In vitro evaluation of Malassezia pachydermatis susceptibility toazole compounds using E-test and CLSI microdilution methods

CLAUDIA CAFARCHIA*, LUCIANA A. FIGUEREDO*, ROBERTA IATTA†, VALERIANA COLAO*, MARIA T. MONTAGNA‡ & DOMENICO OTRANTO*

*Dipartimento di Sanità Pubblica e Zootecnia and †Dipartimento di Scienze Biomediche ed Oncologia Umana, Università degli Studi di Bari, Bari, Italy

Dermatitis caused by Malassezia spp., one of most common skin disease in dogs, requires prolonged therapy and/or high doses of antifungal agents. In the present study, the antifungal susceptibility of M. pachydermatis to ketoconazole (KTZ), fluconazole (FLZ), itraconazole (ITZ), posaconazole (POS) and voriconazole (VOR) was evaluated in vitro using both CLSI reference broth microdilution (CLSI BMD) and E-test. A total of 62 M. pachydermatis strains from dogs with and without skin lesions were tested. M. pachydermatis strains were susceptible to ITZ, KTZ and POS using both test methods, with the highest MIC found in tests of FLZ. Essential agreement between the two methods ranged from 87.1% (VOR) to 91.9% (ITZ), and categorical agreement from 74.2% (FLZ) to 96.8% (ITZ). Minor error discrepancies were observed between the two methods, with major discrepancies observed for KTZ. A higher MIC50 value for FLZ was noted with M. pachydermatis genotype B. The MIC50 of M. pachydermatis genotype B for KTZ, VOR and POS were higher in isolates from dogs with skin lesions than those in isolates from animals without skin lesions. The results suggest a link between genotypes of M. pachydermatis and in vitro drug susceptibility. The categorical agreement for both E-test and CLSI BMD methods found in this investigation confirms the E-test as a reliable diagnostic method for routine use in clinical mycology laboratories.

Keywords Malassezia pachydermatis, antifungal susceptibility, genotype, CLSI reference broth microdilution method, E-test

Introduction

Malassezia pachydermatis is a non-lipophilic, non-mycelial, unipolar budding yeast characterized by a thick cell wall [1]. This yeast is a commensal on animal skin, but may become pathogenic under the influence of predisposing factors [2–5], leading to cases of otitis externa and different clinical forms of dermatitis in domestic animals [1,6–8]. In addition, this yeast has also been reported as a commensal on the skin of dog owners [9] and identified as the causative agent of nosocomial infection in neonatal patients handled by healthcare workers who have had close contact with dogs [10,11].

Malassezia dermatitis is one of most common skin diseases in dogs, with severe infections usually requiring prolonged treatment and/or high doses of antifungal agents [8], including ketoconazole (KTZ) and itraconazole (ITZ) [8,12]. However, in vitro resistance of selected genotypes of M. pachydermatis to azoles has emerged recently [13,14], thus giving rise to the hypothesis that the occurrence of skin lesions may be associated with resistant genotypes of this microorganism [13,14]. In recent studies [15,16], sequencing
of the chitin synthase-2 (chs-2) gene, the first internal transcribed spacer (ITS-1) and the large subunit (LSU) of nuclear rDNA has led to the categorization of *M. pachydermatis* isolated dogs into three major genotypes (i.e., A, B and C) [15,16]. However, to date, no information is available on the in vitro susceptibility of *M. pachydermatis* genetic subpopulations to antifungal agents.

Since the number of human and animal reported skin infections by *Malassezia* is increasing rapidly [1,5], there is a need for in vitro susceptibility testing of this yeast to antifungal compounds. In 2002, the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) developed a reference method for such testing of *Candida* spp. and *Cryptococcus neoformans* [17]. This was subsequently adapted to *Malassezia* yeasts by modifying the media, time of incubation and inocula [13,14,18,19]. The susceptibility of *Malassezia* to antifungal compounds has also been tested using alternative methods, such as the disk diffusion [20–22] and the E-test; to estimate the agreement of results obtained with the E-test and CLSI BMD methods; and (iii) to test the hypothesis of an association between in vitro susceptibility of *M. pachydermatis* to antifungal compounds and the yeast’s genetic subpopulations.

