Differences by Resistotyping Between *C. albicans* Strains Isolated from the Oral Cavity of HIV+ and Seronegative Patients

Diferencias por resistotipificación entre cepas de *C. albicans* aisladas de la cavidad oral de pacientes VIH+ y seronegativos

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ABSTRACT

Candida albicans is the etiological agent most frequently associated with oral candidiasis in human immunodeficiency virus (HIV) carriers. Strain typification is important to disease epidemiology, particularly with simple, low-cost methodologies such as resistotyping. The present study was designed to use resistotyping to identify possible phenotypic differences between *C. albicans* strains isolated from the oral cavity of HIV+ and HIV-seronegative patients. Analyses were run using resistotyping (boric acid, cetrimide, sodium periodate, sodium selenite and silver nitrate) to identify phenotypical differences between *C. albicans.* Descriptive statistics was performed. Of the 149 clones isolated from HIV+ patients the most frequent (47.0%) resistotype was ABCDE. The most frequent resistotype (64.8%) in the 74 clones from HIV-seronegative patients was --CDE. Phenotypic differences were identified between the strains isolated from each group. HIV+ patients exhibited greater strain diversity. Although it has limitations, resistotyping effectively identified differences between *C. albicans* strains.

KEYWORDS

Candida; *Candida albicans*; HIV; Carriers; Resistotyping; Epidemiology.

RESUMEN

Candida albicans es el agente etiológico más frecuentemente asociado con la candidiasis oral en portadores del virus de la inmunodeficiencia humana (VIH). La tipificación de la cepas es importante para conocer la epidemiología de la enfermedad, particularmente con metodologías simples y de bajo costo, como la resistotipificación. El presente estudio fue diseñado para identificar posibles diferencias fenotípicas por el método de resistotipificación entre cepas de *C. albicans* aisladas de la cavidad oral de pacientes VIH+ y seronegativos. Se realizó estadística descriptiva. Los análisis se realizaron utilizando resistotipificación (ácido bórico, cetrimida, peryodato de sodio, selenito de sodio y nitrato de plata) para identificar diferencias fenotípicas entre *C. albicans*. De las 149 clonas aisladas de pacientes VIH+, el resistotipo más frecuente (47.0%) fue ABCDE. El resistotipo más frecuente (64.8%) en las 74 clonas de pacientes seronegativos al VIH fue --CDE. Se identificaron diferencias fenotípicas entre las cepas aisladas de cada grupo. Los pacientes VIH + exhibieron una mayor diversidad de cepas. Aunque tiene limitaciones, la resistotipificación identificó de manera efectiva las diferencias entre las cepas de *C. albicans.*

PALABRA CLAVE

Candida; *Candida albicans*; VIH; Portadores; Resistotipificación; Epidemiología.

INTRODUCTION

Oral candidiasis (OC) has been closely linked to human immunodeficiency virus (HIV) infection since the pandemic began (1,2). It has become an important diagnostic and prognosis parameter, as well as a marker of the success of highly active antiretroviral therapy (3,4).

Extensive research has been done on OC, and prophylactic treatments developed and implemented for treatment eradication, but it continues to be the most frequent opportunistic oral infection in HIV+ patients, regardless of treatment with highly active antiretroviral therapy (4,5).

Candida species, mainly *C. albicans*, are the causal agents of OC. Normally commensal dimorphic fungi, under specific local and systemic circumstances they can become pathogens (6,7). The *Candida* species that colonize the oral cavity of HIV+ subjects exhibit greater antimycotic treatment resistance than strains from HIV-seronegative subjects. Resistance to antimycotic drugs is considered an important risk factor contributing to antimycotic resistance and consequent development of deep mycoses and candidemia (8).

Although *C. albicans* has a clonal mode of reproduction, phenotypic and genotypic traits can distinguish between strains (9). Strain-typing techniques have been developed to generate epidemiological data on *C. albicans*. These describe the behavior of different strains in distinct patient groups, thus identifying associations between genotypes and phenotypical traits, mainly antibiotic resistance and mechanisms of pathogenicity (9-13).

