

# Microevolution of *Candida albicans* Isolate from a Patient with Mucocutaneous Candidiasis and HIV Infection

Gabriel Palma Cortés<sup>1</sup>, Carlos Cabello Gutierrez<sup>1</sup>, Misael González Ibarra<sup>2</sup>,  
Magdalena Aguirre García<sup>3</sup>, Fernando Hernández Sánchez<sup>1</sup>, Haydee Torres Guerrero<sup>3\*</sup>

<sup>1</sup>Departamento de Investigación en Virología y Micología, Instituto Nacional de Enfermedades Respiratoria “Ismael Cosío Villegas”, Ciudad de México, México

<sup>2</sup>Laboratorio de Inmuno Alergología y Micología Médica, División de Investigación, Hospital Juárez de México, Ciudad de México, México

<sup>3</sup>Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Hospital General de México “Dr. Eduardo Liceaga”, Ciudad de México, México

Email: \*haydeet@unam.mx, \*htorresguerrero@yahoo.com

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## Abstract

Candidiasis is the most common opportunistic fungal infection in HIV patients, and its presence is ascribed mainly to the persistence of the original infecting strain. The latter might acquire genetic variations during interaction with the host, reflecting the adaptation of the strain. Here, we report the case of a 32-year-old man complaining of asthenia, irregular hyperpyrexia, and dry cough, who was admitted to the emergency unit. Laboratory examination showed positivity for HIV. Dark violet macular lesions and ulcerated lesions with verrucous erosion were observed at the tip of the nose, whereas an ulcer without exudates was noted in the pubic region. *Candida albicans* was recovered from the skin by scraping these lesions. Cultures from the bronchoalveolar lavage (BAL) were negative for bacteria and opportunistic fungi but were positive for *Candida albicans*. The isolates from the skin and BAL were typed by PCR-RFLP and *Candida albicans* was identified. Analysis by microsatellite length polymorphisms, established that the pubic isolate was a genetic variant of the isolate from the nose and mouth. This suggested a microevolutionary event. Despite clinical support, the patient died of multiple organ failure.

## Keywords

*Candida albicans*, HIV, PCR-RFLP, Microsatellite Genotyping, Microevolution

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## 1. Introduction

*Candida albicans* is a common commensal coloniser of the human gastrointestinal, respiratory, and reproductive tracts, as well as an opportunistic pathogen, responsible for superficial and systemic infections [1]. Mucosal *Candida* infections are common in patients infected with HIV. The infection is thought to result from invasion by endogenous colonising *Candida* in the patient; however, nosocomial outbreaks of *Candida* infections support the exogenous acquisition of *Candida* spp. as an important cause of morbidity and mortality in hospitalised patients [2]. Candidiasis is caused mainly by *C. albicans*, but other species such as *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida dubliniensis* have also been isolated singly and at low frequency (1.6% - 6%) in combination with *C. albicans* [3]. Therefore, the proper identification of the *Candida* species is important for choosing the appropriate therapy, as different *Candida* spp. display different antifungal drug susceptibilities [3] [4]. Biochemical identification of *Candida* spp. has been standardised using different kits; however, these approaches are often limited by variations in the phenotypic expression of different *Candida* species. Alternative molecular methods have been developed, which are mostly based on the amplification of specific regions, such as the 5.8S-ITS region, followed by restriction analysis [5].

Another important aspect is to determine whether the source of colonisation is a result of the selective proliferation of a single variant strain present in the commensal population before invasive infection, or a nosocomial strain acquired by cross-contamination within a hospital [6]. Patients with recurrent infections might be infected by the same strain throughout the infection period, and this strain may undergo a process of microevolution or it might be replaced by other strains [7].

