Trends in frequency and *in vitro* antifungal susceptibility patterns of *Candida* isolates from women attending the STD outpatients clinic of a tertiary care hospital in Northern Italy during the years 2002-2007

Sara Asticcioli[1], Laura Sacco[2], Rossana Daturi[2], Cecilia Matti[2], Elisabetta Nucleo[1], Francesca Zara[1], Laura Pagani[1]

[1]Department of Morphological, Eidological and Clinical Sciences, Section of Microbiology, University of Pavia, Italy; [2]Laboratory of Microbiology, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy

SUMMARY

Vulvovaginal candidiasis is a common mucosal infection caused by saprophytic and opportunistic yeasts belonging to the *Candida* genus. 518 vaginal swabs, with positive fungal culture were collected from unselected women attending the Sexually Transmitted Disease clinic of an Italian tertiary care hospital over a six year period to determine the pathogen prevalence in vulvovaginal candidiasis and to evaluate *in vitro* the antifungal susceptibilities of yeast recovered by Sensititre YeastOne antifungal panel plates according to CLSI document M27-A2.
The isolates belonging to the genus *Candida* were 495 (95.5%) with *Candida albicans* percentage equal to 61.2%. Voriconazole was highly active (MIC_{50} 0.008; MIC_{90} 0.5 µg/ml), regardless of the species tested. On the contrary, fluconazole susceptibility was based upon the species. The intrinsic resistance to fluconazole of *C. krusei* was confirmed.

KEY WORDS: Candida, Vulvovaginal candidiasis, Antifungal susceptibility

Received September 23, 2008 *Accepted* January 28, 2009

Vulvovaginal candidiasis (VVC) is a common problem, causing significant morbidity and affecting women's wellbeing. *Candida* is a saprophytic opportunistic microrganism and condition resulting in a decrease in vaginal pH or alteration of the local defense mechanisms favour the appearance of Candida vaginitis (Candido R.C. *et al.*, 1999). This generally occurs when an imbalance of the normal vaginal flora appears or when pathogens are introduced into the vagina. Proposed risk factors for VVC include pregnancy, diabetes mellitus, contraceptives, antibiotics, tight-fitting clothing, synthetic underwear, various dietary excesses or deficiencies, sexual activity, and the use of feminine hygiene and menstrual products (Otero *et al.*, 1998).
Many studies have reported *Candida albicans* as the main species involved, probably because it belongs to the normal vaginal microbial population, although the emergence of other species of the genus has been observed over the last few decades, involving *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. guilliermondii*, *C. lusitaniae* (Resende Pinho *et al.*, 2002).
Species identification of the yeasts involved in vulvovaginal candidiasis is not only an important step for a better understanding of the distribution of *albicans*/non-*albicans* species in different sub-populations of patients, in particular those with recurrent infections, but the data obtained

Corresponding author
Sara Asticcioli
Department S.M.E.C.
Section of Microbiology
University of Pavia
Via Brambilla, 74 - 27100 Pavia, Italy
E-mail: sara.asticcioli@unipv.it

can provide a very accurate view of antifungal susceptibility rates. In fact, innate or acquired resistance to available antifungal agents is now recognized among pathogenic fungi, particularly the *Candida* species, and the appearance of yeast isolates resistant to certain antifungal compounds may play a role in cases of recurrent vulvovaginal candidiasis (RVVC), particularly when caused by non-*albicans* species (Sojakova *et al.*, 2004).

Access to over-the-counter medications allows women to self-diagnose and treat vaginal symptoms. Although this increases women's autonomy, there are concerns about the accuracy of self-diagnosis and the appropriateness of over-the-counter medication choice, as well as fears of cross-resistance of *Candida* spp. to imidazoles resulting from inappropriate use.

The rank order of occurrence and resistance profiles of the various species of *Candida* causing candidiasis is important in establishing empiric treatment protocols and in judging the potential impact of newer antifungal agents.

The purposes of this study were:

1. to determine the prevalence and distribution of yeasts recovered from the vagina of women examined in an STD outpatients clinic;
2. to evaluate the antifungal susceptibility of the isolates;
3. to examine recurrences within the study period.

Samples were collected from women seen over a 6-year period, 2002-2007, at the Sexually Transmitted Disease outpatients clinic of S. Matteo Hospital Pavia for a physical examination. Use of a speculum allowed direct observation of the upper vaginal tract from which swab samples were obtained for laboratory analysis. Strains were identified by conventional micological methods, including gross inspection for colony morphology and growth on chromogenic media such as CHROMalbicans agar (Biolife), followed by microscopic examination (germ tube production, and chlamydospore formation).