Genotype and phenotype variation in *Candida* species is a possible cause for increased antifungal resistance with consequent treatment failure and persistent infection. This suggests that oral candidiasis in HIV+ patients may be caused by different clones of the same species (12,14). Little data is available on the presence of *C. albicans* clones with different resistance profiles colonizing the oral cavity of HIV+ subjects. In addition, it is still under debate if certain *C. albicans* strains exhibit variation in their virulence capabilities.

The objective of this study was to use resistotyping to identify possible phenotypic differences between *C. albicans* strains isolated from the oral cavity of HIV+ and HIV-seronegative patients.

METHODS

ETHICS STATEMENT

The research protocol was approved by the bioethics committee of the Faculty of Dentistry of the Autonomous University of Yucatan (Universidad Autónoma de Yucatán [UADY]) (code FOCAI 04/02). It was classified as minimal risk in accordance with the Health Research Regulation, Article 17, General Health Law, Health Department, Government of Mexico.

STRAIN ISOLATION AND RECOVERY

A total of 92 consecutive clinical isolates of oral *C. albicans* strains were obtained from oral swabs subsequently frozen at -80°C in heart infusion broth (Becton Dickinson, Mexico) containing 10% glycerol (Sigma-Aldrich, USA). Isolates were collected from 37 healthy subjects (≥ 21 years old) lacking clinical lesions indicating any clinical form of oral candidiasis, and from 55 HIV+ patients (>18-<50 years old) in the O'Horan General Hospital, Merida, Yucatan, Mexico. Inclusion criteria for the HIV+ patients were that they must have been asymptomatic for any clinical form of oral candidiasis at the time of sampling, and not have received any antifungal or antibacterial treatment at least six months prior to oral examination.

Strains were sown on CHROMagar *Candida* (Becton Dickinson Microbiology Systems, USA) and incubated at 37ºC for 48 h. Isolates were identified by standard morphology.

MOLECULAR IDENTIFICATION

1. DNA EXTRACTION

Strains were grown on Sabouraud dextrose agar (SDA) (Becton Dickinson and Company, USA) for 48 hours at 37°C, suspended in 500 µL sterile distilled water, heated for 15 min at 95°C and then immediately frozen at -70°C for 15 min. After thawing to room temperature samples were centrifuged for 5 min at 16,000 x g, the supernatant placed in a sterile Eppendorf tube and stored at -20°C until analysis (6,15).

2. PCR

Two pairs of oligonucleotides (Invitrogen, USA) were simultaneously used in each PCR test: the first pair, specific to *C. albicans*, amplifies a 175 bp fragment of the 25S rRNA gene: CAL5 (5'-TGTTGCTCTCTCGGGGGCGGCCG-3') and NL4CAL (5'-AAGATCATTATGCCAACATCCTAGGTA/TAA-3'). The second pair, present in all *Candida* species, amplifies a 610 bp fragment of the 25S rRNA gene: RNAF (5'-GCATATCAATAAGCGGAGGAAAAG-3') and RNAR (5'-GGTCCGTGTTTCAAGACG-3'). PCR was done following Yang *et al.* (16) as described in Hernández-Solís *et al.* (11).

RESISTOTYPING

Resistogram biotyping of *C. albicans* strains was done following the McCreight and Warnock method as modified by Nakamura et al. (17). Briefly, stock solutions of boric acid, cetrimide, sodium periodate and sodium selenite (Sigma-Aldrich,

USA) were prepared at a 20 mg/mL concentration. A silver nitrate (Sigma-Aldrich, USA) stock solution was prepared at a 2 mg/mL concentration.