The amplification of species-specific microsatellites by PCR has been successfully applied in molecular epidemiology and population studies to differentiate between *C. albicans* strains [8] [9]. The microsatellite loci CAI (CAA repeats), CAIII (GAA repeats), and CAVI (TAAA repeats) present in the *C. albicans* genome have been used for genotyping clinical isolates. This approach facilitates the differentiation of diverse isolates, as well as their epidemiological pathways. High similarity among samples suggests adaptation to environmental conditions and the occurrence of microevolutionary processes [10]. Herein, the three *C. albicans* isolates from the patient were identified using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The genetic relationship among isolates was analysed by CAI, CAIII, and CAVI microsatellite genotyping. The high similarity among the three samples suggests adaptation to the environmental conditions of the isolate from the pubic region, thus confirming a microevolutionary event.

## 2. Case Presentation

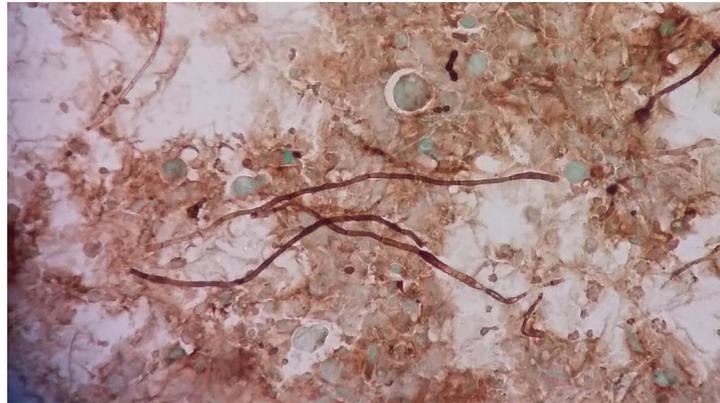
We present the case of a 32-year-old HIV-infected man who was admitted to the

emergency unit because of asthenia, irregular hyperpyrexia, and dry cough. The patient stated that he had lived in the United States for 13 years, and 4 years prior to his admission, he was diagnosed with bacterial pneumonia and treated with antibiotics. Physical examination showed acute illness, with asthenia, tachypnoea, and a small lesion suspected to be Kaposi's sarcoma. Fundoscopy revealed HIV-related retinopathy, with small necrotic spots on the inner retina and pathological dilatations of the small vessels. Dark violet macular lesions and ulcerated lesions with verrucous erosion were observed at the tip of the nose. In the pubic region, an ulcer without exudate was observed. Abundant hyaline oval yeasts, pseudomycelia, and mycelia were observed in bronchoalveolar lavage (BAL) cultures (**Figure 1**). The patient also developed ulcerative mucocutaneous disease due to the *Herpes simplex virus*, with vesicles in the eighth intercostal space of the right hemithorax. Generalised dermatosis, with vesicle-like lesions mainly on the trunk and lower limbs, was observed. Chest radiography showed diffuse bilateral interstitial infiltrates. Methenamine silver staining performed on both sputum and BAL excluded *Pneumocystis jiroveci* pneumonia (PJP). The initial treatment included intravenous hydration and trimethoprim/sulpha methoxazole (20 mg/kg/day in three doses) because there was a high clinical suspicion of *Pneumocystis* pneumonia. Antifungal treatment with fluconazole (200 mg/kg/day) and antiviral treatment with intravenous acyclovir (30 mg/kg/day) were started. Cultures from BAL were negative for bacteria but positive for *Candida* spp. Two weeks after hospital admission, the patient's overall condition continued to deteriorate despite clinical support, and he died of multiple organ failure. The aetiology of the respiratory infection could not be identified, perhaps because of the poor sensitivity of our laboratory assays.

