storage, the strains were maintained in Sabouraud's dextrose broth supplemented with 18% (v/v) glycerol.

Patient history. The patient is a male neonate who was diagnosed with Hirschsprung's disease and necrotizing enterocolitis shortly after birth. He was operated on 2 months later and an ileostomy fashioned. In the meantime, he also developed anaemia and thrombocytopenia for which he needed blood transfusion. Post-operatively, he had been given the antibiotics vancomycin, imipenem and metronidazole, as he developed sepsis caused by methicillin-resistant *Staphylococcus epidermidis* and *Enterobacter* species. The sepsis resolved after removal of the catheter and continued treatment with the same antibiotics. Shortly after, he developed fungaemia with *Trichosporon* species isolated from the blood culture. He was treated with amphotericin B for a week. He remained hospitalized for total parenteral nutrition that was administered through a central venous line (CVL). A month later he again became septic. His blood was taken for culture, *C. tropicalis* was isolated, and he was started on fluconazole empirical

therapy. As the patient's infection did not seem to respond well to this therapy, we decided to investigate the *in vitro* susceptibilities of the isolates to fluconazole.

Species identification. Gram stain, germ tube test and microscopic examination were carried out initially to identify the *Candida* species. Auxanographic tests such as carbohydrate assimilation tests and carbohydrate fermentation tests were also used to identify the species (Ng *et al.*, 2000). In addition, a PCR-based test using *C. tropicalis*-specific primers targeting the internal transcribed spacer 2 (ITS2) region of the 5.8S and 28S rRNA genes (Bougnoux *et al.*, 1999) was used to confirm the identification further.

Antifungal susceptibility to fluconazole. The method was similar to the M2-A6 disc test method for bacteria (NCCLS, 2000, 2002), but the Mueller-Hinton agar was supplemented with 2% glucose and 0.5 µg methylene blue ml⁻¹. Fluconazole discs (25 µg; Pfizer) were used in the antifungal susceptibility test. Five isolated colonies on Sabouraud's dextrose agar were resuspended in 5 ml normal saline and mixed vigorously. The suspension was adjusted to a turbidity of 0.5 McFarland standard corresponding to 1–5 × 10⁶ yeast cells per ml. A Mueller-Hinton agar plate was pre-dried at 35 °C and inoculated evenly with the inoculum suspension by cross-streaking using a cotton swab. The agar plate was allowed to dry at room temperature for 10 min before a fluconazole disc was applied to the designated position on the plate using a paper template placed under the Petri dish. The plates were incubated at 35 °C for 18–24 h. The test results were read electronically by image analysis, interpreted and recorded with a BIOMIC Plate Reader System (Ng *et al.*, 2000). Interpretative breakpoints used for fluconazole disc tests were based on zones that correlated with category breakpoints recommended by the NCCLS for the reference broth dilution method; with the interpretive criteria of ≥19 mm scored as sensitive, 15–18 mm as susceptible dose-dependent and ≤14 mm as resistant.

DNA extraction. *Candida* DNA isolation from broth-harvested culture was conducted with lysis buffer (10 mM Tris/HCl pH 7.5, 0.5% β-mercaptoethanol, 5 mM EDTA and 0.5 mg lyticase ml⁻¹) with incubation at 37 °C for 60 min on a shaker, followed by treatment with proteinase K (50 µg ml⁻¹) and 1% (w/v) SDS at 56 °C for 60 min. The mixture was boiled for 5 min and extracted using a mixture of phenol/chloroform/isoamyl alcohol (25:24:1; pH 8.0). The DNA was precipitated by adding 0.1 vol. 3 M sodium acetate (pH 5.2) and 1 vol. 2-propanol. The pellet was washed with 70% ethanol and resuspended in 20 µl sterile distilled water.

DNA fingerprinting. The RAPD-PCR technique was used to generate unique genotype banding patterns for the different *Candida* clinical isolates. Enterobacterial repetitive intergenic consensus (ERIC) primers were used in combination as described by Metzgar *et al.* (1998). ERIC1 (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTACTGGGGTGAGCG-3') primers were used in the fingerprinting. Another primer, RP02 (5'-GCGATCCCCA-3') (Roilides *et al.*, 2003), was also used separately. All primers were custom-made by Research Biolabs Pte Ltd (Singapore).