**Materials and methods**

*Malassezia isolates and their phenotypic identification*

*Malassezia pachydermatis* isolates (*n* = 62) were collected from 62 dogs with or without skin lesions and/or otitis which comprised two groups, i.e., Group A included 30 isolates from 30 dogs in good general health with no history of skin or ear diseases and that had not been treated in the preceding five months and Group B consisting of 32 isolates from 32 dogs with cutaneous lesions. In the latter group were five dogs that had localized dermatitis characterized by erythema, pruritus at only one site (i.e., two dogs with otitis and pinna hyperkeratosis and three with inguinal dermatitis) and the remaining 27 animals had generalized lesions characterized by pruritus, erythema, alopecia, excoriations, seborrhoea, lichenification and/or hyperpigmentation. Samples were collected from each animal by rubbing sterile swabs on seven different anatomical areas (i.e., left and right ear canals, ventral neck, left and right axilla, and left and right groin). After collection, samples were inoculated onto modified Dixon Agar (mDA) and incubated at 32°C for 10 days. *Malassezia pachydermatis* isolates were identified phenotypically based on their macroscopic and microscopic morphology and their ability to grow on media without lipid supplementation (Sabouraud Dextrose Agar, BioLife-SAB) [25]. Additional tests were conducted which included assessment of assimilation of different Tweens (i.e., Tweens 20, 40, 60, 80; Sigma-Aldrich, Italy) and cremophor EL (PeG 35 castor oil, Sigma-Aldrich, Italy), pigment production on tryptophan-based medium and growth on mDA at 32 and 40°C. Isolates were maintained on mDA until molecular characterization and testing of in vitro antifungal susceptibility. Isolates were deposited in the fungal collection of the Faculty of Veterinary Medicine at the University of Bari, Italy.

**PCR amplification and multilocus sequencing for Malassezia genotyping**

Genomic DNA was extracted from 1 ml culture (containing ∼1–2 × 10⁸ cells) of each isolate, as described previously [15,16]. The chs-2 gene (∼540 bp) was amplified from genomic DNA by the polymerase chain reaction (PCR) using the primers CED1 and CED2, the ITS-1 region (∼282 bp) using the primers 18SF1 and 5.8SR1 and the D1/D2 regions of the LSU rRNA gene (∼640 bp) using the primers F63 and LR3 [15,16]. Genomic DNA (4 μl) was added to the PCR mix (46 μl) containing 2.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3 and 50 mM KCl, 250 μM of each dNTP, 50 pmol of each primer and 1.25 U of AmpliTag Gold (Applied Biosystems, Milan, Italy). The PCR was performed in a thermal cycler (Gene Amp PCR System 2700, Applied Biosystems, Milan, Italy) at 94°C for 12 min (polymerase activation), followed by 25–30 cycles of 94°C for 1 min (chs-2) or 30 sec (LSU and ITS-1) (denaturation), respectively, 55°C (LSU) and 60°C for 1 min (chs-2) or 15 sec (ITS-1) (annealing); 72°C for 2 min (chs-2) or 1 min (LSU) or 15 sec (ITS-1) (extension), followed by 7 min at 72°C (final extension). Amplicons were resolved in 2% w/v agarose (Gelyphor, Euroclon, Milan, Italy) gels, stained with ethidium bromide (10 mg/ml) and then purified using Ultrafree-DA columns (Amicon, Milipore; Bedford, USA) and sequenced directly using the Taq DyeDeoxyTMTerminator Cycle Sequencing Kit (v.2, Applied Biosystems, Milan, Italy) and then sequences available in the GenBank™ database (http://www.ncbi.nlm.nih.gov).
In vitro susceptibility testing

The in vitro susceptibility of *M. pachydermatis* strains to antifungal compounds was assessed employing the reference CLSI M27-A2 method, using the Sabouraud dextrose medium (Liofilchem Diagnostici, Roseto degli Abruzzi, Italy-SDB) with 1% of Tween 80 (Sigma Co, Milano, Italy) [14]. In particular, Sabouraud dextrose medium with 1% of Tween 80 was employed since *M. pachydermatis* genotype B grows better in this medium as it is lipid dependent [15].

The E-test strips (AB BIODISK, Solna, Sweden) for KTZ, ITZ, VOR, POS (concentrations ranging from 0.002–32 mg/l) and for FLZ (from 0.16–256 mg/l) were used in the testing. Stock inoculum suspensions were prepared from 7-days-old *M. pachydermatis* colonies incubated on modified Dixon agar at 32°C. The final concentration of the stock inoculum suspensions in sterile distilled water was adjusted to an optical density of 2.4 using a turbidimeter (DEN-1 McFarland Densitometer, Biosan) which was equivalent to 1–5 × 10⁶ colony forming unit (CFU)/ml, as validated by quantitative plate counts of CFU on Sabouraud Dextrose agar 1% of tween 80.

