Oropharyngeal Yeast Colonization in HIV-infected Outpatients in Southern Taiwan: CD4 Count, Efavirenz Therapy and Intravenous Drug Use Matter

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Abstract

To understand the status of oropharyngeal yeast colonization in human immunodeficiency virus (HIV)-infected outpatients in the era of highly active antiretroviral therapy (HAART), we conducted a prospective, cross-sectional study from October 2009 to January 2010 at a medical center in southern Taiwan. Fungal cultures of the oropharyngeal swabs were performed on 327 enrolled patients. At enrollment, 258 (79%) patients had been receiving HAART, and 42 (12.8%), 73 (22.3%) and 212 (64.8%) patients had CD4 cell counts ≤ 200, 201-350, and > 350 cells/mm^3, respectively. Oral yeast colonization was detected in 193 (59%) patients, among whom 157 (81.3%), 25 (13.0%), and 11 (5.7%) were colonized by a single, two, and more than two species, respectively. Among the 241 isolates recovered, *Candida albicans* accounted for 69.7%, followed by *C. dubliniensis* (9.5%), *C. glabrata* (8.3%), *C. tropicalis* (3.3%), *C. intermedia* (2.1%), *C. parapsilosis* (1.7%), and 11 other species (5.4 %). Overall, 230 (95.4%), 236 (97.9%), and 240 (99.6%) isolates were susceptible to fluconazole, voriconazole, and amphotericin B, respectively. In conclusion, colonization by *C. dubliniensis* has emerged in recent years. In addition to CD4 cell counts ≤ 200 cells/mm^3, a known risk factor for oropharyngeal yeast colonization in HIV-infected patients identified in our previous studies, two risk factors, non-receipt of efavirenz-based combinations and intravenous drug use, were first identified in the present study. Fluconazole remained effective *in vitro* against the yeasts colonizing the oropharynx in this population.

Keywords: HIV, yeast colonization, efavirenz, antifungal susceptibility, *Candida dubliniensis*

Introduction

Oropharyngeal colonization by *Candida* species in human immunodeficiency virus (HIV)-infected patients [1-4] may evolve to oroesophageal candidiasis during progressive immunodeficiency [5], which can subsequently hamper the nutritional intake of patients and increase the medical cost of patient management. In HIV-infected patients, *Candida albicans* is the main species of colonization [3, 6]. However, colonization by *C. dubliniensis* has increased in recent years [3, 7-8]. Oral candidiasis usually responds to effective antifungal agents, but the antifungal susceptibility profile varies with *Candida* species and prior use of antifungal agents [9].

In Taiwan, the overall prevalence of HIV infections increased from 0.16 ‰ in 2001 to 0.89 ‰ in 2010 [10]. The government has been providing free highly active antiretroviral therapy (HAART) to HIV-infected patients in Taiwan since 1997. Consequently, the number of patients receiving HAART has increased in the recent decade. Although the risk factors and species distribution of oropharyngeal yeast colonization in HIV-infected patients had been discussed in our previous studies conducted in 1999~2002 and 2005 [1, 6], the effect of different antiretroviral agents on colonization was not addressed. Moreover, there was a dramatic increase in the population of HIV-infected intravenous drug users (IDUs) between 2004 and 2006 in Taiwan [11]. Facing a changing epidemiology, we conducted another survey between 2009 and 2010 to investigate the risk factors and species distribution of oropharyngeal yeast colonization in HIV-infected patients and the antifungal susceptibility of isolates recovered from the survey, with the hope of providing useful information to assist the population.

Methods

Study population and data collection

This prospective cross-sectional study was conducted from October 2009 to January 2010.

The study was approved by the Human Experiment and Ethic Committee of National Chen-Kung University Hospital (NCKUH), a medical center in southern Taiwan. HIV-infected patients at the outpatient infectious diseases clinic were enrolled after informed consents had been obtained. A standardized data collection form was used to retrieve demographic characteristics (age, gender and types of HIV transmission), known period of HIV infection, the underlying medical conditions, and information within 6 months prior to enrollment, including the presence of oral thrush, the latest CD4 cell counts and HIV viral loads, history of hospitalization and residence in jails or rehabilitation centers, and recent receiving of antibacterial/antifungal treatments for ≥ 1 day, and antiretroviral agents for ≥ 2 weeks within 3 months of enrollment.