### 3. Material and Methods

#### 3.1. Isolation and Identification of *Candida albicans*

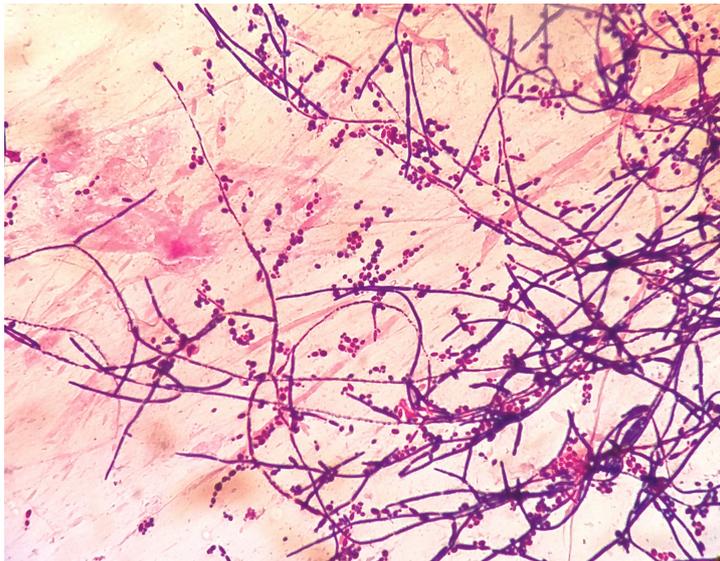
Three specimens, including BAL and skin lesion samples, were obtained from the patient for *in vitro* culture and pathogen identification via microscopic analysis. Lesion samples were taken from the tip of the nose and the pubic region using sterile swabs (Culturette, Becton, Dickinson, USA) and were processed at the Mycology Laboratory. Specimens were initially analysed by routine procedures comprising direct microscopic examination of samples with 5% KOH mount, and periodic-acid-Schiff and Grocott-Gomori-methenamine-silver-stained smears highly specific for *P. jirovecii*. Additional microbiological evaluations were conducted per standard procedures. Morphology testing for the presence of chlamydo spores, pseudohyphae, true mycelia, and blastospore arrangement was performed on corn meal agar. The three samples were inoculated on Sabouraud Dextrose Agar (SDA) (Difco, USA) with chloramphenicol (50 µg/mL) and incubated at 28°C for 72h. For presumptive identification of *Candida* species, the isolates were grown on CHROMagar™ *Candida* plates (CHROMagar, France).



(a)



(b)



(c)

**Figure 1.** Cytological smears: (a) Nose skin scraping stained with Grocott's methenamine silver (GMS), hyphae, budding yeast and blastoconidia were identified; (b) Direct microscopic examination with 10% KOH of pubic ulcer smear showed blasto conidia, and hyphae; (c) Periodic Acid-Schiff (PAS) stained BAL showed pseudohyphae and budding yeast.

### 3.2. DNA Purification for PCR-RFLP and Microsatellite Typing

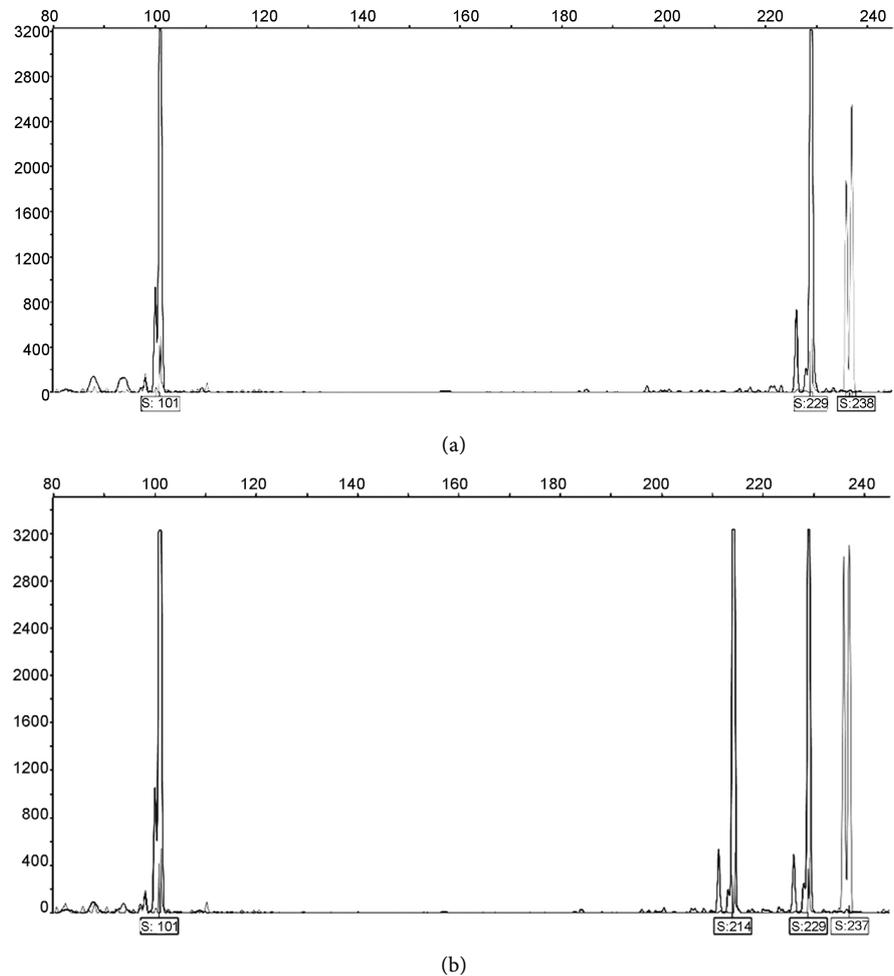
DNA was extracted and purified using YeaStar Genomic DNA (Zymo Research, USA). The primers used in the PCR reactions were ITS1-F (5'TCC GTAGGTGAACCTGCGG) and ITS4-R (3'TCCTCCGCTTATTGATATGC).

