

## CLINICAL STUDY

# Detection of *Candida* species in vaginal samples in a clinical laboratory setting

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### Abstract

**Objective.** To present the detection rates of *Candida* species in vaginal samples from patients visiting physicians.

**Methods.** The presence of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* in 3978 vaginal swabs from patients in six US states was detected by PCR amplification.

**Results.** *Candida* DNA was detected in 33.1% of the population studied. Of the 1316 positive samples, 80.2% contained *C. albicans*, 14.3% contained *C. glabrata*, 5.9% contained *C. parapsilosis* and 8.0% contained *C. tropicalis*. Comparing samples by patients' state of residence revealed an association with the detection of *C. glabrata* ( $p = 0.029$ ). Comparing samples by patients' age revealed a decrease in the overall detection of *Candida* ( $p < 0.001$ ) and *C. albicans* ( $p < 0.001$ ), concomitant with an increase in the detection of *C. glabrata* ( $p < 0.001$ ) and *C. parapsilosis* ( $p = 0.025$ ).

**Conclusions.** These results provide geographic- and age-specific data on four *Candida* species associated with vaginitis.

**Keywords:** *Candida*, DNA, age, detection rates

### Introduction

*Candida* species cause 20% to 25% of the cases of infectious vaginitis, second only to the 40% to 50% of cases caused by bacteria [1]. Candidal vaginitis (CV) is marked by pruritis, soreness, a change in discharge, dyspareunia, vulvar erythema, edema and fissures [1, 2]. The condition is rare before puberty but by the age of 25 years, nearly 50% of all women will have had at least one clinician-diagnosed episode of CV [3,4]. The condition is less common in post-menopausal women. Overall, it is estimated that 75% of all women will experience an episode of CV in their lifetime [1, 5].

*Candida albicans* accounts for 80% to 92% of all cases of CV worldwide [1, 6]. Vaginal colonization by non-*albicans* species is more common in immunodeficient women. However, an increased frequency of non-*albicans Candida* species, particularly *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, has

been reported in healthy women [7]. The widespread use ofazole antifungal drugs is postulated to have promoted the shifting of vaginal colonization and selection of more naturally resistant species, such as *C. glabrata* [8–11].

We investigated the detection rate of four different *Candida* species isolated from women in Florida (FL), Georgia (GA), Louisiana (LA), New Jersey (NJ), Pennsylvania (PA) and Texas (TX). The use of rapid PCR-based assays for the specific detection of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* allowed large-scale surveillance of the results obtained from these vaginal samples. The established PCR methods have high sensitivity and specificity and have a shorter turn-around time in comparison with current microscopy and culture techniques[12]. This study, using the PCR method, will provide detection data to help guide clinical evaluation and therapy.

## Materials and methods

### *Clinical samples*

Between April and November 2003, vaginal swabs from a total of 3978 women from FL, GA, LA, NJ, PA, and TX were submitted to our laboratory for *Candida* PCR testing. Tests were performed with informed consent as ordered by the evaluating physician following the guidelines of the laboratory's federal, state, and Clinical Laboratory Improvement Amendments (CLIA) certifications. Patient anonymity was strictly protected in accordance with the federal Health Insurance Portability and Accountability Act (HIPAA) of 1996. Subjects were informed of the results of the testing procedures. None of the samples was accompanied by information regarding the medical history or clinical presentation of the participant. Only the woman's state of residence and age were obtained for the purposes of this retrospective study. The specimens were collected from a vaginal sampling performed by the evaluating physician with a Cellmatics swab (BD, Sparks, MD, USA), which was then placed in 2 ml of transport medium (BD, Sparks, MD, USA). Upon receipt, swabs were immediately processed for PCR analysis.

### *DNA extraction*

Established procedures for DNA extraction were used. In brief, swabs were thoroughly mixed in the transport media contained within the transport vials; 470  $\mu$ l of transport media was mixed with 25  $\mu$ l of 10% sodium dodecyl sulfate and 12  $\mu$ l of freshly prepared DNase-free proteinase-K (10 mg/ml), then incubated for 2 h at 55°C. DNA was extracted in phenol:chloroform:isoamyl alcohol and recovered by ethanol precipitation, pelleted, dried in a speed vacuum, and re-suspended in 20  $\mu$ l TE buffer. Absorbance was monitored by 260/280 readings with a spectrophotometer, and the DNA concentration was adjusted to 0.2  $\mu$ g/ $\mu$ l before PCR analysis.

