FLUCONAZOLE SUSCEPTIBILITY OF ITALIAN Candida dubliniensis CLINICAL ISOLATES DETERMINED BY REFERENCE AND SIMPLIFIED TESTS

GIAMMANCO, G.M.*, PECORELLA, S.*, DISTEFANO, S.*, PECORARO, V.*, MILICI, M.E.*, and PIZZO, G.*

*1Dipartimento di Igiene e Microbiologia "G. D’Alessandro", Università di Palermo, Via del Vespro 133, 1-90127 Palermo, Italy, E-mail: gmgiamm@libero.it;
2A.U.S.L. 6, P.O. Guadagna, I Divisione di Malattie Infettive, Via Villagrazia 46, 90124 Palermo, Italy;
3Dipartimento di Scienze Stomatologiche "G. Messina", Università di Palermo, Via del Vespro 129, 90127 Palermo, Italy

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SUMMARY

Candida dubliniensis is an opportunistic pathogen mainly associated with oral candidiasis in human immunodeficiency virus (HIV)-infected individuals. We recently recovered the first Italian clinical isolates of C. dubliniensis from the oral cavities of seven HIV-seropositive subjects. The in vitro susceptibility to fluconazole (FLCZ) of these isolates was determined according to the National Committee for Clinical Laboratory Standards (NCCLS) M27-A broth microdilution method for yeasts. All seven isolates of C. dubliniensis were susceptible to FLCZ (MICs ≤0.5 μg/ml). Results of this reference method were compared to those obtained with simplified tests, more adapted to routine evaluation in hospital laboratories. Fungitester and Sensititre YeastOne colorimetric microplate-based methods have been evaluated. The agar disk diffusion method has also been tested on two different media: RPMI 1640-2% glucose and High Resolution-2% glucose-0.5 μg/ml methylene blue. All of the simplified methods tested were able to correctly identify FLCZ-susceptibility of this group of Italian C. dubliniensis isolates.

KEY WORDS: Candida dubliniensis, Italy, fluconazole, susceptibility, NCCLS M27-A protocol

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INTRODUCTION

Fungal infections are particularly important in immunocompromised patients. *Candida albicans* is still the species most frequently involved in oral candidiasis both in immunocompromised and immunocompetent patients. However, over the last two decades, the relative frequency of *C. albicans* infections has decreased compared to those caused by other *Candida* species, including *C. tropicalis*, *C. glabrata*, and *C. krusei* (Pfaller, 1996). This epidemiologic shift also involves the recent emergence of a new opportunistic pathogen: *Candida dubliniensis* (Sullivan et al., 1995). Starting from the end of the '80s, AIDS pandemic produced a new population of immunocompromised patients, which are highly subjected to serious fungal infections (Dupont et al., 1992). Oropharyngeal candidiasis occurs in up to 90% of patients infected by the human immunodeficiency virus (HIV) (Feigal, 1991), and the isolation of *C. dubliniensis* has been most often associated with the colonization and infection of the oral cavity, mainly of HIV-infected persons (Sullivan et al., 1997). However, *C. dubliniensis* has also been isolated from anatomical sites other than the oral cavity as well as from HIV-negative subjects (Jabra-Rizk et al., 2000) or from patients undergoing antibiotic chemotherapy (Polacheck et al., 2000). The involvement of this new species in systemic disease has also been recently documented in patients receiving immunosuppressive treatment, after bone-marrow transplant, suffering end-stage liver disease, or HIV-infected (Meis et al., 1999; Brandt et al., 2000).

The azole antifungal agents have long been used to treat chronic mucocutaneous candidiasis. Fluconazole (FLCZ) has become the drug of choice for long-term therapy and prophylaxis of AIDS-associated opportunistic infections due to high oral bioavailability and weak toxicity (Galgiani, 1990). Unfortunately, extensive use of this drug has led to an increasing number of treatment failures due to the selection of resistant strains (Tumbarello et al., 1997). *C. dubliniensis* deserves special interest since many clinical isolates have been found to be resistant to the azoles (Moran et al., 1997) and it has been demonstrated that *C. dubliniensis* is able to develop resistance to FLCZ following direct exposure to this antifungal drug in vitro (Moran et al., 1997) and after long-term treatment in vivo (Ruhnke et al., 2000). Antifungal therapy is frequently selected empirically, but in vitro susceptibility testing of the isolate should guide the selection. Moreover, monitoring of the patient through repeated sampling at regular intervals during long-term treatments may help to determine whether the selection of resistant variants might explain mycological persistence.