PCR reactions were performed in 20 µl reaction mixtures containing 30 ng of *C. albicans* DNA; 1 µM of each primer; 0.6 U of Taq DNA (Fermentas Inc., Canada); 200 µM each of dATP, dCTP, dGTP, and dTTP; 2 µl of 10×Taq polymerase buffer (Fermentas Inc.); and 2 mM MgCl<sub>2</sub>. The amplification products were digested with MspI enzyme to achieve length patterns. Restriction fragments were analysed by 2% agarose gel electrophoresis in TAE buffer; gels were stained with ethidium bromide and then photographed. *C. albicans* 90028, *C. tropicalis* 0750, *C. glabrata* 90030, *C. krusei* 6298, *C. parapsilosis* 22019, and *Candida guilliermondii* 9058 were used as control strains. Genotyping with microsatellites multiplex PCR was performed in a 25-µl final volume of PCR buffer (20 mM Tris HCl, pH 8.4, 50 mM KCl), 0.2 mM dNTPs, 2 mM MgCl<sub>2</sub>, 50 ng of genomic DNA, and 0.5 U of *Taq* polymerase (Applied Biosystems, USA). Forward primers used in the PCR amplification were labelled with 6-carboxyfluorescein (6-FAM) (for CAI and CAIII) and hexachlorofluorescein (HEX) (for CAVI) fluorochromes [10]. The PCR products were run on an ABI 310 Genetic Analyser (Applied Biosystems) together with the GeneScan-500 (TAMRA) size standard (Applied Biosystems). Fragment sizes were automatically determined by GeneScan 3.7 Analysis software. Alleles were designated according to their size (bp) and number of motif repeats.

## 4. Results

To identify the species of the *Candida* isolates, DNA was extracted from samples and control strains, after which the 5.8S-ITS region was analysed by PCR-RFLP [11]. The three isolates were identified as *C. albicans*. Antifungal susceptibility tests were performed with FUNGITEST® and results were read after incubating each isolate for 24 h. The three *Candida* isolates were found to be susceptible to fluconazole (8 - 64 µg/mL), itraconazol (0.5 - 4 µg/mL), ketoconazole (0.5 - 4 µg/mL), myconazol 0.5 - 8 µg/mL), amphotericin B (2 - 8 µg/mL) and 5-fluorocytocine (2 - 32 µg/mL).