Sample collection and fungal cultures

Oropharyngeal swabs were obtained using a dry sponge swab (EZ Culturette, Becton Dickinson, Sparks, MD). All swabs were maintained at room temperature and transported to the central laboratory at the National Health Research Institutes within 24 hours. The swabs were then streaked onto Chromagar Candida agar media (CHROMagar, Paris, France). All plates were incubated at 30ºC. Three, if present, colonies from each plate were selected for further analyses. Additional colonies were selected from the plates when there were more than one morphotypes present. All isolates were subjected to the VITEK Yeast Biochemical Card (YBC) (bioMérieux, Marcy l'Etoile, France) for species identification. When the YBC identification probability was less than 90% or when uncommon species were reported, the sequences of the internal transcribed spacer (ITS) region and/or the D1/D2 region of ribosomal DNA were used for species identification. The ITS regions were amplified by the primers ITS1, 5'-TCCGTAGGTGAACCTGCGG-3, and ITS4 5'-TCCTCCGCTTATTGATATGC-3', and the D1/D2 regions were amplified by the primers NL1 5'-GCATATCAATAAGCGGAGGAAAAG-3' and NL4

5’-GGTCCGTGTTTCAAGACGG-3’ [12-13].

Antifungal susceptibility tests

The minimum inhibitory concentrations (MICs) of antifungal drugs were determined according to the guidelines of M27-A3 recommended by the Clinical and Laboratory Standards Institute [14]. The RPMI medium 1640 (31800-022, Gibco BRL) was used for the dilution and growth of the yeast culture. Strains from American Type Culture Collection (ATCC), including *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), and *C. parapsilosis* (ATCC 22019), were used as the standard controls. Growth of each isolate was measured by the Biotrak II plate spectrophotometric reader (Amersham Biosciences, Biochrom Ltd., Cambridge England) after incubation at 35ºC for 48 hours.

The MICs for amphotericin B and azoles were defined as the lowest concentrations capable of preventing any discernible growth and of reducing the turbidity of cells more than 50%, respectively. For amphotericin B, MICs of ≥ 2 μg/ml were considered resistant and ≤ 1 μg/ml susceptible. For fluconazole, MICs of ≥ 64 μg/ml were considered resistant and ≤ 8 μg/ml susceptible. The isolates with MICs in the range of 16–32 μg/ml were referred to as susceptible-dose dependent (SDD). For voriconazole, MICs of ≥ 4 μg/ml were considered resistant and ≤ 1 μg/ml susceptible [14]. Generally, only one isolate of each patient was analyzed. Nevertheless, when a patient was colonized by more than one species, one isolate of each species was included.

Statistical methods

The results were analyzed with SPSS software for Windows, version 12.0. Variables of those collected in the data collection form were tested for association with yeast colonization and infection. The Chi-squared test was applied for categorical variables, and the Student’s t-test for continuous variables. Logistic regression was applied to assess the independent effects of

factors found significant in the univariate analysis. A *p* value less than 0.05 was considered significant.

Results

Patients

During the study period, a total of 327 patients were enrolled. Their demographic data are shown in Table 1. They were predominately males (299, 91.4%) and 36 (11%) patients were IDUs. Overall, 42 (12.8%), 73 (22.3%), and 212 (64.8%) patients had a CD4 cell count ≤ 200, 201-350, and > 350 cells/mm^3, respectively. At enrollment, 258 (79%) patients were receiving HARRT.