The resistance profile was assessed based on different concentration series of each stock solution added to SDA: boric acid 1.15, 1.3, 1.45 and 1.6 mg/mL; cetrimide 0.06, 0.08, 0.1 and 0.12 mg/mL; silver nitrate 0.0075, 0.01, 0.0125 and 0.015 mg/mL; sodium periodate 0.01, 0.02, 0.03 and 0.04 mg/mL; and sodium selenite 0.1, 0.2, 0.3 and 0.4 mg/mL. *Candida albicans* (5 µL CFU), previously incubated for 24 h at 37°C and with an optical density ranging from 0.45 to 540 nm, were inoculated onto the agar plates containing one of the chemical reagents and incubated for 40h at 37°C.

Resistance was tested using a concentration for each stock solution that had exhibited clear differentiation between the studied strains: 0.3 mg/mL for sodium selenite; 1.6 for boric acid; 0.6 for cetrimide; 0.03 for sodium periodate; and 0.015 for silver nitrate (Table 1). Growth patterns were described following the criteria suggested by Khan et al. (18): confluent growth (resistance) with capital letter identifying reagent; non-confluent growth (sensitivity) with lowercase letter; and absence of growth indicated by a hyphen (-).

Table 1. Chemical inhibitor codes and concentrations used in resistotyping technique.

Chemical inhibitor	Code	Concentration (mg/mL)
Sodium selenite	A	0.3
Boric acid	B	1.6
Cetrimide	C	0.06
Sodium periodate	D	0.03
Silver nitrate	E	0.015

STATISTICAL ANALYSIS

Descriptive statistics and possible associations between resistotype profiles and any study group were identified using a X2 test at a 95% confidence level ($p < 0.05$, CI 95%) in the Statgraphics plus 5.1 software.

RESULTS

Control group. Thirty-seven *C. albicans* isolates were collected from the HIV-seronegative patients. From these, 74 clones were obtained (2 clones per strain) and seven different resistotypes identified (Table 2). The most frequent (n=48 clones; 64.8%) resistotype profile was --CDE, followed by -bCDE (n= 8; 10.8%) and ---DE (n= 6 cases; 8.1%). One strain in this study group had two clones with different resistotype profiles.

Table 2. Resistotype patterns of *C. albicans* isolated from HIV-seronegative patients.

Resistotype	Distribution	%
--CDE	48	64.8
-bCDE	8	10.8
---DE	6	8.1
-b-d-	4	5.4
--cDE	4	5.4
a-CDe	3	4.1
-bcDe	1	1.4
Total	**74**	**100.0**

HIV+ group. Three clones were obtained from each of the 55 *C. albicans* isolates from this group. Sixteen clones were lost during processing, leaving 149 clones to determine the resistotypes. Eleven different resistotype profiles were identified in this study group. The most frequent (n=70;

47%) was ABCDE, followed by A-CDE (n=37; 24.9%) and --CDE (n= 14; 9.4%). Twenty oral *C. albicans* strains were identified with two different resistotype profiles. The remaining strains had clones with the same resistotype profile (Table 3).

The frequency of the serotype --CDE was significantly higher in the HIV-seronegative group (64.8% versus 9.4%, $p<0.05$).

Table 3. Resistotype patterns of *C. albicans* isolated from HIV+ patients.

Resistotype	Distribution	%
ABCDE	70	47.0
A-CDE	37	24.9
--CDE	14	9.4
-BCDE	13	8.7
aBCDE	5	3.4
AB-DE	2	1.3
abCDE	2	1.3
-BC--	2	1.3
--C--	2	1.3
a-CDE	1	0.7
--C-E	1	0.7
Total	**149**	**100.0**

DISCUSSION

Candida strain pathogenicity has been correlated to certain resistotypes (19). Variations in *C. albicans* clone biotype are known to exist in recurrent oral candidiasis in HIV+ patients (17,20). Antifungal therapy can lead to replacement of an initial biotype by a new one, even one resistant to antifungal drugs. Though infrequent, shifts in the resistogram biotypes of oral *C. albicans* isolates may also occur to a certain extent in normal subjects (17).