The genetic relationship among the isolates was established with three polymorphic microsatellites: CAI, CAIII, and CAVI. Microsatellite-based genotyping was performed by multiplex PCR using automated fluorescent genotyping (Applied Biosystems & Laragen Inc., USA) according to the protocol by Sampaio *et al.* [10]. For each marker and for a given isolate, one or two bands were observed. Given that *C. albicans* is diploid and each marker tested a single locus, each observed band was assigned to an allele. The genotypes observed in the patient isolates by the multiplex assay were highly related, suggesting a clonal origin. The isolates obtained from the nose and BAL were homozygous and had the same allelic combination of microsatellites as revealed by the GeneScan profiles (Figure 2). However, the genotype of the strain obtained from the pubic area



**Figure 2.** Gen Scan profiles representing the results of automatic fragment sizing for the microsatellite multiplex analysis for: (a) BAL and nose isolates and (b) pubic isolate (CAI) is heterozygous.

was heterozygous, suggesting that a microevolutionary event occurred at the CAI locus between genotype 38-38 and genotype 33-38 (Table 1).

## 5. Discussion

Mucocutaneous candidiasis, including oropharyngeal, oesophageal, and vulvovaginal forms, produced by *C. albicans* is the most common fungal infection in individuals with HIV [5]; however, the involvement of other *Candida* species has also been reported [3]. In this study, PCR-RFLP analysis of the 5.8S-ITS region clearly demonstrated *C. albicans* infection in the three analysed clinical samples. Molecular typing of these *C. albicans* isolates with CAI, CAII, and CAVI microsatellites revealed high similarity among the genotypes; two were identical, whereas one strain displayed a minor difference in the CAI locus. The observed allele size variation was apparently due to the deletion of five dinucleotide units (from 38 to 33). This suggests adaptation to environmental conditions and confirms a microevolution event [10]. Different studies have reported that microsatellite markers have high discriminatory power and reproducibility and

**Table 1.** Alleles have been assigned according to the fragment size in bp; in parenthesis is the number of motif repeats. Genotype showing microevolution is in bold.

Genotype of the three isolates from the patient, based on analysis of microsatellite markers						
Isolate origin	CAI		CAIII		CAVI	
	Allele	Allele	Allele	Allele	Allele	Allele
	1	2	1	2	1	2
Pubis	<b>214 (33)</b>	<b>229 (38)</b>	101 (07)	101 (07)	237 (06)	237 (06)
Nose	229 (38)	229 (38)	101 (07)	101 (07)	237 (06)	237 (06)
BAL	229 (38)	229 (38)	101 (07)	101 (07)	237 (06)	237 (06)

are useful for studying the genetic diversity of *Candida* spp. [12].

Opportunistic pneumonias are the major cause of morbidity and mortality associated with HIV patients. Pulmonary complications during the evolution of the disease are mainly of infectious aetiology. The spectrum of involved pathogens is broad and includes bacteria, mycobacteria, fungi, viruses, and parasites [13]. These are responsible for opportunistic infections and malignancies, which progressively increase as T CD4<sup>+</sup> cell counts fall below 200 cells/ $\mu$ L. The aetiology of the pulmonary disease in the present patient could not be determined probably because of the poor sensitivity of the assays. However, *C. albicans* was clearly identified upon direct examination of BAL, where yeasts and mycelia were observed. The existence of *Candida* pneumonia as a clinical entity has been questioned. Because different species are frequently found in tracheal aspirate specimens in healthy people, the presence of *Candida* in sputum or other respiratory tract specimens in immunocompromised conditions is generally considered a contamination from colonisation in the upper respiratory tract. The most reliable method to confirm the clinical entity would be lung biopsy [14] [15]; however, owing to the patient's condition, this approach was not advisable.

DNA typing techniques have been extensively used for epidemiological studies in nosocomial infections and for the identification of routes of colonisation by *C. albicans*. The analysis of endogenous *Candida* isolates is also important because they can become invasive in immunodeficient individuals. Knowledge of the genetic relations among clinical *C. albicans* isolates involved in infections is important for the development and application of appropriate treatment strategies and to better understand the epidemiology of these infections.

## 6. Conclusion

Microsatellite analysis of these three isolates revealed that two of them shared the same genotype and that of the third one was also very similar. These findings suggest a common clonal origin followed by a microevolutionary event in the CAI locus.

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