

Distribution of yeast species associated with oral lesions in HIV-infected patients in Southwest Uganda

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Oropharyngeal candidiasis remains a significant clinical problem in HIV-infected and AIDS patients in regions of Africa where anti-retroviral therapy isn't readily available. In this study we identified the yeast populations associated with oral lesions in HIV-infected patients in Southwest Uganda who were receiving treatment with nystatin and topical clotrimazole. Samples were taken from 605 patients and 316 (52%) of these yielded yeast growth following incubation on Sabouraud dextrose agar. Samples were subsequently re-plated on CHROMagar Candida medium to facilitate identification of the yeast species present. The majority (56%) of culture-positive samples yielded a mix of two or more species. *Candida albicans* was present in 87% (274/316) of patient samples and accounted for 87% (120/138) of single species samples. *Candida glabrata*, *Candida tropicalis* and *Candida norvegensis* were also found in cultures that yielded a single species. No *Candida dubliniensis* isolates were identified in this population.

Keywords prevalence, yeast species, *Candida*, HIV, oropharyngeal candidiasis

Introduction

Candida species are frequently found as commensal organisms in the oral and gastrointestinal flora of normal healthy individuals [1]. In addition, they are also the most common cause of opportunistic fungal infections in humans [1]. In particular, oropharyngeal candidiasis (OPC) is the most common opportunistic infection in human immunodeficiency virus (HIV)-infected patients and in those with acquired immunodeficiency syndrome (AIDS) [2,3]. The incidence and prevalence of OPC has fallen dramatically in the developed world, largely due to the introduction of highly active antiretroviral therapy (HAART) treatment, which reduces viral replication with a subsequent increase in the levels of CD4 T lymphocytes [4,5]. However, since the prevalence of AIDS remains high throughout much of Africa because HAART is still not widely available. OPC

is still a common opportunistic infection in these individuals [6,7].

There are several different manifestations of OPC in AIDS patients, including pseudomembranous candidiasis (PC) which is characterized by white pseudomembrane-like lesions that are mainly composed of fungal and host cells. In addition, there is erythematous candidiasis (EC) which is distinguished by red lesions and angular cheilitis which is an infection affecting the angles of the mouth [3].

Candida albicans is widely recognized as the most pathogenic yeast in humans and is the most common cause of fungal infections [1]. However, other *Candida* species, such as *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* are also commonly identified in samples from patients with OPC [8]. During the 1990s a new species, *Candida dubliniensis*, was identified for the first time in Irish HIV-infected individuals [9]. Despite the high prevalence of OPC in African HIV-infected and AIDS patients there have been relatively few comprehensive studies investigating the aetiology of these infections in countries where HAART is relatively rarely prescribed. In

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a recent study, Agwu *et al.* [10] described the high prevalence of oral lesions in HIV-infected and AIDS patients in Southwest Uganda. The aim of the present study was to isolate and identify the yeast species present in samples recovered from the oral lesions of these patients, with the ultimate goal of generating a data-base for use in the design of effective interventions to treat oral yeast infections in this region.

Materials and methods

Geographical sampling area and participant inclusion criteria

The AIDS Support Organization (TASO) HIV/AIDS clinics in four districts of Southwest Uganda (i.e., Masaka, Rukungiri, Bushenyi [Mitoma, Katungu, Kigarama outreach clinics] and Mbarara metropolis [including Nyihanga outreach clinics]) were included in this investigation. Other non-TASO HIV clinics from where samples were collected included Uganda Cares in Masaka, Komboni Hospital in Bushenyi, and Ruhoko clinic in Ibanda (formerly in Mbarara) district. All participants included in this study had previously tested positive for HIV infection by enzyme-linked immunosorbent assay, had HIV/AIDS clinical staging (using the World Health Organization criteria [11], performed within 1 month of sample collection) and were current registered patients clinically diagnosed with an oral lesion before the commencement of sample collection. Individuals with all clinical stages of HIV were included in the analysis. At the time of this study, all patients with oral lesions were receiving treatment with cotrimoxazole, nystatin and topical clotrimazole cream. The age and gender profile of patients was similar in the four districts studied, i.e., 22.5% were male and 77.5 % were female. Patient ages ranged from 6–75 years (median = 39 years), with the majority (72%) aged between 31 and 50.