Identification was also confirmed by biochemical methods with Vitek Card System and API 20C AUX products (BioMérieux, France). Isolates were stored as suspensions in sterile BHI +20% glycerol at -80°C until the study was performed. Prior to testing, each isolate was subcultured on Sabouraud dextrose agar to ensure purity and optimal growth.

Antifungal susceptibility testing of isolates of *Candida* spp. was performed by the colorimetric method Sensititre YeastOne (Trek Diagnostic System, East Grinstead, UK). This system is based on microdilution methodology with RPMI 1640 medium supplemented with a pH indicator (Alamar blue). Panels enable testing of *in vitro* susceptibility to a panel of antifungal agents, including voriconazole, with MICs being determined by color changes. It is a standardized investigational method and correlates well with the CLSI method M27-A2 (National Committee For Clinical Laboratory Standards, 2002) for a large number of experimental variables. Disposable trays were precoated with six antifungal agents: amphotericin B (AB), fluconazole (FZ), itraconazole (IZ), ketoconazole (KZ), 5-flucytosine (FC), and voriconazole (VOR). The individual trays contained dried concentrations for each compound: AB, IZ, KZ, VOR ranging from 0.008 to 16 µg/ml (twofold serial dilutions), FZ ranging from 0.125 to 256 µg/ml, and FC from 0.03 to 64 µg/ml. The inoculum size was adjusted turbidimetrically and standardized to a 0.5 McFarland to the M27-A2 guidelines. Results were read after 24 h of incubation at 35°C. Yeast growth was evident as a change in the Alamar blue growth indicator; the change from blue to pink facilitated clearer identification of breakpoints than the turbidimetric method, thus reducing the trailing effect characteristic of azole antifungal agents that hinders the interpretation of results when dilution techniques are used. Two reference yeast-like fungal strains with known *in vitro* susceptibility were used as quality controls: *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258. All clinical isolates grew on the drug-free growth controls when the test method were used; MICs for quality control reference strains were within accepted limits for antifungals (Linares *et al.,* 2004).

Susceptibility results were analyzed using the criteria published in the CLSI document M27-A2 for FZ (S: ≤8 µg/ml; SDD: 16-32 µg/ml, and R: ≥64 µg/ml), IZ (S: ≤0.12 µg/ml; SDD: 0.25-0.5 µg/ml, and R: ≥1 µg/ml) and FC (S: ≤4 µg/ml; I: 8-16 µg/ml, and R: ≥32 µg/ml). For VOR, a breakpoint of ≤1 µg/ml was used based on previous pharmacokinetic studies (Sheehan *et al.*, 1999; Pfaller *et al.*, 2003; Pfaller *et al.*, 2006).

The CLSI breakpoints were used for IZ and FZ. Establishing a clear correlation between AB MIC

and outcome is known to be difficult and official CLSI interpretative breakpoints are not available. Yet it has been suggested that susceptible isolates have an AB MIC of ≤1 μg ml^{-1} so it was used as cutoff value to separate the susceptible from the less susceptible and resistant isolates (National Committee for Clinical Laboratory Standards, 2002). KZ is known to have a similar mode of action and similar pharmacokinetics to previously studied itraconazole, so we might expect that the breakpoints would be similar (Candido *et al.*, 1998).

During the study period, 518 strains positive for yeast (equal to 14% of all samples collected) were isolated from vaginal samples. The isolates belonging to the genus *Candida* were 495 (95.5%) and specifically: *C. albicans* (61.2%), *C. glabrata* (28.8%), *C. parapsilosis* (2.9%), *C. tropicalis* (1.8%), *C. krusei* (1.8%), *C. lambica* (1.1%), *C. guilliermondii* and *C. kefyr* (0.6%), *C. zeylanoides*, *C. lusitaniae* and *C. famata* (0.4%). The genus *Saccharomyces*, *Trichosporon* and *Yarrowia* were isolated with a lower percentage (4.5%).

Table 1 shows the distribution of the different species in every year considered: an increased rate of isolation of *C. albicans* was noted during the years 2004 and 2007 while a decreased percentage of strains belonging to this species was observed during 2005; *C. glabrata* and *C. parapsilosis* are the only two species non-*albicans* constantly present in every year; noticeably increased rates of isolation were noted when the years of the study period were compared. In particular, *C. glabrata* regularly represented the second species isolated.