Status and risk factors for yeast colonization

Of the 327 patients, 193 (59%) were colonized by yeasts, among whom 157 (81.3%), 25 (13.0%), and 11 (5.7%) were by single, two, and more than two species, respectively. A higher proportion of patients with low CD4 cell counts were colonized by yeasts than those with higher ones (81% in ≤ 200/mm^3 vs. 56% in > 200/mm^3, *P* =0.002). By univariate analysis, IDUs, prior exposure of antibacterial agents, and patients who received mycostatin oral suspension in prior 6 months were at risk for colonization, whereas a CD4 cell count > 200 cells/mm^3 and receipt of lamivudine or lamivudine/zidovudine, or efavirenz protected patients from colonization. Use of protease inhibitors (atazanavir or lopinavir/ritonavir) had no significant effect on colonization (*P*=0.26). By multivariate analysis, receipt of efavirenz and a CD4 count > 200 cells/mm^3 protected patients from colonization, while IDUs were at risk for colonization (Table1). Furthermore, the protective effect of efavirenz-based antiretroviral therapy on colonization remained significant even in a subgroup analysis of 258 patients receiving HAART. Receipt of efavirenz (OR, 0.474; 95% CI 0.28-0.804; *P*=0.006) and a CD4 count > 200 cells/mm^3 (OR, 0.362; 95% CI 0.139-0.942; *P*=0.037) were two

negative predictors for colonization based on multivariate analysis.

As for oral yeast infections, 11 of the 327 patients had experienced oral thrush within 6 months prior to the study and 5 patients had oral thrush at the time of enrollment. Overall, 15 (4.7%) patients had recent or current oral thrush. Patients with a CD4 cell count ≤ 200 cells/mm^3 had a higher rate of recent or current oral thrush than those with a CD4 cell count > 200 cells/mm^3 (28.6% vs.1%, $P < 0.001$), and multivariate analysis showed that a CD4 cell count ≤ 200 cells/mm^3 was the only independent factor associated with the development of oral thrush (OR, 12.8; 95% CI 1.08-150.4; P=0.043). Among the 193 patients with yeast colonization, a CD4 cell count ≤ 200 cells/mm^3 (OR, 9.37; 95% CI 1.48-59.2; P=0.017) and prior exposure to antibacterial agents (OR, 9.9; 95% CI 1.50-65.4; P=0.017) were the two variables associated with oral thrush based on multivariate analysis.

Species distribution and antifungal susceptibility of yeasts

Of the 241 yeast isolates characterized, 12 isolates needed to be speciated by DNA sequencing of the ribosomal DNA fragments. Seven isolates, including 3 *C. glabrata*, 2 *C. albicans*, 1 *C. guilliermondii*, and 1 *C. membranifaciens*, were identified by sequencing the ITS fragments and four isolates, including one each of *C. glabrata*, *C. inconspicua*, *C. membranifaciens*, and *C. tropicalis* were identified by sequencing the D1/D2 fragments. The remaining isolate, *Metschnikowia* spp, could not be identified to species level even by both ITS and D1/D2 sequence. The colonization species were *C. albicans* (168, 69.7%), *C. dubliniensis* (23, 9.5%), *C. glabrata* (20, 8.3%), *C. tropicalis* (8, 3.3%), *C. intermedia* (5, 2.1%), *C. parapsilosis* (4, 1.7%), and *Saccharomyces cerevisiae* (3, 1.2%), and others (10, 4.0%) (Table 2). Overall, *Candida* species accounted for 97.5% of these isolates. All 15 patients with previous or current oral thrush were colonized by *C. albicans* at enrollment, and two were simultaneously colonized by *C. tropicalis*, one by *C. dubliniensis*, and one by *C. glabrata.*

Patients with *C. albicans* colonization were significantly younger than those with non-*C. albicans* yeasts (39.6 *vs.* 43.6 years, *P*=0.037). Risk factors for *C. dubliniensis* colonization were not identified. Furthermore, patients colonized with multiple species were associated with a longer known period of HIV infection (4.6 *vs.* 3.5 years, *P*=0.011) and prior exposure of penicillin derivatives (OR, 9.0; 95% CI 1.28-63.92; *P*=0.027).

