A molecular epidemiological study of prevalence of Candida spp. in women in the City of Tuxtla Gutierrez, Chiapas.


1 Facultad de Medicina Humana, Universidad Autónoma de Chiapas, México.
2 Cuerpo Académico de Investigación y Desarrollo Agroindustrial, Universidad Politécnica de Chiapas, México.
3 Departamento de Biotecnología y Bioingeniería, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. Unidad Zacatenco, México.

*To whom all correspondence should be sent.


ABSTRACT

Vaginitis caused by Candida spp., is a frequent fungal infection of epidemiological significance that affects particularly to women of childbearing age. Historically vaginitis has been associated with C. albicans; nevertheless an important increase in incidence of other species such as C. glabrata, C. parapsilosis and C. krusei has been recently reported. Our main objective was to characterize differentially through biochemical, microbiological and molecular assays four species of Candida in order to determine its prevalence in Tuxtla Gutiérrez region. Samples were obtained from vaginal exudates of 167 women of 15 to 45 years residing in Tuxtla Gutiérrez from August 2007 to October 2008. The species identification was performed through chromogenic medium and induction of fungal germ tube formation. The genus identification was done by PCR analysis using oligonucleotide primers (5′–AAGTATTTGGGAGAAGGGAAAGGG–3′ and 5′–AAAATGGGCATTAAGGAAAAGGC–3′), which were designed based on an intron of a ribosomal gene of Candida albicans. The prevalence of vaginal candidiasis in the women population studied was 21%. The isolated species were: C. glabrata (13%, n = 21), C. albicans (6%, n = 10), C. krusei (1%, n = 2) and C. parapsilosis (1%, n = 2). The symptoms associated with vaginal candidiasis are caused by four different species; thus, the identification of species in the diagnosis of candidiasis with antifungal susceptibility testing is crucial for effective treatment. The PCR amplification produced bands of 100–350 bp in different species of Candida. Changes in vaginal ecology caused by candidiasis were observed in 43% of clinical isolates. A written test was applied to the population under study to find out the relation between clinical, demographic and socioeconomic factors. According to the results, most of the candidiasis cases were found in asymptomatic women. There is a higher prevalence of C. glabrata (12.6%) than other species, and the disease incidence is associated to low-income women with active sexual life.

*Contact address: Peggy E. Alvarez-Gutiérrez. Cuerpo Académico de Investigación y Desarrollo Agroindustrial, Universidad Politécnica de Chiapas. Eduardo J Selvas S/N, Col. Magisterial, Tuxtla Gutiérrez, Chiapas, México. 29010. Phone & fax: +52 961 6120484, e-mail: peggy.alvarez@hotmail.com

Abbreviations: VC, Vaginal candidiasis; ENCB-IPN, Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional; YPD, yeast extract-peptone-dextrose; SSPP: Statistical Package for the Social Sciences; OR: odds ratio; UW: Unpaid works; OUr: other unpaid works; SAH: Systemic Arterial Hypertension; DM Diabetes mellitus; ID, immune disease; CVE, cervicovaginal erythema; AWP, Adherent withish plaques; STI, sexually-transmitted infections; S, sensibility; E, specificity; PV, predictive value; INT2, primer 5′-AAGTATTTGGGAGAAGGGAAAGGG-3′; INT2, primer 5′-AAAATGGGCATTAAGGAAAAGGC-3′; CaTX1 to CaTX35, vaginal exudates of 167 women who visited the cytology vaginal module at “Tuxtla” Health Center (Ministry of Health) in Tuxtla Gutiérrez, Chiapas, México.