Different methods for evaluating the susceptibility of yeasts to antifungal agents have been the subject of many studies during the last fifteen years. Recently, a standard reference procedure, approved standard M27-A, has been described by the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 1997). This procedure is a macroplate dilution technique that is too cumbersome for use in most clinical laboratories. A broth microdilution technique, which can be performed in 96-well microtiter plates, has been found to be more acceptable (NCCLS, 1997). For the M27-A procedure 48 h readings are specified and MIC breakpoints for defining fluconazole-susceptible and fluconazole-resistant categories have been established. The MICs obtained by this procedure are generally reproducible within a range of ±1 dilution interval (Barry et al., 2000). The M27-A reference method is, however, labour-intensive, costly, and uses a difficult to read endpoint for azoles. Thus, simpler to perform commercial miniaturized colormetric methods have recently been developed according to the principles of the NCCLS procedure for the determination of susceptibility to different antifungal agents, including FLCZ (Davey et al., 1998; Posteraro et al., 2000; Willinger et al., 2000). Agar disk diffusion methods for testing antifungal agents susceptibility are still in their infancy, but recent studies have demonstrated that disk testing with a method similar to the NCCLS M2-A6 disk test for bacteria (NCCLS, 1999) can
reproducibly and accurately determine susceptibility of yeasts to FLCZ (Barry et al., 1996; Kirkpatrick et al., 1998). All of these simplified methods for susceptibility testing of yeast isolates have been validated on a number of *Candida* species including *C. albicans*, which phenotypically resembles *C. dubliniensis*, but no data are yet available on colorimetric or disk testing performed on the latter species.

In Italy, the first clinical isolate of *C. dubliniensis* was reported in Catania (Giammanco et al., 2000) from the oral cavity of an HIV-seropositive subject without symptoms of oral candidiasis. Afterwards, another six strains of *C. dubliniensis* were isolated in Palermo from oral samples from HIV-positive individuals or AIDS patients (Giammanco et al., 2001).

In this study, we tested these seven Italian *C. dubliniensis* isolates for FLCZ susceptibility using the reference M27-A microdilution NCCLS protocol. Results were compared with those obtained by simpler methods such as two miniaturized colorimetric tests and agar disk diffusion testing performed on two different media.

**MATERIALS AND METHODS**

**Strains**

Seven clinical isolates of *C. dubliniensis* recovered from the oral cavity of HIV-seropositive subjects or AIDS patients in Catania (Giammanco et al., 2000) and Palermo (Giammanco et al., 2001), Italy, were tested. Only two of these subjects showed symptoms of oral candidiasis at the time of sampling and one of them had previously received FLCZ therapy. *C. krusei* type strain (ATTC 6258), whose MIC for FLCZ has been defined and is between 16 and 64 μg/ml, was used as quality control.

**Inoculum**

Isolates were stored at −80°C and subcultured twice onto Sabouraud dextrose agar to ensure purity and viability prior to testing. Five isolated colonies were then suspended in sterile saline. The turbidity of the suspension was adjusted spectrophotometrically to obtain an inoculum containing between 0.5 x 10^6 and 2.5 x 10^6 CFU/ml. Dilutions of the inoculum suspension were prepared and quantitatively subcultured to confirm the actual number of CFU per milliliter.

**Reference method**

The M27-A NCCLS microplate dilution procedure (NCCLS, 1997) was used as the standard method for the evaluation of the other methods. FLCZ testing perfusion solution was purchased from Pfizer Central Research, Sandwich, UK. Two-fold serial dilutions of the drug were prepared in RPMI 1640 liquid medium (Sigma-Aldrich, Milan, Italy) supplemented with L-glutamine and buffered to pH 7 with morpholinepropanesulfonic acid (MOPS) organic buffer and the glucose content was increased to a final concentration of 2% (RPMI-G). Testing was performed in 96-well flat-bottomed microtiter plates (Biospa, Milan, Italy). For every isolate tested, 0.1 ml volumes of 2x serial dilutions were dispensed in each of eleven wells in a row of twelve leaving the twelfth well for the inoculum growth control. Microplates were then frozen at −80°C until needed. At the time of testing, each well received 0.1 ml of standardized yeast inoculum that diluted the drug to final concentrations ranging from 0.125 to 128 μg/ml. Microplates were incubated at 35°C and results scored after 24 and 48 h incubation, but 48-h MICs were used as a reference for evaluation. The MICs were determined as the lowest concentration of the drug inhibiting at least 80% of the growth (NCCLS, 1997). That was determined by comparing each well to a 1:5 dilution of the growth control well. Interpretative criteria defined by NCCLS were adopted for microplates testing. According to these criteria MIC breakpoints of ≤8 μg/ml for susceptibility and of ≥64 μg/ml for resistance were chosen. An intermediate category groups the strains whose MIC is 16 or 32 μg/ml. Their susceptibility to FLCZ is dose dependent (SDD); they are not fully susceptible, nor are they truly resistant. The introduction of this intermediate category derives from the wide range of dosage options available for therapy (NCCLS, 1997).