### *PCR analysis*

The oligonucleotide primers used for species-specific amplification of *Candida* ribosomal DNA and reaction conditions were previously described [12]. Primers were synthesized by Invitrogen (Carlsbad, California) and purified by reverse-phase cartridge chromatography. All PCR reactions were carried out in 0.2-ml tubes using a T3 Thermocycler (Biometra, Göttingen, Germany) with 1  $\mu$ g of extracted DNA in 50  $\mu$ l total volume. Thermocycling was performed as follows: initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C to 62°C for 30 s, and 72°C for 30 s, with a final elongation at 72°C for 10 min. PCR products were

analyzed by electrophoresis through a 2% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide and UV visualization using a MultiGenius gel documentation and analysis system (Syngene, Frederick, MD, USA). Positive controls were included in each PCR experiment and consisted of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* (ATCC 14053, 2001, 22019 and 13803, respectively). Negative controls in each experiment consisted of the substitution of nuclease and pyrogen-free water for DNA; 100% specificity and 100% sensitivity of these PCR amplifications were previously determined [12]. Additionally, PCR amplifications with each primer pair exhibited no cross-reactivity among the four *Candida* species or a panel of genomic DNA extracted from 35 known bacterial, fungal, and viral pathogens (data not shown).

### *Protection against contamination*

DNA extraction and PCR were performed under sterile conditions and in separate rooms. Water for all experiments was nuclease- and pyrogen-free. Microcentrifuge tubes were sterilized in an autoclave and UV-irradiated. Nuclease-free PCR tubes (Costar, Corning, NY, USA) were UV-irradiated. Pipettes were used solely with filter tips for PCR. PCR reactions were prepared in a UV-irradiated biological safety cabinet (Labconco, Kansas City, MS, USA).

### *Statistical analysis*

Chi-square and chi-square for trend tests were used for comparisons as noted; northeastern and southern geographic regions were determined by 2000 US census bureau mapping. Unpaired *t* tests compared means and Spearman *r* tests determined correlations as noted. A two-tailed *p* value of less than 0.050 was considered to be significant. All tests were performed using GraphPad InStat version 3.0b for Macintosh (GraphPad Software, San Diego, CA, USA).

## Results

### *Characteristics of the study population*

A total of 3978 samples originating from women residing in FL, GA, LA, NJ, PA and TX were included in the present study (distribution shown in Table I). These states represent those from which evaluating physicians routinely send vaginal swabs to our laboratory for *Candida* PCR testing. Participants' ages at the time of sample collection ranged from 12 to 97 years. The mean age was  $34.5 \pm 13.0$  years. Data were divided into five age groups, containing women of ages 12 to 25 years, 26 to 35 years, 36 to

45 years, 46 to 55 years and 55 to 97 years (distribution shown in Table II).

**Geographic differences in detection rate of *Candida***

Table I summarizes the results obtained by the *Candida* PCR test categorized by the subjects' state and region of residence. Chi-square analysis revealed no significant association of the overall positivity rate (determined by detection of any of the four *Candida* species) or detection rates (determined out of all samples in a group) of *C. albicans*, *C. parapsilosis*, *C. tropicalis* and multiple colonizing *Candida* species with the state of residence. However, the detection of *C. glabrata* was determined to be significantly associated with the state of residence, with the lowest detection rate in PA (2.5%) and the highest in TX (5.7%). Grouping the states into two regions, northeast (NJ and PA) and south (FL, GA, LA, and TX), revealed a significant association of the detection rate of *C. glabrata* and the region of residence ( $p=0.032$ ). The detection rate was 3.1% (95% CI, 2.0% to 4.7%) in the northeast and 5.1% (95% CI, 2.5% to 5.9%) in the south, with a difference of  $2.0 \pm 0.87\%$ .

**Age differences in detection rate of *Candida***

Table II summarizes the results obtained by the *Candida* PCR test categorized by participants' age. Chi-square analysis revealed no significant association of the detection rates of *C. tropicalis* and multiple colonizing *Candida* species with age. However, the overall positivity rate and the detection of *C. albicans*, *C. glabrata* and *C. parapsilosis* were determined to be significantly associated with the subject age groups: as age increased, the overall positivity rate and the detection rate of *C. albicans* gradually decreased concomitant with an increase in the detection rate of *C. glabrata* and *C. parapsilosis*. Each of these changes was determined to have a significant linear trend among the sequential age groups by chi-squared tests for trend.