Keywords: Candida spp., C. glabrata, PCR, sexually-transmitted diseases, Vaginal candidiasis.
INTRODUCTION

Vulvovaginitis is one of the major causes for gynecologic consultation in the world. Among them, vaginal candidiasis (VC) is considered as the leading cause of vulvovaginitis in Mexico and Central America (1). It is estimated that 75% of the women in their reproductive age have presented this infection, at least once in their lifetime, and 5% presented recurrence (2,3). In Mexico, in 2007, the Ministry of Health reported prevalence below 1% in women of reproductive age, but other authors have found increased prevalence of the disease (1,4). It is well known that Candida albicans is the main agent of vaginal fungal infections (5) and other gynecological disorders (6). Furthermore, less frequently there have been involved another Candida species known as “non-albicans”, like C. glabrata, C. tropicalis, C. parapsilosis and C. krusei, among others (7). Nevertheless, in recent decades non-albicans species had increased their frequency and gained greater clinical significance (5,7).

Since not all species of the genus Candida share the same antifungal susceptibility pattern, it is necessary to identify the genus and species and their associated clinical characteristics for epidemiological studies in order to provide reliable data to carry out preventive programs for disease control. (7) The objective of this study was to characterize differentially through biochemical, microbiological and molecular assays four species of Candida in order to determine its prevalence in Tuxtla Gutiérrez region. A pair of oligonucleotide primers (INT1 and INT2) were used in this study, because the sequence from which they were designed has a high homology among Candida species.(8).

MATERIAL AND METHODS

Patients and methods

Women who participated in this study were selected according to the following criteria: (1) age between 15 to 45 years old; (2) sexually active women (3) residence in Tuxtla Gutiérrez, Chiapas (4) a 72h period of sexual abstinence (5) informed written acceptance. Women were excluded when using antimicrobials and antifungals in past three weeks prior to the date of sample collection, or showing menstrual and/or abnormal uterine bleeding. All women were informed about the study, agreed to answered a questionnaire and gave the consent to all clinical procedures. The questionnaire collected data on demography (age, place of residence, occupation, level of education, family income), sexual risk behavior (pregnancy, number of sexual partners ever, sexual activity), potential risk factors associated to candidiasis (drug use, history of sexual transmitted infections, associated diseases) and the clinical details relating to infection recorded by the attending physicians (signs and symptoms of candidiasis, characteristic of vaginal exudates, vaginal abnormalities, speculoscope observations).

Clinical isolates (CaTX1 to CaTX35) were obtained from vaginal exudates of 167 women who visited the Cytology Vaginal Module at “Tuxtla” Health Center (Ministry of Health) in Tuxtla Gutiérrez, Chiapas, México, from August 2007 to October 2008. C. albicans (CAL1), C. parapsilosis (CPA4), C. tropicalis (CTR34) strains were kindly provided by Dr. César Hernandez from ENCB-IPN, México.

Sample collection

Vaginal exudates were collected from secretions of the bottom of the sac and vaginal walls with sterile swabs and inoculated on YPD medium with 0.05% chloramphenicol. Vaginal pH and Gram staining were also registered.

Biochemical and microbiological assays

Biochemical identification of all isolates was performance with Whiff (10% KOH) and urine test (9). Isolates were considered presumptive for Candida spp according to Amsbe criteria (14). In addition, isolates were subcultured in rich and chromogenic medium for further identification according to their morphology on solid medium. Candida species were identified through colonial growth on YPD medium and CHROMagar Candida® (11,12). Isolates of Candida spp were identified as positive of Candida when colonies grown on solid YPD medium presented yeast-like morphology and Gram positive stain. Candida species were defined as albicans, krusei, glabrata or parapsilosis using chromogenic media CHROMagar Candida® (CHROMagar Microbiology, France) and CandiSelect® (Bio-Rad) according to morphology, as described in the manufacturer’s instructions. Germ tube formation of isolates cultured on 0.5ML of human serum, and incubated at 37 °C for 2-3 h, was also determined.