From women with subsequent episodes we isolated *C. glabrata* in 52.3% of cases, *C. albicans* and *C. krusei* in 36.4% and in 11.3% respectively of the remaining cases. Non-*albicans* species percentage, accounting for 63.6% of all strains, was particularly high (higher than that observed for women with sporadic candidiasis).

All patients with subsequent episodes were Italian, only one came from Albania. Patients' ages ranged from 18 to 60 years, with a mean of 36 years. Two of 16 patients had undergone menopause, while one had had a hysterectomy. Two women used oestrogenic-progestogenic contraceptives. None of these patients had used over-the-counter medications before examination, but five of 16 women reported exposure to antifungal drugs in a previous therapy, specifically azole compounds.

During the first examination and the subsequent ones, six of 16 patients had a mixed culture with different microrganisms: *Gardnerella*, *E. coli*,

TABLE 1 - *Species distribution by year.*

	2002	*2003*	*2004*	*2005*	*2006*	*2007*
C. albicans	57,3%	60,6%	73,6%	55,5%	60.5%	68.4%
C. glabrata	25%	27%	22,3%	37,3%	30.5%	27.5%
C. tropicalis	5,2%	3,4%	0	1,8%	0	0
C. krusei	5,2%	2,3%	0	0	0	1.6%
C. parapsilosis	2,1%	2,3%	1,4% 1	,8%	4,5%	2.5%
C. lambica	2,1%	0	2,7%	1,8%	0	0
C. lusitaniae	2,1%	0	0	0	0	0
C. guilliermondii	1%	1,1%	0	0	0	0
C. zeylanoides	0	1,1%	0	0	1.5%	0
C. kefyr	0	1,1%	0	1,8%	1.5%	0
C. famata	0	1,1%	0	0	1,5%	0

Streptococcus group B, *Chlamydia*, *Mycoplasma* and *Ureaplasma*.

In vitro susceptibility results for the 518 isolates tested with AB, FZ, IZ, KZ, FC, and VOR, expressed as the overall MIC range, the MICs at which 50% of isolates are inibited (MIC_{50}) and at which 90% of isolates are inhibited (MIC_{90}) are shown in Table 2. Even though *C. albicans* revealed a high susceptibility to fluconazole, FZ-resistant isolates were recovered from women during the study period. By comparison (Table 3) VOR was considerably more active than FZ showing a MIC_{50} and MIC_{90} ≤1 µg/ml either for fluconazole-susceptible or fluconazole-resistant *C. albicans*.

In our study vaginal mycotic infections were caused in 95.5% of cases by yeasts of the genus *Candida*. Although *C. albicans* represented the dominant species of pathogenic yeast isolated from women attending the STD outpatients clinic during the period 2002-2007; the frequency of non-*albicans* species was elevated, with an average of 41%, with *C. glabrata* as the most prevalent species (29.1%).

This situation changed considering the isolates of subsequent episodes in which the ethiologic agent was *C. glabrata* in 52.3%, *C. albicans* in 36.4% and *C. krusei* in 11.3% of cases.

The reasons for such differences could lie in the fact that vulvovaginal candidiasis caused by non-*albicans* species, particularly *C. glabrata* and *C. krusei*, are commonly associated with lower susceptibility to over-the-counter antifungal agents and 5 of the 16 women that experienced repeated episodes of vulvovaginal candidiasis had been exposed to courses of a wide array of antifungals, predominately azoles (Sojakova *et al.*, 2004). Therefore, it can be assumed that repeated exposure to antifungals, including topical agents, over a prolonged period of time may cause a shift in the vaginal mycoflora from the more drug-susceptible *C. albicans* to the less drug-susceptible species. Numerous reports suggest that these non-*albicans* species are generally more resistant to imidazole and polyene therapy than *C. albicans* and that the therapy itself may create the non-*albicans* selection (Sojakova *et al.*, 2004).

Antifungal agents can effectively treat mucosal candidiasis. However, their use can lead to colonization with less susceptible species and to resistance among normally susceptible strains. The prevalence of non-*C. albicans* strains is increasing over time, and these strains were more likely among women reporting recurrent vulvovaginal candidiasis. *C. albicans* isolates were sensitive to the polyene AB and to the pyrimidine FC. Among the azole compounds MIC_{90} were elevated, except for VOR (MIC_{90} 0.25 µg/ml). Even

TABLE 2 - *Summary of MIC_{50} and MIC_{90} of all strains isolated and of the two most frequent species.*