In that the increase in the detection rate of *C. glabrata* was associated with increase in age, the geographical differences in the detection rate of *C. glabrata* might reflect the age distribution in each state. In Table III, the mean age and standard deviation for each state and for the northeast and south regions are shown. A Spearman  $r$  test showed no significant correlation between the mean age and the detection rate of *C. glabrata* in each state

Table I Distribution of samples and detection rate of *Candida* species by subjects' state of residence.

Region	State	Samples	Positive (% <sup>a</sup> )	<i>C.alb.</i> (% <sup>a</sup> ;% <sup>b</sup> )	<i>C.glab.</i> (% <sup>a</sup> ;% <sup>b</sup> )	<i>C.para.</i> (% <sup>a</sup> ;% <sup>b</sup> )	<i>C.trop.</i> (% <sup>a</sup> ;% <sup>b</sup> )	Multiple (% <sup>a</sup> ;% <sup>b</sup> )
Northeast	NJ	570	194 (34.0)	163 (28.6;84.0)	19 (3.3;9.8)	13 (2.3;6.7)	10 (1.8;5.2)	10 (1.8;5.2)
	PA	163	49 (30.1)	41 (25.2;83.7)	4 (2.5;8.2)	3 (1.8;6.1)	5 (3.1;10.2)	4 (2.5;8.2)
South	FL	1286	419 (32.6)	323 (25.1;77.1)	69 (5.4;16.5)	30 (2.3;7.2)	36 (2.8;8.6)	34 (2.6;8.1)
	GA	362	117 (32.3)	100 (27.6;85.5)	11 (3.0;9.4)	6 (1.7;5.1)	8 (2.2;6.8)	8 (2.2;6.8)
	LA	189	51 (27.0)	40 (21.2;78.4)	5 (2.7;9.8)	3 (1.6;5.9)	5 (2.7;9.8)	2 (1.1;3.9)
	TX	1408	486 (34.5)	389 (27.6;80.0)	80 (5.7;16.5)	22 (1.6;4.5)	41 (2.9;8.4)	45 (3.2;9.3)
Total		3978	1316 (33.1)	1056 (26.6;80.2)	188 (4.7;14.3)	77 (1.9;5.9)	105 (2.6;8.0)	103 (2.6;7.8)
$p$ Value <sup>c</sup>			0.344	0.252	0.029	0.741	0.754	0.345

For each individual species, samples with multiple species are included. <sup>a</sup>Percentage of the number of samples from each row category; <sup>b</sup>percentage of the number of positives from each row category; <sup>c</sup>the two-tailed  $p$  value is calculated from chi-square testing of column data, excluding the total.

Table II Distribution of samples and detection of *Candida* species by subjects' age in years.

Group	Age	Samples	Positive (% <sup>a</sup> )	<i>C.alb.</i> (% <sup>a</sup> ;% <sup>b</sup> )	<i>C.glab.</i> (% <sup>a</sup> ;% <sup>b</sup> )	<i>C.para.</i> (% <sup>a</sup> ;% <sup>b</sup> )	<i>C.trop.</i> (% <sup>a</sup> ;% <sup>b</sup> )	Multiple (% <sup>a</sup> ;% <sup>b</sup> )
I	12-25	1116	417 (37.4)	349 (31.3;83.7)	44 (3.9;10.6)	20 (1.8;4.8)	35 (3.1;8.4)	30 (2.7;7.2)
II	26-35	1311	441 (33.6)	378 (28.8;85.7)	41 (3.1;9.3)	20 (1.5;4.5)	31 (2.4;7.0)	28 (2.1;6.4)
III	36-45	847	264 (31.2)	205 (24.2;77.7)	51 (6.0;19.3)	16 (1.9;6.1)	19 (2.2;7.2)	24 (2.8;9.1)
IV	46-55	413	119 (28.8)	80 (19.4;67.2)	31 (7.5;26.1)	8 (1.9;6.7)	8 (1.9;6.7)	8 (1.9;6.7)
V	56-97	291	75 (25.8)	44 (15.1;58.7)	21 (7.2;28.0)	13 (4.5;17.3)	12 (4.1; 16.0)	13 (4.5; 17.3)
$p$ Value <sup>c</sup>			< 0.001	< 0.001	< 0.001	0.025	0.262	0.193
$p$ Value <sup>d</sup>			< 0.001	< 0.001	< 0.001	0.025	0.886	0.319

For each individual species, samples with multiple species are included. <sup>a</sup>Percentage of the number of samples from each row category; <sup>b</sup>percentage of the number of positives from each row category; <sup>c</sup>the two-tailed  $p$  value is calculated from chi-square testing of column data; <sup>d</sup>the two-tailed  $p$  value is calculated from chi-square for trend test of column data.