DNA extraction and amplification

Total DNA was extracted with Aqua Pure® extraction kit (Bio-Rad). Polimerase Chain Reaction was done according to Sambrook et al. (13). INT1 and INT2 primers were designed by Baquero et al. (8) from a intron sequence of a ribosomal gene of Candida albicans (CyST1). The reaction mixture was as follows: 500 ng of DNA, 1U Taq Polimerase (TaqKara Bio USA ®), 0.3 mM of INT1 primer (5′-AAGTTTGGGAAGGAAAGGC-3′), 0.3 mM of INT2 primer (5′-AAAATGCGTTAAGGAAAGGC-3′), 0.2 mM
dNTP’s Mix (Promega®), 2 μL of 10X EX buffer extraction. DNA was amplified in a PCR thermal cycler (Cycler Bio-Rad®), by using 1 cycle at 95 °C 3 min, and then 40 cycles as follows: 60 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 45 s of primer extension at 72 °C. At the final cycle, an additional 5 min of incubation at 72 °C was carried out ensure complete polymerization of any remaining PCR products.

Data processing, analysis and results
Data were analyzed using SPSS version 15. Frequency distribution of demographic data, characteristics of the population, sexual history and clinical manifestations were analyzed. The relationship between selected risk factors and the prevalence of candidiasis was determined by using unadjusted Odds Ratios (OR) and Chi square tests. In this study, a transversal, analytical and experimental design with a confidence level of 95% and 80% power was used. To determine the disease prevalence, we used the operational definition of positivity for Candida spp.

Ethical considerations
Ethical approval for the study was obtained from the Ethics Committee, Faculty of Medicine, Autonomy University of Chiapas. Informed verbal consent was obtained from all participants, after explaining them the purpose of the study. Patient identity was kept confidential.

RESULTS

Epidemiological analysis
Women’s profiles were consistent in this study. According to our results, one half were mature young women among 35 and 45 years old (48%; 80 cases). As shown in Table I, they lived in urban areas (56%; 94 cases) without any remuneration to their job (76%; 126 cases); thus, almost all have a low socioeconomic status (87%; 145 cases). These women were sexually active (99%; 166 cases) within a low risk profile, because most of them had one sexual partner during their lifetime (69%; 115 cases). Among them, only 14% (23 cases) were pregnant. In general, they were healthy women: a minority of them presented a history of sexually-transmitted infections (4%; 6 cases), had a common Candida spp. associated disease, were on treatments for systematic hypertension (4%; 7 cases), diabetes mellitus (2%; 4 cases), or other chronic diseases (13%; 22 cases), or reported used drugs like hormonal contraceptives (12%; 20 cases).

These women showed vaginal complains as leucorrhea (34%; 57 cases), pruritus (14%; 23 cases), heat (5%; 8 cases) and dyspareunia in (3%; 6 cases). Remarkably, many of them declared to be asymptomatic (44%; 73), but vaginal speculoscopry showed the opposite. An 88% (147 cases) of them had some kind of vaginal discharge, only 8% (13 cases) presented the “characteristic” vaginal exudates (kind off-white,
<table>
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<th>Study Variables</th>
<th>Studies women (n=167)</th>
<th>Candidiasis (n=35)</th>
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<td><strong>Age ranges</strong></td>
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<td>35-45</td>
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<td>(OR, 0.9; IC 95% 0.4-1.9; (p=0.9)</td>
<td>(OR, 0.7; IC 95% 0.4-6.4; (p=0.7)</td>
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UW= Unpaid works, OUW= Other UW, SAH= Systemic Arterial Hypertension, DM= Diabetes mellitus, ID Immune disease, CVE= Cervicovaginal erythema, AWP= Adherent whitish plaques, STI= Sexual Transmitted Infections
semisolid and lumpy of "Cottage cheese" appearance; refs. 1, 5) and most of them presented “non characteristic” leucorrhrea (80%; 134 cases). Additionally, erythema was present in 85% of cases at the cervix (50%; 84 cases), vaginal mucosa (17%; 28 cases), or both areas (16%; 26 cases). Only 2% (3 cases) presented adherent whitish macroscopic plaques, in the vaginal epithelium (Table 1).