Ethical considerations

Permission to conduct the study was sought and obtained from the Uganda National Council of Science and Technology, the Kampala International University Research and Ethics Committee and TASO at both local and national levels. Informed consent was obtained from all participants. Letters explaining the project were written in English and interpreted into local languages spoken in the various districts where TASO and non-TASO clinics were located. Participants who agreed to participate in the study were expected to sign or have their thumb printed to show approval for sample collection. Where the participants could not read or write in English or local languages, the

letter outlining the benefits of the research study was read aloud and interpreted to the clients. Six hundred and five patients fulfilled the inclusion criteria and gave informed consent to participate in the study.

Sample collection and species identification

The prevalence of oral lesions associated with HIV/AIDS among participating patients recruited for this study was determined and calibrated by previously trained oral clinicians using standard methods described in previous studies [6,10]. Oral lesions were also grouped according to WHO clinical staging of oral infections associated with HIV/AIDS [12]. Sterile cotton swabs (Evepon, Nigeria) were used to collect samples aseptically from the oral lesions identified in each patient. Duplicate samples were collected; one for culture and one for wet mount and direct Gram's staining. After collection, the samples were immediately taken to the laboratory unit, Microbiology Department of Kampala International University, Western Campus (KIU-WC), Bushenyi, Uganda for culture. In cases where there was a delay in getting the samples to the laboratory, the swabs were transported within 2 hours in sterile distilled water to the laboratory in a vaccine box carrier under ice. Oral swab samples were inoculated on to Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, USA) supplemented with 2% (w/v) chloramphenicol and incubated at 37°C for 3–4 days. Due to the lack of resources, assessment of yeast growth was not quantitative, but >90% of culture-positive samples yielded semi-confluent or confluent growth. Multiple suspect yeast colonies on each plate exhibiting growth were taken in a sweep using a sterile inoculating loop and aseptically sub-cultured onto SDA slants at 37°C for storage prior to being transported to the Microbiology Research Unit, Division of Oral Bioscience, Dublin Dental University Hospital for analysis. There, suspect yeast isolates from the SDA slants were streaked for single colony growth on CHROMagar Candida medium and incubated at 37°C for 24–48 h [13]. Following incubation, plates were examined visually and the species present were presumptively identified on the basis of colony colour. The identity of 65 representative yeast isolates (i.e., 20 green colonies (suggestive of *C. albicans* or *C. dubliniensis*) and 45 pink colonies (suggestive of species such as *C. glabrata*, *C. norvegensis*, *C. parapsilosis*, *C. sake* and *Saccharomyces* spp.), including all non-*C. albicans* single species samples, was determined using the ID32C carbohydrate assimilation yeast identification system (BioMérieux, Paris, France) according to the manufacturer's instructions. All isolates putatively identified as *C. albicans* or *C. dubliniensis* on the basis of light to dark green colony coloration following growth on CHROMagar Candida medium were subjected to germ tube and chlamydosporulation tests [9].

In order to identify potential *C. dubliniensis* isolates in the collection, all germ tube-positive isolates were also subcultured on Pal's medium [14] and CHROMagar Candida medium supplemented with Pal's medium [15] to test for the presence of a hyphal fringe which is characteristic of *C. dubliniensis* when grown under these conditions. All putative *C. albicans* and *C. dubliniensis* isolates were also sub-cultured on SDA plates and incubated at 45°C for 4–5 days and 100 isolates were tested by PCR using *C. dubliniensis* and *C. albicans*-specific primers [16].