Of the 241 isolates, 230 (95.4%), 236 (97.9%) and 240 (99.6%) isolates were susceptible to fluconazole, voriconazole, and amphotericin B, respectively (Table 2). There were 7 (2.4%) and 5 (2.1%) isolates, mostly *C. albicans* (4 and 3 isolates), were resistant to fluconazole and voriconazole, respectively. Eight *Candida* isolates, including 6 *C. albicans* isolates, 1 *C. dubliniensis*, and 1 *C. glabrata*, were recovered from 6 patients who had received fluconazole for oral thrush. Of these 8 isolates, only the *C. glabrata* isolate had increased MICs of fluconazole (8 μg/ml) and voriconazole (0.25 μg/ml) whereas the other 7 isolates remained susceptible to fluconazole (MIC ≤ 1 μg/ml) and voriconazole (≤ 0.03 μg/ml), irrespective of prior fluconazole exposure.

Discussion

The demographical and microbiological characteristics of oropharyngeal yeast colonization in HIV-infected and non-HIV-infected populations in our previous reports [1, 6, 15] and the present study were compared. With the introduction of HAART, the mean CD4 cell counts gradually increased over time (from 208 cells/mm^3 in 1999-2002 to 478 cells/mm^3 in 2009-2010, respectively), which coincided with a reduced rate of patients with recent or current oral thrush (from 12.9% in 1999-2002 to 4.7% in 2009-2010). There were more HIV-infected IDUs enrolled in the present study. Previous studies have found the rate of oral *Candida* colonization among HIV-infected individuals to range from 44 to 82.8% [1-4]. In our earlier surveys, more than half of HIV-infected patients were colonized by yeasts [1, 6] compared to only 15.2% of the healthy individuals in 2007 [15]. These findings suggested

that HIV-infected patients are at risk for oropharyngeal yeast colonization, and this continues despite wide use of HAART. Among the isolates from HIV-infected patients, 87% in 1999-2002 [6] and 70% in the present study were *C. albicans*. Hence, the prevalence of non-albicans *Candida* species has indeed increased in the past decade.

Clinical information about the effects of different antiretroviral agents on the risk of oropharyngeal yeast colonization or infection is limited. In 2000, HIV protease inhibitors were the first to be associated with a lower rate of oropharyngeal *Candida* colonization and candidiasis [16]. The positive impact of protease inhibitors has been attributed to a better immunological function with their use, or with their antifungal activity resulting from the similar structure of *Candida* secreted aspartic protease with the targeted protein, ***i.e.*** HIV aspartic protease [17]. However, such an effect of protease inhibitors was not found in the present study as well as in another recent study [2]. Instead we found that receipt of efavirenz-containing regimen was significantly associated with a lower frequency of oropharyngeal yeast colonization both in all enrolled patients and in patients receiving HAART. This is the first study showing the potential effect of efavirenz on oropharyngeal yeast colonization. Despite the statistical significance of efavirenz on reduced colonization, further clinical investigations involving more patients to determine the impact of the drug on colonization and *in vitro* antifungal activity are warranted.

In our previous study conducted in 2005 [1], we found that all six IDUs were colonized by yeasts and had a CD4 count of more than 450 cells/mm^3. This observation suggested that IDU may be at risk for yeast colonization. In the present study, we were able to reach a statistical significant association between IDU and yeast colonization. An earlier report had a similar finding, showing a higher prevalence of *Candida* lesions and oral yeast colonization among the HIV-infected IDU group than the HIV-infected heterosexual and the homosexual groups [18]. Further, studies showed that IDUs, regardless of HIV serostatus, were more likely than homosexual men to present with oral candidiasis [19-21]. Several factors of the

lifestyle, access to health care, and the hygiene conditions of the oral cavity before HIV infection, influenced the development of oral lesions in HIV-infected populations [20]. Whether those same factors contribute to oropharyngeal yeast colonization needs to be examined.

Candida albicans was the major species, accounting for 70% of all yeast isolates colonizing the oropharynx of HIV-infected patients, a finding similar to previous studies in which *C. albicans* accounted for 60-83% of colonized isolates [3, 22-23], though a decreased isolation rate was found compared with that (87%) of our earlier study during 1999~2002 [6]. Another shift of species distribution in our studies was noted in *C. dubliniensis,* which was not recovered during 1999~2002 [6] but was increasingly detected in 2005 (4.3%) [1] and in the present study (9.5%). Little is known about the risk factors for *C. dubliniensis* colonization in HIV-infected patients, except for a higher prevalence in the European descent (9%) than in the African descent individuals (1.5%) in South Africans [24]. Neither did our study identify the risk factors for *C. dubliniensis* colonization. The clinical implication of these findings remains to be elucidated.