Based on this phenomenon it is possible that differences may be linked to selection of a more virulent switched phenotype, or that a switched phenotype of the same strain can exhibit variations in virulence in response to changing environmental conditions (18,21).

This being the case, *C. albicans* populations are mainly clonal in origin with gene content being apparently constant and stable between strains (9,22). Phenotypic differences between *C. albicans* strains are therefore probably due mainly to changes in expression levels of associated genes, and/or minimal modifications in their sequences, which may influence variations in the function of their encoded proteins (9). Determining if changes in genetic expression are a possible cause of the presence of different *C. albicans* strains in the oral cavity of HIV+ patients is beyond the scope of the present study and provides ample opportunity for future research. This will need to focus on identifying what factors are associated with changes in *C. albicans* phenotypes and biotypes, concentrating on these strains' intergenic regions and protein coding sequences (9).

Resistotype --C-- is reported to be the most common of the highly pathogenic *C. albicans* isolates (23). This does not coincide with the present results in which only two strains exhibited this resistotype; the rarity of --C-- resistotypes has been reported elsewhere (23). In a study of sixteen different resistograms from 198 oral isolates from 22 normal subjects (24), it was found that a particular strain tends to persist in the oral cavity of normal subjects although some changes can occur in these *C. albicans* biotypes. Differences in oral colonization by Candida species may be strongly influenced by ethnic, geographical and sociodemographic factors (19,25,26).

A broad range of fungi-typing techniques currently exist with variations in throughput, cost, processing time and discriminatory power. What method is chosen will depend largely on the epidemiological data needed and available laboratory infrastructure (10,12,27). Several

technologies are currently available for biotyping and genotyping of *C. albicans* strains, molecular methods being the most precise methods for yeast biotyping (9,28-30). However, molecular biology methods are more expensive than phenotypic methods and are often only available in research or epidemiological reference laboratories (19,30). Phenotyping methods such as auxonotyping, enzymotyping and resistotyping may be easy to run but do not have the discriminatory power of molecular methods (19,29,31). Recurrent *C. albicans* isolates have been shown to exhibit a high frequency of morphological variation that is not related to genomic DNA fingerprinting (32).

The resource limitations of routine laboratories can make genotyping unfeasible, and morphotyping tests more promising (18,19). However, discrimination between strains and results reproducibility require molecular typing methods to compare and confirm results. Most genotypic methods are expensive and involve highly specific infrastructure, as well as highly trained laboratory staff. This is a vital concern in developing and undeveloped countries, which have the highest HIV+/AIDS prevalence, since it prevents use of these highly specific techniques for routine strain identification. The resistotyping method used in the present study is an easy-to-run, comparatively inexpensive method applicable to all *C. albicans* strains. It is a near ideal system in developing countries for phenotypic and genotypic identification of *Candida* strains since it allows for testing a large number of isolates, provides rapid results, involves relatively inexpensive procedures, and can be automated.

The usefulness of resistotyping for phenotypic discrimination of *C. albicans* strains is well known (18,23,31), as are the problems of using phenotypic tests, such as lengthy time requirements, difficulty in automating and inaccurate strain identification (33). The resistotype technique has been successful in identifying different *C. albicans* strains from areas other than the oral cavity. It has also been used to identify four different *C. albicans* resistotypes (-B--F- was the most common) from the respiratory tract of TB-positive and TB-negative patients (34), and to identify *C. albicans* resistotypes from feces and vaginal mucus (A-C-F-, ABC-F- and A- --F- were the most frequent) (23). One promising approach for improving results accuracy is to use different analyses in conjunction (e.g. resistograms in addition to an API system, or Odds and Abbott's method) to increase discriminatory capacity beyond what each technique can offer individually (18,31).

CONCLUSIONS

In this study, we have demonstrated the differences between strains isolated from HIV+ and seronegative patients. Resistotyping is easy to perform in laboratories with lower economic resources, and useful for epidemiological purposes.

FOOTNOTE

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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