DNA of the eight clinical isolates of *C. tropicalis* was extracted and diluted 100-fold. For the PCR set-up using the ERIC primers, 2.5 µl 10 × PCR buffer, 2 mM dNTPs, 1.5 mM MgCl₂, 1.0 µl ERIC1 or ERIC2 primer (40 pmol µl⁻¹), 0.5 µl SuperTherm *Taq* polymerase (1 U µl⁻¹) and 0.02–0.2 µg DNA template were placed in a PCR tube and sterile distilled water was added to the final volume of 25 µl. The cycling conditions were initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 25 °C for 3 min and extension at 74 °C for 2 min. The reaction was followed by a final extension step at 72 °C for 8 min and a hold at 4 °C.

For the PCR set-up using the RP02 primer, 2.5 µl 10 × PCR buffer, 2mM dNTPs, 1.5 mM MgCl₂, 1.0 µl RP02 primer (20 pmol µl⁻¹), 0.5 µl SuperTherm Taq polymerase (1 U µl⁻¹) and 0.05–0.5 µg DNA template were placed in a PCR tube and sterile distilled water was added to the final volume of 25 µl. The cycling conditions consisted of four cycles of denaturation at 94 °C for 5 min, annealing at 36 °C for 5 min and extension at 72 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. The reaction was followed by a final extension step at 72 °C for 10 min and a hold at 4 °C.

Agarose gel electrophoresis. PCR products generated from RAPD-PCR were separated based on size by electrophoresis in 1.5 % (w/v) agarose gel in TBE buffer at 30 V for 5 h. Markers (Gene Ruler[®] 100bp plus DNA ladder and Gene Ruler[®] DNA ladder mix; Fermentas) were included for size estimation of the amplicons. The results were visualized using a UV-light transilluminator, and pictures were captured digitally with the Gene Genius BioImaging System (Syngene).

Analysis of RAPD patterns and statistical test. The RAPD-PCR technique employed in this study produces a myriad of PCR products of varying lengths and multiple bands in the agarose gel upon electrophoresis. When PCR products generated from two or more samples were compared, the total number of unique bands produced by a particular DNA sample compared to all the other samples was determined. The presence or absence of every unique band in each sample was recorded on a chart (see Supplementary Tables S1 and S2 in JMM Online). Subsequently, the results obtained using the ERIC primers as well as those obtained using the RP02 primer were analysed, aided by specialized software. The RAPDistance program (Armstrong *et al.*, 1994) was used to compare amplified DNA profiles between the different strains. To inspect the band patterns, the uniqueness of each band was assessed using a previously described method (Chong *et al.*, 2003). The Dice metric (Dice, 1945) was used to calculate similarity coefficients (S_{AB}) of pairs of samples. Phylogenetic and molecular evolutionary analyses were also conducted using MEGA version 2.1 (Kumar *et al.*, 2001) to construct the phylogenetic tree.

RESULTS

Culture identification and antifungal susceptibility

Over a period of 3 months, a male neonatal patient diagnosed with Hirschsprung's disease and accompanying thrombocytopenia had persistent, recurrent febrile episodes suggestive of microbial infections. Blood samples were taken from this patient and upon microbiological culture and conventional biochemical speciation methods, the causative pathogen was identified as *C. tropicalis*. A total of eight isolates of *C. tropicalis* species were isolated.

In order to confirm the identity of the eight isolates as *C. tropicalis*, genomic DNA of all cultured samples was subjected to PCR amplification using universal fungal primers, ITS3 and ITS4 (White *et al.*, 1990), followed by *C. tropicalis* species-specific primers (Bougnoux *et al.*, 1999). The PCR assay using universal fungal primers is also able to detect the presence of co-infection by more than one *Candida* species because each different *Candida* species gives rise to amplicons of distinct sizes of around 300–420 bp due to the variable sequences and lengths of the ITS2 regions of each species. The results (not shown) showed that all eight

isolates were identified correctly as *C. tropicalis* and that no other *Candida* species were involved.

As for the *in vitro* antifungal resistance, we found that all the isolates were resistant to fluconazole, except isolate CT3, chronologically the second isolate obtained from the patient, which was scored as susceptible dose-dependent or intermediate in susceptibility (Table 1). CT3 was isolated after the institution of the amphotericin B regimen.

Case history

Our patient had been given empirical treatment with fluconazole before a fluconazole-resistant *C. tropicalis* isolate was cultured from his blood. Fluconazole therapy was replaced with amphotericin B. However, four subsequent blood cultures yielded uniformly fluconazole-resistant *C. tropicalis* isolates (we have no data on the susceptibility of these isolates to amphotericin B).