In conclusion, compared to our previous studies, CD4 cell counts ≤ 200 cells/mm^3 remained as a risk factor for oropharyngeal yeast colonization in HIV-infected patients, whereas non-receipt of efavirenz-based combinations and intravenous drug use were first identified as another two risk factors in the present study. The mechanism contributing to the effect of efavirenz on oropharyngeal yeast colonization needs further investigations. An increasing proportion of colonization by *C. dubliniensis* was also observed during the decade. Three tested antifungal agents, fluconazole, voriconazole, and amphotericin B, remained effective against more than 95% of the colonized yeasts.

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Authorship and Disclosures

C. J. Wu collected specimens and medical information of patients, analyzed the data and drafted the manuscript. H. C. Lee, C. M. Chang, N. Y. Lee, Y. L. Wang, N. Y, Ko, and W. C. Ko collected specimens and medical information of patients. H. T. Chen, C. C. Lin, and W. L. Chu performed experiments of identification of isolates and drug susceptibilities. L. Y. Hsieh and F. C. Tseng analyzed data. Y. L. Yang, W. C. Ko, T. L. Lauderdale, and H. J. Lo designed the study and edited the manuscript. All authors made a significant contribution to this work.

Transparency declaration

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Table 1. Characteristics of 327 HIV-infected patients enrolled for oropharyngeal fungal cultures and predictors of positive culture for yeasts.

Characteristic	All (n=327)	Yeast culture		Univariate	Multivariate
		Positive (n= 193)	Negative (n=134)	*P* value	*P* value, OR (95% CI)
Age, years (mean ± SD)	38.7 ± 12.8	39.6 ± 12.7	37.5 ± 13.0	0.146	
CD4, cells/mm^3 (mean ± SD)	477.8 ± 283.7	455.3 ± 246.5	524.7 ± 325.3	0.013	
HIV viral load, log (copies/mm^3) (mean ± SD)	2.05 ± 1.27 (n=324)	2.15 ± 1.32 (n=190)	1.91 ± 1.19	0.092	
Known period of HIV infection, years (mean ± SD)	5.21 ± 3.72	5.32 ± 3.81	5.05 ± 3.61	0.53	
No. of subjects with indicated transmission type (%)					
Men having sex with men or bisexual	179 (54.7%)	103 (53.4%)	76 (56.7%)	0.574	
Heterosexual	101 (30.9%)	57 (29.5%)	44 (32.8%)	0.545	
Intravenous drug user	36 (11%)	28 (14.5%)	8 (6%)	0.019*	0.031 2.53 (1.09-5.86)
Males, no (%)	299 (91.4%)	175 (90.7%)	124 (92.5%)	0.689	
CD4 counts > 200 cells/mm^3, no. (%)	285 (87.2%)	159 (82.4%)	126 (94%)	0.002*	0.002 0.27 (0.12-0.62)
Diabetic mellitus, no. (%)	11 (3.4%)	8 (4.1%)	3 (2.2%)	0.535	
Chronic kidney diseases, no. (%)	2 (0.6%)	2 (1%)	0 (0%)	0.515	
Hospitalization within 6 months, no. (%)	111 (33.9%)	60 (31.1%)	51 (38.1%)	0.194	
Residence in a jail or rehabilitation center within 6 months, no. (%)	2 (0.6%)	1 (0.5%)	1 (0.7%)	1	
Medications					
Antiretroviral therapy within 3 months	258 (78.9%)	146 (75.6%)	112 (83.6%)	0.098	
lamivudine/zidovudine	134 (41.0%)	71 (36.8%)	63 (47.0%)	0.068	
zidovudine or lamivudine/zidovudine	136 (41.6%)	72 (37.3%)	64 (47.8%)	0.068	
lamivudine or lamivudine/zidovudine	239 (73.1%)	132 (68.4%)	107 (79.9%)	0.023*	
stavudine	14 (4.3%)	10 (5.2%)	4 (3%)	0.413	
abacavir	96 (29.4%)	53 (27.5%)	43 (32.1%)	0.389	
didanosine	33 (10.1%)	22 (11.4%)	11 (8.2%)	0.36	
efavirenz	101 (30.9%)	45 (23.3%)	56 (41.8%)	< 0.001*	0.005 0.48 (0.29-0.80)
nevirapine	18 (5.5%)	10 (5.2%)	8 (6%)	0.808	
atazanavir	21 (6.4%)	15 (7.8%)	6 (4.5%)	0.26	