Results

The identity of yeast species recovered from the oral cavity of 605 HIV-infected individuals with oral lesions in Southwest Uganda was investigated. Pseudomembranous candidiasis was the most common lesion observed (51.4 %) followed by EC (13.9 %), Kaposi's sarcoma (4.6%), angular cheilitis (3.1%), recurrent aphthous ulceration (2.5%) and other unspecified lesions (24.5%), respectively.

Duplicate swabs were taken from the main oral lesion in each patient to test for the presence of yeast species; one swab was used for microscopic analysis while the other was used for culture. In cases where patients presented with more than one lesion, the most prominent one was sampled. Out of the 605 individual patient samples taken, 316 (52.2%) yielded yeast growth following culture on SDA at 37°C for 3–4 days. Culture-negative and culture-positive samples were confirmed as such by microscopic analysis. In order to estimate the range of yeast species present in the 316 culture-positive samples, colonies were sub-cultured, stored on SDA slants and replated onto CHROMagar Candida medium. This provided a presumptive species identification on the basis of colony color and morphology. Definitive species identification of 65 representative yeasts (including all of the single culture non-*C. albicans* species) was obtained by substrate assimilation analysis using the ID32C yeast identification system (BioMérieux, France). Growth on CHROMagar Candida medium revealed that 138/316 (43.7%) isolates represented single yeast species, while 178/316 (56.3%) of samples yielded two or more yeast species. The distribution of species within the single species samples is presented in Table 1. *Candida albicans* accounted for 120/138 (87%) of the monoculture yeast samples. The remaining samples were mainly comprised of *C. glabrata* (5/138 (3.6%)), *C. tropicalis* (5/138 (3.6%)) and *C. norvegensis* (4/138 (2.9%)) isolates. Small numbers of samples yielded *Candida parapsilosis*, *Candida sake* and *Saccharomyces cerevisiae*. *Candida albicans* was also found in 154/178 (86.5%) of the samples comprised of more than one species. Despite thorough phenotypic analysis (e.g., incubation on SDA at 45°C and on Pal's medium at 30°C) of all

Table 1 Distribution of yeast species among the 138 individual patient samples yielding a single species.

Yeast species	Number of isolates (%)
<i>Candida albicans</i>	120 (87.0)
<i>Candida glabrata</i>	5 (3.6)
<i>Candida tropicalis</i>	5 (3.6)
<i>Candida norvegensis</i>	4 (2.9)
<i>Candida parapsilosis</i>	2 (1.5)
<i>Candida sake</i>	1 (0.7)
<i>Saccharomyces cerevisiae</i>	1 (0.7)
<i>Candida dubliniensis</i>	0 (0.0)
<i>Candida krusei</i>	0 (0.0)

germ tube/chlamyospore-positive yeasts and species-specific PCR analysis of 100 selected isolates that grew as dark green colonies on CHROMagar Candida, no *C. dubliniensis* isolates were identified in this population.

Discussion

The prevalence of HIV and its associated opportunistic infections remains high in Africa [6,7]. To date there have been few studies of the etiology of oral candidiasis in African HIV-infected patients, particularly in a patient cohort routinely receiving some form of antifungal treatment. The purpose of the present study was to determine the distribution of yeast species associated with oral lesions in HIV-infected patients in Southwest Uganda, with a view to developing treatment strategies to improve the quality of life of these patients.

Out of 605 patients swabbed, samples of 316 (52.2%) were found to be positive for oral yeasts, while 289 (47.8%) samples failed to yield any yeast growth. One possible reason for the high level of culture-negative samples, despite the presence of oral lesions, is that many of the lesions were not caused by yeasts, but instead may have been of bacterial or viral aetiology (e.g., ulcers, Kaposi's sarcoma, Herpes simplex, etc.) or due to trauma. In particular, this might be the case for the lesions categorized as 'unspecified lesions', which accounted for 24.5% of the lesions. In addition, since all of the patients were receiving antifungal therapy (e.g., nystatin and topical clotrimazole) it is likely that this treatment reduced the fungal burden in the lesions and in many cases the oral lesions may have been in the process of being resolved. Other reasons for culture negative samples may have been related to the patient cohorts sampled, the sampling procedure used, or to problems associated with sample handling and transport in isolated communities in rural Africa. It is also worth noting that lesions identified as EC by oral physicians in some cases of APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) were recently found to be culture-negative, suggesting that there may be alternative