**Biochemical and microbiological tests**

The cellular morphology of the 167 exudates showed Gram staining positive cells (27%; 46 cases) and yeast like cells (19%; 32 cases), and clue cells in the stained frots (13%; 22 cases; Fig. 1). Amines test and pH greater than 4.5 were positive in 43% (72 cases) of the exudates. Only one exudate was positive to urease test (3%). Biochemical analysis and the related optical microscopy showed neither the sensitivity, nor the specificity to discriminate the clinical relevance (Table 2). All 167 vaginal exudates were grown on YPD solid medium and subjected to biochemical and microbiological tests. Thirty five isolates were positive to *Candida* spp. with characteristics according to Ausubel et al. (10). Positive isolates had three different *Candida* spp. presumptive colonial morphologies, as described in Table 3.

Chromogenic media culture allowed *Candida* species identification, through differential features on solid media. *C. glabrata* (CaTX1, CaTX4, CaTX5, CaTX7, CaTX8, CaTX9, CaTX12, CaTX13, CaTX14, CaTX15, CaTX16, CaTX18, CaTX20, CaTX23, CaTX26, CaTX27, CaTX28, CaTX31, CaTX32, CaTX34 and CaTX35), *C. albicans* (CaTX3, CaTX10, CaTX17, CaTX19, CaTX21, CaTX22, CaTX24, CaTX25, CaTX29 and CaTX30), and *C. krusei* (CaTX6, CaTX11) were identified with CandiSelect® and CHOROMagar®. The last two isolates were identified as *C. parapsilosis* (CaTX2 and CaTX33) in CHOROMagar®. These results showed that *Candida glabrata* was the primary causative agent of vaginal candidiasis in low income women of Tuxtla Gutiérrez, Chiapas.

**Molecular assays**

Primers INT1 and INT2 deduced form CaYSTI intron sequence produced a 310 pb amplicon from different *C.albicans* laboratory strains (8). PCR amplification seven isolates previously identified as *C. albicans* (CaTX10, CaTX17, CaTX19, CaTX22, CaTX24, CaTX29 and CaTX30) and reference strain CAL1 generated a 310 bp amplicon (Figure 2A). Meanwhile, PCR amplification of *C. glabrata* strains generated a polymorphic pattern. CaTX7, CaTX14, CaTX15, CaTX16, CaTX18, CaTX20, CaTX26, and CaTX27 generated a 350 bp and 100 pb bands. CaTX1, CaTX23, CaTX28, and CaTX32 generate a single 350 bp band and CaTX4, CaTX5, CaTX13, CaTX31, CaTX34, and CaTX35 amplified a single 100 bp band (Fig. 2B). In the case of PCR amplification of CaTX11 (C. *krusei*) and CaTX33 (C. *parasilosis*) both strains generated a 310 bp single (Figure 2C).

**Epidemiological study**

Vaginal candidiasis (VC) was identified in 35 women (21%, n = 167) who had a mean age of 30 ± 8.7 years old, range 16-45 years old, and a coefficient of variation of 29%. The highest proportion (40%, 14 cases) of them were located in the range of 35-45 years old. It was found that VC was prevalent among...
women living in urban areas, with unpaid work and a low socioeconomic status (Table I). Regarding their sexual behavior, all of them were sexually active, 80% (28 cases) had one sexual partner and 23% (8 cases) were pregnant. Diabetes mellitus was prevalent in 6% (2 cases) of women with VC, and 17% (6 cases) of them consumed contraceptives. The main causes of vaginal discomfort were leucorrhoea 34% (12 cases) and vaginal pruritus 17% (6 cases). The 46% (16 cases) did not mention any discomfort. However, the vaginal speculoscopv showed that 83% (29 cases) of them had some kind of vaginal discharge, manifesting the non characteristic leucorrhoea in 66% (23 cases). Likewise, in 84% (29 cases) there was local erythema.