lopinavir/ritonavir	117 (35.8%)	73 (37.8%)	44 (32.8%)	0.412
PI[a] (atazanavir or lopinavir/ritonavir)	21 (6.4%)	15 (7.8%)	6 (4.5%)	0.26
Antibacterials within 6 months[b]	42 (12.0%)	33 (17.1%)	9 (6.7%)	0.007*
Antifungals within 6 months				
fluconazole	6 (1.8%)	6 (3.1%)	0 (0%)	0.085
amphotericin B	2 (0.6%)	1 (0.5%)	1 (0.7%)	1
mycostatin oral suspension	10 (3.1%)	10 (5.2%)	0 (0%)	0.006*

[a] PI: protease inhibitor; OR: odds ratio, CI: confidence interval; SD: standard deviation.

[b] Use of antibacterial agents, i.e. penicillin derivatives, cephalosporins, trimethoprim/sulfamethoxazole, clindamycin, macrolides, or anti-tuberculosis agents, was not associated with oropharyngeal yeast colonization.

* Variables entered in the multivariate analysis.

Table2. Species distribution and antimicrobial susceptibilities of yeasts recovered from the oropharynx of HIV-infected patients.

Yeast	No. of isolates (%) with indicated minimum inhibitory concentrations (μg/ml)								Total no. of isolates (%)
	Fluconazole			Voriconazole			Amphotericin B		
	S, ≤ 8	SDD, 16-32	R, ≥ 64	S, ≤ 1	SDD, 2	R, ≥ 4	S, ≤ 1	R, ≥ 2	
Candida albicans	163	1	4 (2.4)	165	0	3 (1.8)	168	0	168 (69.7)
Candida dubliniensis	22	0	1 (4.3)	22	0	1 (4.5)	22	1 (4.3)	23 (9.5)
Candida glabrata	19	1	0	20	0	0	20	0	20 (8.3)
Candida tropicalis	7	0	1 (12.5)	7	0	1 (12.5)	8	0	8 (3.3)
Candida intermedia	5	0	0	5	0	0	5	0	5 (2.1)
Candida parapsilosis	4	0	0	4	0	0	4	0	4 (1.7)
Saccharomyces cerevisiae	3	0	0	3	0	0	3	0	3 (1.2)
Candida famata	1	0	0	1	0	0	1	0	1 (0.4)
Candida galeiformis	0	1	0	1	0	0	1	0	1 (0.4)
Candida guilliermondii	1	0	0	1	0	0	1	0	1 (0.4)
Candida inconspicua	0	1	0	1	0	0	1	0	1 (0.4)
Candida krusei	0	0	1 (100)	1	0	0	1	0	1 (0.4)
Candida lusitaniae	1	0	0	1	0	0	1	0	1 (0.4)
Candida rugosa	1	0	0	1	0	0	1	0	1 (0.4)
Cryptococcus neoformans	1	0	0	1	0	0	1	0	1 (0.4)
Kodamaea ohmeri	1	0	0	1	0	0	1	0	1 (0.4)
Metschnikowia spp.	1	0	0	1	0	0	1	0	1 (0.4)
Total	230 (95.4)	4 (1.7)	7 (2.9)	236 (97.9)	0 (0)	5 (2.1)	240 (99.6)	1 (0.4)	241 (100)

Note: S = susceptible, SDD = susceptible-dose dependent, R = resistant.