aetiological reasons for EC-like lesions, other than *Candida* infection [17]. A majority of culture-positive samples (56%) yielded more than one yeast species. The rate of mixed yeast cultures identified is higher than that described in other studies of African populations [7,18] and is likely due to the use of CHROMagar *Candida* medium in this study, which facilitates the identification of multiple species in clinical samples. It is also worth noting that patients with WHO stage three and four clinical staging of HIV/AIDS were most likely to have multiple species present. Among the 316 patients with positive yeast cultures, *C. albicans* was the most commonly identified species present and was found in 87% of single species samples and in 86.5% of samples containing multiple species. This high prevalence of *C. albicans* is not surprising as this species is widely recognized as the most pathogenic yeast species and similar levels have been observed in previous studies [7,18,19]. Similarly, the finding that *C. glabrata* and *C. tropicalis* were, jointly, the second most commonly identified *Candida* species is also in agreement with previous studies. However, the next most common species found was *C. norvegensis*. This species has only been reported very rarely in cases of oropharyngeal candidiasis [20]. Isolates of *C. norvegensis* have previously been found to be resistant to fluconazole [21]. Although the patients in this study were not routinely treated with fluconazole, it is possible that the topical clotrimazole therapy that many of the patients regularly received may have aided in the selection of this species. Future studies in this region should include attempts to accurately record antifungal usage by patients and to determine the susceptibility of isolates to the antifungals used to treat patients (e.g., clotrimazole). It would also be informative to compare the species distribution present in the oral cavities of non-HIV-infected individuals in this region.

Another unexpected finding from this study was the complete absence of *C. dubliniensis* in this patient group. This species was originally identified in cases of oral candidiasis in Irish HIV-infected patients and subsequently in many other HIV-infected cohorts [9,22]. However, it has only been recognized as a rare cause of systemic infection around the world [21,23]. *Candida dubliniensis*, can be difficult to discriminate from *C. albicans* due to its close phylogenetic relationship and shared phenotypic traits with *C. albicans*, and is often misidentified as *C. albicans* in epidemiological studies [24,25]. Thus, the prevalence of this species tends to be underestimated. To ensure that any *C. dubliniensis* isolates present in the population included in the current study were correctly identified, every putative *C. dubliniensis* yeast as determined on the basis of one or more phenotypic tests was screened using *C. dubliniensis*-specific PCR primers [16]. There have been conflicting reports describing the prevalence of this species in Africa.

In Tanzania, only one *C. dubliniensis* isolate was recovered in a survey of 292 patients [7], while in one South African study, 26% of institutionalized pediatric HIV/AIDS patients were found to harbour this yeast [26]. Interestingly, this same group also reported that the prevalence of this yeast in healthy white South Africans was higher than in healthy and in HIV-infected black individuals [27].

In conclusion, this prevalence study confirms that *C. albicans* is by far the most common cause of oral candidiasis in this region in Africa and that late-stage HIV-infection often results in mixed cultures, including unusual non-*C. albicans* *Candida* species such as *C. norvegensis*. Further in-depth analysis, using antifungal susceptibility testing is required to investigate if the routine use of antifungal drugs can lead to shifts in the prevalence of *Candida* species. Already, as a result of this study, oral clinicians are now part of the team that visits patients in outlying and remote areas, thus improving the detection and diagnosis of oral infection. In addition, plans are currently underway to improve the laboratory services of TASO to include improved facilities to allow the culture of oral samples and with the ultimate aim of targeting the appropriate treatment to culture-positive patients.

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