**Colorimetric tests**

Two different colorimetric commercial tests were used in this study:

- The *Fungitest* (Sanofi-Pasteur, Marnes la Coquette, France) determines susceptibility to six different antifungal agents including FLCZ and is based on the demonstration of growth in a liquid medium containing two different concentrations of each drug and incorporating an oxidation-reduction indicator. Concentrations used for FLCZ are 8 and 64 μg/ml. Fungitest was used according to the manufacturer's instructions and growth assessment was based on color change of the redox
RESULTS

Table 1 summarises the results obtained with the five methods used for FLCZ susceptibility determination. Results of all tests were scored after 24 and 48 h incubation. The seven \textit{C. dubliniensis} isolates showed MICs between $\leq 0.125$ and 0.5 $\mu$g/ml both with the reference NCCLS protocol and with the microplate Sensititre YeastOne method. A slight increase in MICs for fluconazole (1 dilution) was observed for some strains after 48 h incubation with both methods. \textit{C. krusei} ATCC 6258 quality control MIC was within the expected range with both methods and incubation times, though one dilution increase was observed at 48 h. With Fungitest too all \textit{C. dubliniensis} were susceptible to FLCZ (MIC $\leq 8$ $\mu$g/ml) both after 24 and 48 h incubation, while \textit{C. krusei} ATCC 6258 was scored in the intermediate category (8$<$MIC$\leq 32$ $\mu$g/ml) corresponding to the expected range of MICs. The diameters of growth inhibition zones around 25-$\mu$g FLCZ disks were interpreted on the basis of breakpoints defined in literature for \textit{C. albicans} on the agar media used (Barry & Brown, 1996; Meis \textit{et al.}, 2000). On RPMI-G agar medium, all of the \textit{C. dubliniensis} strains showed inhibition zones larger than the 19 mm breakpoint for susceptibility when read after 24 h, with the exception of strain 417 which needed 48 h incubation to show its susceptibility. However, 48 h incubation produced an “inside-zone” growth for three isolates that were misidentified as resistant strains. On HR-GMB agar medium as well, inhibition zones larger than the 19 mm breakpoint for susceptibility were seen for all our \textit{C. dubliniensis}, but only after 48 h incubation. On both agar media \textit{C. krusei} ATCC 6258 was not inhibited by the FLCZ disk (expected diameter (Barry & Brown, 1996): 6 to 17 mm), but on HR-GMB growth required 48 h incubation.

DISCUSSION

\textit{C dubliniensis} is most often associated with recurrent candidiasis in HIV-infected patients probably as a result of selection due to wide-
### Table 1

<table>
<thead>
<tr>
<th>Isolate</th>
<th>NCCLS</th>
<th>YeastOne</th>
<th>RPMI-G 24h</th>
<th>HR-GMB 24h</th>
<th>RPMI-G 48h</th>
<th>HR-GMB 48h</th>
<th>Fungistest</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. dubliniensis 417</td>
<td>0.25 µg/mL</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>R</td>
</tr>
<tr>
<td>C. dubliniensis 428</td>
<td>0.25 µg/mL</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>R</td>
</tr>
<tr>
<td>C. dubliniensis 356</td>
<td>0.25 µg/mL</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>R</td>
</tr>
<tr>
<td>C. dubliniensis 342</td>
<td>0.25 µg/mL</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>R</td>
</tr>
<tr>
<td>C. dubliniensis 254</td>
<td>0.25 µg/mL</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>R</td>
</tr>
<tr>
<td>C. dubliniensis 15a CT</td>
<td>0.25 µg/mL</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>R</td>
</tr>
<tr>
<td>C. dubliniensis 603</td>
<td>0.25 µg/mL</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.25 (S)</td>
<td>R</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>16 (SDD)</td>
<td>16 (SDD)</td>
<td>16 (SDD)</td>
<td>16 (SDD)</td>
<td>16 (SDD)</td>
<td>16 (SDD)</td>
<td>R</td>
</tr>
</tbody>
</table>

S: susceptible; R: resistant; f: intermediate; SDD: susceptible-dose-dependent; NR: not readiable due to weak growth.