Organism (N° isolates)	*Antifungal*	*MIC (µg/ml) Range*	*MIC_{50} (µg/ml)*	*MIC_{90} (µg/ml)*
C. albicans (303)	Amphotericin B	0.03-1	0.125	0.25
	Fluconazole	≤0.125-64	0.25	2
	Voriconazole	≤0.008-16	0.008	0.25
	Itraconazole	≤0.008-16	0.06	4
	Ketoconazole	≤0.008-16	0.008	4
	5-flucytosine	≤0.03-16	0.125	1
C. glabrata (143)	Amphotericin B	0.008-0.5	0.125	0.25
	Fluconazole	≤0.125-64	16	32
	Voriconazole	≤0.008-16	0.5	1
	Itraconazole	≤0.008-16	0.5	8
	Ketoconazole	≤0.008-16	0.5	1
	5-flucytosine	≤0.03-16	0.03	0.125
All strains (495)	Amphotericin B	0.008-1	0.125	0.25
	Fluconazole	≤0.125-64	1	32
	Voriconazole	≤0.008-16	0.008	0.5
	Itraconazole	≤0.008-16	0.125	2
	Ketoconazole	≤0.008-16	0.03	2
	5-flucytosine	≤0.03-16	0.06	2

TABLE 3 - *Voriconazole MIC_{50} and MIC_{90} of different species.*

C. spp	*VORI*	
Tot. 495	*MIC_{50} (µg/ml)*	*MIC_{90} (µg/ml)*
C. albicans (303) 97.7% S FZ 2.3% R FZ	 0.008 0.125	 0.25 1
C. glabrata (143)	0.5	1
C. parapsilosis (14)	0.03	0.06
C. tropicalis (9)	0.03	0.06
C. krusei (9)	0.5	0.5
C. lambica (5)	-	-
C. guilliermondii (3)	-	-
C. kefyr (3)	-	-
C. famata (2)	-	-
C. lusitaniae (2)	-	-
C. zeylanoide (2)	-	-

though among *C. albicans* strains resistance to azoles was infrequent, FZ-resistant isolates were recovered from women during the study period. The frequency of these isolates was lower (2.3%) than that found by Linares *et al.*, Sojakova *et al*. and Bauters *et al.* (Sojakova *et al.*, 2004; Linares 2004; Bauters Tiene G.M., 2002), similar to Alexander *et al.* (Alexander *et al.*, 2007) and higher than the 1.5% reported by Pfaller *et al*. (Pfaller *et al.*, 2007).

MICs of IZ, FZ and VOR (MIC_{90} 8 µg/ml, 32 µg/ml and 1 µg/ml respectively) in *C. glabrata* isolates were considerably higher than those for *C. albicans*.

Therefore, in addition to a truly representative picture of *Candida* species and strain distribution, the data obtained in the course of this study period have provided an accurate overview of antifungal susceptibility rates. Knowledge of local susceptibility patterns with that of local prevalence or identification of more resistant species, such as *C. krusei*, *C. glabrata* and *C. lusitaniae*, can significantly aid in selecting effective antifungal agents for empiric use. If local susceptibilities to these and other common *Candida* species are known, this information can allow intelligent decisions on antifungals selection, especially that used empirically (Hospenthal Duane, 2004).

In summary, our results show that *C. albicans* remains the *Candida* species most frequently implicated in vulvovaginal candidiasis followed by *C. glabrata* in our local geographic setting of Pavia. Isolates intrinsically resistant (*C. krusei*), or with decreased susceptibility to FZ (*C. glabrata),* were susceptible to the triazole compound VOR. Our *in vitro* antifungal susceptibility data are in agreement with the findings reported by other authors (Alexander *et al.*, 2007; Linares *et al.*, 2004; Marco *et al.*, 2003; Pfaller *et al.*, 2004; Cheng *et al.*, 2004; Sobel *et al.*, 2003; Swinne *et al.*, 2004) and VOR results support the good activity of this compound for the treatment of *Candida* spp. infections.

REFERENCES

Alexander B.D., Byrne T.C., Smith K.L., Hanson K.E., Anstrom K.J., Perfect J.R., Reller L.B. (2007). Comparative evaluation of Etest and Sensititre YeastOne panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing *Candida* susceptibility to seven antifungal agents. *J. Clin. Microbiol.* **45** (3), 698-706.

Bauters Tiene G.M., Dhont A., Temmerman Marleen I.L., Nelis Hans J. (2002). Prevalence of vulvovaginal candidiasis and susceptibility to fluconazole in women. *American Journal of Obstetrics and Gynecology.* **187**, 569-574.