The continuous source of candidaemia was suspected to be the CVL. It was thought that amphotericin B would eradicate the *Candida* without removal of the central line. However, upon failure to eliminate the candidaemia, it was considered necessary to remove the line. The CVL tip was sent for culture and sensitivity testing, and this yielded *C. tropicalis* that was resistant to fluconazole, confirming our suspicion. Insertion of a new CVL catheter was delayed for a week and the patient was started on amphocil (amphotericin B and cholesteryl sulfate complex) for 14 days, as it is considered less toxic than amphotericin B alone. A day after stopping the administration of amphocil, he again started to develop fever. Blood culture was undertaken and again *C. tropicalis* was isolated. Amphocil was resumed and continued for another 21 days. During this time, two blood cultures from the patient taken a week apart became positive for *C. tropicalis*. The central line had been removed and insertion of a new line was delayed for fear of colonization by *Candida* and a subsequent infection. Amphocil was stopped after 21 days as repeated blood cultures remained negative.

Table 1. *In vitro* fluconazole-susceptibility profile of *C. tropicalis* bloodstream isolates analysed by disc diffusion tests

Isolate	Mean zone of inhibition (mm)	Susceptibility category	Date of collection
CT1	14	Resistant	12 December 2002
CT2	12	Resistant	6 January 2003
CT3	16	Susceptible dose-dependent	4 January 2003
CT4	14	Resistant	14 January 2003
CT5	14	Resistant	16 January 2003
CT6	12	Resistant	5 February 2003
CT7	14	Resistant	8 February 2003
CT8	14	Resistant	13 February 2003

Genetic relatedness of the isolates

Although we had proven that the recurrent systemic candidiasis in this patient was caused by the same *Candida* species, we were interested to find out whether the eight isolates were identical or different strains. Therefore, RAPD-PCR was carried out in order to determine the genotypes of these isolates. Fig. 1 shows the electrophoresis result of RAPD-PCR obtained using ERIC1 and ERIC2 primers and Fig. 2 shows the electrophoretic banding patterns of the isolates obtained using RP02. The RAPD-PCR experiments were repeated to ensure reproducibility and reliability. Pairwise distance values computed by the RAPDistance software were exported into the MEGA 2.1 software for constructing the phylogenetic tree. A neighbour-joining tree was constructed according to the algorithm of Saitou & Nei (1987) (Fig. 3).

Analysis of the phylogenetic tree and the distance values shows that the eight strains were highly related: the first three isolates, CT1, CT2 and CT3, were clustered together in a subgroup; CT4 and CT5 were in a different group; and CT6, CT7 and CT8 were clustered in a subgroup that belonged to the same parent group as the first three isolates (see Supplementary Table S3 in JMM Online). Analysis of the pairwise S_{AB} of the isolates revealed that the most related pair of isolates were CT2 and CT5 ($S_{AB}=0.943$), whereas the least related pair were CT4 and CT8 ($S_{AB}=0.80$). The mean S_{AB} was 0.893 ± 0.041 . This result implies that there was a progressive minor genetic evolution that occurred from the first to the eighth isolate, but, in general, these

Fig. 1. Electrophoretic separation of RAPD-PCR products amplified from eight bloodstream *C. tropicalis* isolates by using primers ERIC1 and ERIC2. Lanes: M, 100 bp ladder marker (Fermentas); 1–8, RAPD-PCR products generated from *C. tropicalis* isolates CT1–CT8; C, negative control in which the DNA template was replaced with sterile distilled water.

Fig. 2. Electrophoretic separation of RAPD-PCR products amplified from eight bloodstream *C. tropicalis* isolates by using primer RP02. Lanes: M, GeneRuler 1 kb DNA ladder marker (Fermentas); 1–8, RAPD-PCR products generated from *C. tropicalis* isolates CT1–CT8; C, negative control in which the DNA template was replaced with sterile distilled water.

isolates were closely related and were likely to have originated from the same source.

DISCUSSION

The global trend for the distribution of *Candida* species isolated from the blood of immunocompromised patients has shown a decrease in *C. albicans* concomitant with the increase in other species, in particular *C. tropicalis* and *C. parapsilosis*. Kontoyiannis *et al.* (2001) investigated the risk factors for *C. tropicalis* fungaemia in comparison with those of *C. albicans* and found that patients with leukaemia and prolonged neutropenia had significant susceptibility to *C. tropicalis* fungaemia. Other established risk factors for breakthrough fungaemia include catheter insertion, antimicrobial and antifungal prophylaxis with quinolones (Krcmery *et al.*, 1998), mucositis and underlying haematological malignancy. Kontoyiannis *et al.* (2001) also noted

Fig. 3. Phylogenetic tree constructed by the neighbour-joining method for clustering analysis of the *C. tropicalis* isolates. Bar, branch length of 0.01; branch lengths are proportional to the genetic distance.

that, when *C. tropicalis* fungaemia represented a breakthrough infection rather than a de novo infection, the response rate was lower. Our patient did not have cancer, nor was he immunocompromised. The only aetiological risk factor that could explain the persistent candidaemia was exogenous, most likely the catheter, and so we investigated the cause for the recurrent candidaemia from this angle.