Table III. Mean age in years of study population by state and region

State/region	Mean age $\pm$ SD
NJ	35.2 $\pm$ 12.2
PA	38.5 $\pm$ 13.3
FL	35.6 $\pm$ 14.2
GA	33.1 $\pm$ 11.0
LA	31.3 $\pm$ 13.0
TX	33.2 $\pm$ 12.4
Northeast	36.0 $\pm$ 12.5
South	34.0 $\pm$ 12.5
Total	34.4 $\pm$ 13.0

( $p = 0.497$ ). However LA, which had the lowest average age, also had the lowest detection rate of *C. glabrata*. When samples were grouped by region, an unpaired  $t$  test showed the average subject age was significantly greater in the northeast ( $p < 0.001$ , 95% CI 1.0 to 3.0 years). Therefore, the geographical differences in the detection rate of *C. glabrata* were probably not due to biases in the age distribution of the study populations.

## Discussion

In this retrospective study, we analyzed PCR-based tests performed at our facility to estimate the detection rate of *Candida* species present in vaginal samples from patients of diverse geographic areas and various ages within the USA. The analysis benefits from the large-scale surveillance of samples by sensitive and specific PCR methods. However, it is recognized that the data do not represent a random sampling within these states and age groups, but rather a more specific population of women visiting submitting physicians for aid in diagnosis of abnormal conditions.

Although there was no significant association between the subjects' region of residence and the overall positivity rate for *Candida*, there was a significant association with the detection of *C. glabrata*. The 1.6-fold increase in the detection of *C. glabrata* in the south region suggested that regional differences in study population might favor colonization or infection by this particular species. Since age distribution in each state was probably not an attributing factor, lifestyle, behavior, and environmental conditions might be associated with the differences in the detection rate of *C. glabrata*. However, further investigation is necessary to directly address this observation and discriminate unanticipated subject selection biases.

Our results analyzing the detection of *Candida* in vaginal swabs by patients' age were in agreement with previous reports of clinician diagnosis of CV [1, 3–5]. We showed that women younger than 26 years

of age (Table 2, Group I) had the highest rate of detectable *Candida*, and that this rate was lowest after the age of 56 years (Table 2, Group V). In addition, our data identifying the *Candida* species revealed interesting trends associated with participants' age. As the overall detection of *Candida* decreased with age, changes in the distribution of individual species occurred. Although the dominant *Candida* species found in vaginal swabs remained *C. albicans*, the detection rate of *C. glabrata* increased 2.1-fold in the age group 36 to 45 years (Table 2, Group III) and continued to increase in the age groups 46 to 55 and 56 to 97 years (Table 2, Groups IV and V). Also, the rate of *C. parapsilosis* increased 2.6-fold in the age group 56 to 97 years (Table 2, Group V). It could be postulated that these changes in distribution were related to selection of resistant species by widespread use of azole-based anti-fungal drugs (particularly in the case of *C. glabrata*) [8, 9, 13], hormonal changes due to menopause and estrogen treatments [14, 15], the effects of aging on protective immune responses [16, 17] and prolonged stays in hospital [11]. These factors might change the vaginal environment and the characteristics of vaginal flora to favor the growth and virulence of non-*albicans* species, or might increase subject exposure to non-*albicans* species.

Finally, of interest is the overall detection rate of non-*albicans* species, particularly *C. glabrata*, which is more naturally resistant to common azole-based antifungal drugs than *C. albicans* [13, 18]. One recent worldwide study comparing the susceptibilities of *Candida* species to fluconazole indicated that, in 2001, 0.6% of *C. albicans* isolates from genitalia were resistant, compared with 30.7% of *C. glabrata* isolates [19]. Considering that our results showed a 9.3% non-*albicans* species detection rate and a 4.7% *C. glabrata* detection rate, current routine diagnostic tests that only identify *C. albicans* might be inadequate. It is important to include non-*albicans* species in testing vaginal samples to accurately diagnose abnormal conditions, as well as assist physicians in choosing drug treatment for CV and to monitor the trends of resistance.

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