The analysis of the data was obtained with conventional studies in exudates, which were positive for C. albicans, (77%; 27 cases) had pH values above 4.5, (28%; 10 cases) resulted in a positive amine test, and 3% (1 case) of the samples were uracase positive. Only in 46% (16 cases) of the samples which were positive for Candida spp., yeast cells were observed by direct microscopy, and when stained with the Gram technique, (77%; 27 cases) were identified as Gram positive. The latter technique allowed the identification of key cells in 11% (4 cases) of the samples which were positive for Candida spp. The 26% (9 cases) of primary isolates corresponding to 90% (9 cases) of those determined as C. albicans by biochemical tests, produced germinative tubes (See table 2).
DISCUSSION

 Mostly, VC was detected in women who had some of the following factors: age 35-45 years, low socioeconomic status, with use of hormonal contraceptives, diabetes mellitus and pregnancy status. However, an alpha level of significance (X^2 0.05) showed that there was not statistical significance to assume these clinical traits had a relationship with the presence of candidiasis. Moreover, the results of this study suggest that to succeed in the infection, the fungi might not require these conditions a priori, because candidiasis was found in some women without the above characteristics, even though other authors have documented a close relationship with these factors (1, 6).

The presence of significant alterations in the vaginal ecosystem of women was notable in this study because there is a 43% with a positive amine test and vaginal pH values above 4.5, which according to Casanova et al. (1) are indicative of replacement of the normal vaginal microbiota by anaerobic bacteria. Imbalance of few bacteria such as Döderlein bacillus might alter vaginal ecosystem. In addition, 11% (4 cases) of women with VC meet 3 or more criteria for the diagnosis of bacterial vaginosis. According to Amsel (14) bacterial vaginosis showed pH> 4.5, positive amine, the presence of cell contacts, homogeneous vaginal discharge, which indicate the presence of mixed infections, as described by Medina et al. (15) and Bucemi et al. (3). These changes in the vaginal microenvironment allow for the establishment of VC, because there occur also biochemical, microbiological and physiological changes such as increased epithelial glycogen, alkaline pH, decreasing of the bacilli flora and overcrowding anaerobic flora (16). As shown in Table 2, the statistical indicators used to evaluate the efficiency inherent to conventional diagnostic tests were rather sensitive, and specific for establishing the diagnosis of VC, as mentioned in Baquero et al. (8), at least for the germ tube test, which is excellent for discriminating C. albicans from other non-albicans species (E = 100%). Therefore, a negative value gives us 83% confidence that the species analyzed could be a non-albicans case, and a high probability (VP+ = 100%) that a result with positive value could be infected by C. albicans. However, the evidence is inadequate to confirm the non-albicans species (S = 26%).

In agreement with the results obtained in this work, presence of clinical symptoms such as vaginal discharge, vaginal pruritus and heat, not necessarily determine the VC nor the high rate of infection candidiasis (48%) in asymptomatic women (p> 0.05). Medina et al. (3) had studied the same behavior in women with these symptoms (including whitish vaginal fluid) could have other types of vaginitis like in the bacterial type (15). Histological changes associated with VC in this study are related to inflammatory processes in the cervicovaginal epithelium that may be correlated to the adhesion and invasiveness capability of C. albicans and C. glabrata, as described in Castaño et al. (17). Whitish plaques adherent to the vaginal epithelium in 1.8% (3) of the women studied were not statistically significant (p> 0.05) although Torres et al. (18) related them as a suggestive indication of infection with Candida spp.