It is still debatable whether *C. tropicalis* is a more virulent species than *C. albicans* (Walsh & Merz, 1986; Wingard, 1995). However, it is more likely that *C. albicans* is more adept at taking advantage of the host conditions. *C. tropicalis* was shown to have a negligible level of fluconazole resistance in a large surveillance study conducted by Pfaller *et al.* (2000), but it can acquire resistance to fluconazole swiftly upon exposure to increasing concentrations of the drug, through upregulation of efflux transporters (Barchiesi *et al.*, 2000).

Clancy *et al.* (2000) studied closely the factors contributing towards late recurrent candidaemia. They found a prolonged time of relapse for candidaemia ranging from 1 to 8 months in patients and attributed this to the time it takes for non-*albicans* *Candida* species of lower virulence to accumulate to a clinically evident infectious load. Strain typing of the recurrent episodes of candidaemia showed that the same strain of *Candida* was responsible for the initial and subsequent episodes. One possible reason for this was the inability of antifungal therapy to eliminate all invasive organisms fully, although it was sufficient to resolve signs and symptoms. Another reason could be the presence of asymptomatic foci of organisms at intravascular or deep tissue sites, poorly reached by the drug, that might serve as the origin of recurrent infections.

The eight isolates in this study that were investigated for their genetic relatedness were highly similar strains. We used three different arbitrary primers in two distinct PCR experiments in order to display a large number of polymorphic bands and hence increase the discriminatory power of the genotyping method. One of the primers used, RP02, was used by Roilides *et al.* (2003) to genotype *C. tropicalis* strains that caused an outbreak in a neonatal intensive care unit. The ERIC1 and ERIC2 primers were used by Metzgar *et al.* (1998) to determine the relatedness of *Candida* species isolated from HIV-infected patients. In combining the RAPD patterns generated using these different primers, we were able to distinguish 15 polymorphic bands from RP02 and 15 from ERIC primers, thus producing a total of 30 polymorphic bands. Some of the bands were weaker in intensity than others, but the results were analysed scrupulously by increasing the brightness and contrast of the digitally captured images to visualize these weak bands. For very bright bands of high intensity, we reduced the brightness of the image to ascertain whether there were any doublets.

The results from the molecular epidemiology coupled to the antifungal susceptibility test showed that the second isolate cultured from the patient's blood, CT3, was distinct from

the other isolates in that it was scored as susceptible dose-dependent to fluconazole. Interestingly, this isolate was situated on a separate branch of the cluster from CT1 and CT2 in the phylogenetic tree (Fig. 3). The fourth and subsequent isolates were located on a different cluster from the first three isolates, with CT4 and CT5 in a subcluster and CT6, CT7 and CT8 in a different subcluster. These results seem to suggest that the strains causing the persistent candidaemia were non-identical but highly related. We postulate two possible explanations here: (i) there is a pool of genetically related strains of *C. tropicalis* co-existing in the patient's body that serves as a reservoir for infection but, at any given time, only one strain becomes dominant and causes a breakthrough infection depending on the host conditions; (ii) there is only one strain of *C. tropicalis*, perhaps from an external source infecting the patient, but this strain undergoes rapid and progressive minor genetic variations in order to suit the host environment.

Clearly, this case shows that a persisting source of infection, most likely the intravascular catheter, served as the origin of the repeated candidaemia despite aggressive treatment. Upon removal of the central venous catheter, resolution of the infection was achieved. This reinforces the importance of removing any intravascular catheter as a precautionary step in the management of recurrent fungaemia, especially for neonatal patients who are at increased risk of developing candidaemia. In addition, clinicians should strongly consider the use of maximal sterile barriers during insertion of the central venous catheter, as this has been shown to be advantageous in reducing the incidence of catheter-related infections (Hu *et al.*, 2004). This study also demonstrates the usefulness of molecular typing techniques for determining the source of infection in cases of recurrent candidaemia and the clinical importance of antifungal susceptibility tests in aiding the clinician's decision for appropriate therapy.

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