Fluconazole susceptibility of Italian C. dubliniensis strains. However, our results seem to confirm that both colorimetric spread use of antifungal therapy, mainly with FLCZ (Moran et al., 1997). Unlike *Candida albicans*, it is able to develop stable resistance to FLCZ following exposure to the drug *in vitro* (Moran et al., 1997) and after long-term treatment *in vivo* (Ruhnke et al., 2000). Despite this fact, there have been few studies evaluating the *in vitro* susceptibility of *C. dubliniensis* isolates to FLCZ. Two studies on limited numbers of clinical isolates found rates of susceptible strains of 80 and 91%, respectively in Ireland and Texas (Moran et al., 1997; Kirkpatrick et al., 1998b). Larger studies showed that 5.2% of isolates in an European yeast stock collection were resistant to FLCZ (Odds et al., 1998) and 97% of 71 *C. dubliniensis* collection strains and clinical isolates from different geographic locations were susceptible (Pfaller et al., 1999). More recently, 79.3% of 29 clinical strains mostly isolated in Spanish hospitals were susceptible to FLCZ (Quindós et al., 2000).

A few clinical *C. dubliniensis* strains have recently been isolated in Italy from the oral cavity of HIV-infected subjects (Giammanco et al., 2000; 2001). Thus, Italy can be added to the list of countries where this opportunistic pathogen has already been reported (Sullivan et al., 1997). In this study, these clinical isolates have been tested for FLCZ resistance and they were all susceptible to the drug *in vitro* by the NCCLS reference method. This result is not surprising since in previous studies isolates with reduced susceptibility to FLCZ were mainly recovered from AIDS patients who had already been treated with the drug (Moran et al., 1997; Kirkpatrick et al., 1998), while none of our patients but one had received FLCZ treatment. Since all of our test strains were susceptible to FLCZ, we cannot speculate on the ability of the different simplified methods used to identify fluconazole-resistant *C. dubliniensis* strains. However, our results seem to confirm that both colorimetric
and agar diffusion tests are able to correctly identify C. dubliniensis isolates susceptible to FLCZ. Microplate colorimetric tests like Fun- gitest and Sensititre YeastOne are by far the easiest to use and read, but doubts have been raised in the past on the ability of the first to correctly identify Candida resistant strains (Wittnuck et al., 1999). On the contrary, disk diffusion tests are difficult to interpret due to incomplete growth inhibition linked to the fungistatic effect of the drug. Thus, a certain amount of subjectivity risks being introduced into zone determination. However, both media we used for disk diffusion testing correctly identified our C. dubliniensis isolates susceptible to FLCZ. Inhibition zones were better read after 24 h incubation on RPMI-G medium to avoid misinterpretation due to "inside-zone" growth, while HR-G-MB medium required 48 h incubation for reading.

Results of susceptibility testing of the limited number of available Italian isolates seem to corroborate the finding that C. dubliniensis isolates are generally susceptible to FLCZ. However, a larger number of strains should be studied for a correct evaluation of the importance of FLCZ-resistance in C. dubliniensis clinical isolates in Italy.

The introduction of simple and accurate methods for antifungal susceptibility testing that can be routinely used in hospital laboratories and for global surveillance would be of help for guiding clinical therapy and collecting valuable epidemiological information on drug susceptibility of yeast isolates. Systematic evaluation of FLCZ susceptibility of C. dubliniensis isolates would be of special interest due to the still incomplete definition of the pathogenic role of this species. Colorimetric microplate determination, and agar disk diffusion methods have already proved affordable for the study of C. albicans and other Candida species and are likely to be of value for C. dubliniensis too. Our results represent a first step in the direction of such an evaluation. Large comparative studies including a significant number of FLCZ resistant strains would be needed for appropriate testing of the ability of these methods to correctly determine the susceptibility of C. dubliniensis isolates.

REFERENCES


among oral yeast isolates from an Italian population of human immunodeficiency virus-infected (HIV+) subjects. *Oral Microbiology and Immunology*, accepted for publication.