Species identified in this study were: C. glabrata (12.6%), C. albicans (6.0%), C. krusei (1.2%) and C. parapsilosis (1.2%). The species most frequently found was C. glabrata, as reports by Iglesias et al. (21), Paul et al. (22). But these results differ from those obtained in other studies describing C. albicans species as the principal organism in infection statistics (19, 20). This transition may suggest that C. glabrata incidence can be associated with the use of antifungal azoles for topical use, empirical and indiscriminate chemotherapy treatment or increased use of flucanazole as prophylaxis for enteric route, which could probably determine the presence of recurrent vaginitis (17, 23). VC caused by C. glabrata examined in this study is consistent with the low percentage of white plaques adherent to the vaginal mucosa, since this species lacks properties of filamentation. This observation led us to consider C. glabrata as a fungus of low virulence. However, its production of proteases encoded by a family of subtelomeric genes and hydrophobicity of the cell surface, which confers adherence property on it, in comparison to C. albicans, and its association with high mortality and the increasing reported cases, allowed us to propose C. glabrata as an emerging pathogen (22, 24). Although C. glabrata has higher phylogenetic relationship with Saccharomyces cerevisiae rather than C. albicans, it shares some features of virulence with the latter. Both fungi have high capacity of adhesion to epithelial cells and the ability to form biofilms that allow them to adapt to different conditions in the host. Besides C. glabrata has the ability to develop higher virulence by mutation of subtelomeric genes, homologous to the gene EPA1 (17). Indeed, it can also undergo morphogenetic changes as adaptation to the environment where it grows, creating variations in the colony appearance when growing in culture media supplemented with copper sulfate (25). This behavior could arise in other culture media, as was observed in all three types of colonies obtained in this study. Evidences obtained in this study demonstrated that chromogenic media permits the adequate discrimination between Candida species, results consistent with those described by Houang and Yücesoy et al. (12). But they can not be conclusive.

Molecular assays offered a particular trend in the population studied. Amplification products were obtained from the isolates of C. glabrata (amplicons of 100 and 350 bp), C. albicans, C. krusei and C. parapsilosis (310 bp amplicons). These results differed from the findings obtained by Baquero et al. (8), who obtained amplifications only in C. albicans (310 bp), C. pseudotropicalis (1,200 bp), Kluyveromyces marxianus (1,250 bp) and C. neoformans (1,200 bp). These results are consistent
with the hypothesis that there is a significant degree of genetic polymorphism in the intron sequence CaYST1 findings of Baquero et al. (8); however, they suggest that these genetic variability results in inconsistent conservation of species in the genus Candida. It is prudent to mention that our population of study is part of a genetic mix of various ethnic groups that could influence the molecular findings of this study. However, it should be noted that in her study, Baquero et al. (8), presented evidences of alternative tests for confirmatory identification of the non-albicans species they used.

Prevalence of Candida spp. in the analyzed population was 21.0%, which agrees with the results obtained by Flores et al. (26) and Reyna et al. (27). Although it was higher when compared to the one observed by Jiménez et al. (28) in a population of women with cervical dysplasia in Tuxtla Gutiérrez (15%); who used the germ tube test as the sole study of discrimination of the species albicans from non-albicans. However, it is necessary to take into account that this test does not identify the 5-10% of C. albicans not forming germinative tubes. Moreover, the prevalence obtained in this study was significantly (p > 0.05) higher than the prevalence reported in 2007, in Mexico (1%).

Since inclusion of VC condition as part of an epidemiological report is not strictly mandatory, it is possible that the records in the national system of Public Health Institutions contain an underrepresentation of new cases of diseases, leading to a dangerous inaccuracy in the statistical evaluations. The situation is more critical because private medical clinics do not report accurately to the health authorities the morbidity of the population they serve.

**CONCLUSIONS**

The identification of Candida species was done successfully through the isolation of fungi by conventional culture and biochemical tests. Confirmation of the genus was done by PCR analysis using primers INT1 and INT2 and classification of species using chromogenic media. The results of this study allow us to conclude that the prevalence of VC was 21.0%. The isolated species were C. glabrata, C. albicans, C. krusei and C. parapsilosis. The most frequent species isolated as a causative agent of vaginal infections corresponded to C. glabrata. The high frequency of alterations in the vaginal ecosystem of the women studied was risk of vaginitis. Determination of prevalence levels in women relying on common vaginal candidiasis symptoms lacks of reliability because, as demonstrated in this study, a lot of women also have an asymptomatic infection.

Vaginal candidasis is one of the most common infections in women. In Tuxtla Gutiérrez one in five women suffers this infection even when they are asymptomatic. However, in our days public and private medical services lack of accurate clinical, biochemical, microbiological and molecular of tools for diagnostic, that allows an opportune and appropriate treatment in order to improve women health. It is imperative to generate scientific information and combine it with responsible health public policies to improve the quality of life of women in the region of Tuxtla Gutiérrez.

**REFERENCES**