Candido R.C., Torqueti Toloi M.R., Franceschini S.A., Garcia F.R., Manini Minto E.C. (1999). In vitro activity of antimycotic agents determined by E-Test method against vaginal shape Candida species. *Mycopathologia*. **144**, 15-20.

Cheng M-F., Yu K-W., Tang R-B, Fan Y-H., Yang Y-L., Hsieh K-S., Ho M., Lo H-J. (2004). Distribution and antifungal susceptibility of Candida species causing candidemia from 1996 to 1999. *Diagn. Microb. and Infect. Dis.* **48**, 33-37.

Clinical and Laboratory Standards Institute (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard M27-A2. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Hospenthal Duane R., Murray Clinton K. Rinaldi Michael G. (2004). The role of antifungal susceptibility testing in the therapy of candidiasis. *Diagn. Microb. Infect. Dis.* **48**, 153-160.

Linares M.J., Charriel G., Solis F., Casal M. (2004). Comparison of two microdiluition methods for testing susceptibility of *Candida* spp. to voriconazole. *J. Clin. Microb.* **42**, 899-902.

Marco F., Danés C., Almela M., Jurado A., Mensa J., de la Bellacasa J.P., Espasa M., Martinez J.A., Jiménez de Anta M.T. (2003). Trends in frequency and in vitro susceptibilities to antifungal agents, including voriconazole and anidulafungin, of Candida bloodstream isolates. Results of a six-yaer study (1996-2001). *Diagn. Microb. and Inf. Dis.* **46**, 259-264.

National Committee For Clinical Laboratory Standards (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. 2nd ed, M27-A2. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Otero L., Palacio V., Carreño F., Méndez F.J., Vázquez F. (1998). Vulvovaginal candidiasis in female sex workers. *Int. J. STD AIDS*. **9**, 526-530.

Pfaller M.A., Diekema D.J., Messer S.A., Boyken L., Hollis R.J. (2003). Activities of fluconazole and voriconazole against 1,586 recent clinical isolates of *Candida* species determined by broth microdilution, disk diffusion, and etest methods: report from the ARTEMIS global antifungal susceptibility program, 2001. *J. Clin. Microbiol.* **41**, 1440-1446.

Pfaller M.A., Messer S.A., Boyken L., Hollis R.J., Rice C., Tendolkar S., Diekema D.J. 2004). *In vitro* activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of *Candida* spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. *Diagn. Microb. Infect. Dis.* **48**, 201-205.

Pfaller M.A., Diekema D.J., Rex J.H., Espinel-Ingroff A., Johnson E.M., Andes D., Chaturvedi V., Ghannoum M.A., Odds F.C., Rinaldi M.G., Sheehan D.J., Troke P., Walsh T.J., Warnock D.W. (2006). Correlation of MIC with outcome for *Candida* species tested against voriconazole: analysis and proposal for interpretive breakpoints. *J. Clin. Microbiol.* **44**, 819-826.

Pfaller M.A., Diekema D.J., Gibbs D.L., Newell V.A., Meis J.F., Gould I.M., Fu W., Colombo A.L., Rodriguez-Noriega E., Global Antifungal Surveillance Study (2007). Results from the ARTEMIS DISK global antifungal surveillance study, 1997 to 2005: an 8.5-year analysis of susceptibilities of *Candida* species and other yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J. Clin. Microbiol.* **45** (6): 1735-1745.

Resende Pinho J.C., de Resende M.A., Saliba J.L. (2002). Prevalence of *Candida* spp. in hospitalized patients and their risk factors. *Mycoses.* **45**, 306-312.

Sheehan D.J., Hitchcock C.A., Sibley C.M. (1999). Current and emerging azole antifungal agents. *Clin. Microbiol. Rev.* **12**, 40-79.

Sobel J.D., Zervos M., Reed B.D., Hooton T., Soper D., Nyirjesy P., Heine M.W., Willems J., Panzer H. (2003). Fluconazole susceptibility of vaginal isolates obtained from women with complicated *Candida* vaginitis: clinical implication. *Antimicrob. Agents. Chemother.* **47**, 34-38.

Sojakova M., Liptajova D., Borovsky M., Subik J. (2004). Fluconazole and itraconazole susceptibility of vaginal yeast isolates from Slovakia. *Mycophatologia*. **157**, 163-169.

Swinne D., Watelle M., Van der Flaes M., Nolard N. (2004). *In vitro* activities of voriconazole (UK- 109, 496), fluconazole, itraconazole and amphotericin B against 132 non-*albicans* bloostream yeast isolates (CANARI study). *Mycoses.* **47**, 177-183.