Sterile cotton swabs were dipped into the inoculum suspension and then streaked on Sabouraud dextrose agar (SDA; Liofilchem Diagnostici, Roseto degli Abruzzi, Italy) containing 1% of Tween 80 with E-test strips placed in the centre of the plate. The inoculated plates were incubated at 32°C and read after 48 h. The drug concentration shown on the E-test strip at the outer border of the elliptical inhibition halo was recorded as MIC. The growth of microcolonies within this inhibition zone was disregarded [27] and the MIC data reported as values at which 50% (**MIC**₅₀) and 90% (**MIC**₉₀) of isolates growth were inhibited.

Quality control strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258; American Type Culture Collection, Manassas, VA, USA) were included on each day to check the accuracy of the drug dilutions and the reproducibility of the results [28].

Tentative breakpoints have been established for azole compounds, allowing isolates of *Candida* spp. tested in accord with CLSI guidelines to be classified as susceptible, susceptible dose-dependent, or resistant [29]. However, since these breakpoints have not yet been established for *M. pachydermatis*, the following criteria were used in both tests to categorize *M. pachydermatis* isolates: susceptible (S) = MIC sample ≤ **MIC**₅₀; intermediary susceptible (I) = **MIC**₅₀ < MIC sample ≤ **MIC**₉₀; and resistant (R) = MIC sample > **MIC**₉₀ [13,14,23,24,30,31]. The categories (i.e., S, I, R) obtained with the E-test method were compared to those obtained using the CLSI BMD reference procedure. Essential agreement (EA) was defined as discrepancies in MIC results of no more than two-fold dilutions between the methods. Categorical agreement (CA) was assigned to susceptibility testing results falling within the same category of interpretation [32].

The discrepancies between tests were classified as: (i) ‘minor’, isolates classified as S or R by one of the two tests, and as I by the other method; (ii) ‘major’, isolate classified as S by the CLSI BMD and ‘R’ by the E-test; and (iii) ‘very major’, isolate classified as ‘R’ by the CLSI BMD and ‘S’ by the E-test [33].

**Statistical analysis**

Agreement between the MICs determined by two different susceptibility-testing methods was assessed using Bland Altman test. In particular, the test was applied on log₂ transformed data.

KTZ, ITZ, VOR, POS and FLZ, CLSI BMD MIC values for genetic population of *M. pachydermatis* were screened by the one-way analysis of variance (ANOVA I).

MIC values of genotype A, B and C coming from animals with and without lesions were compared using Student’s *t*-test and the homoscedasticity of variances was evaluated using the Fisher’s test.

The χ² test was employed to compare the prevalence of *M. pachydermatis* strains with genotype A, B and C resulting susceptible (S), intermediary susceptible (I) and resistant (R) to the fungal drugs tested. Data were statistically analysed using R software (version 2.8.1). A *P*-value less than 0.05 was considered significant.

**Results**

Fifty isolates were phenotypically identified as not lipid dependent *M. pachydermatis* and 12 as lipid dependent (LD) as previously reported [15]. In particular, all LD isolates were catalase positive, did not grow on Sabouraud agar but did grow on modified Dixon agar (at 32, 37 and 40°C), assimilated Cremophor EL, Tween 20, 40, 60 and 80, did not hydrolyze esculin and did not consume tryptophan as a nitrogen source.

The PCR amplification of the *chs*-2 gene, ITS-1 and LSU of nuclear rDNA from individual DNA samples of *M. pachydermatis* isolates resulted in amplicons of the expected sizes [15,16]. Sequencing of amplicons revealed three sequence-types for *chs*-2 (namely *A*, *B*, *C*), and LSU (*A*₁, *B*₁, *C*₁) and four for ITS-1 (*A*₂, *B*₂, *C*_1I, *C*_3I). All *chs*-2, LSU and ITS-1 sequence-types of *M. pachydermatis* matched previously determined sequences of isolates available in GenBank and DDBJ databases (accession numbers: DQ915500–DQ915509; EU158826, EU158827 and EU158829) [15,16]. Genotypic grouping (A–C) was concordant between *chs*-2, LSU and ITS-1 [15,16] and three major *M. pachydermatis* genotypes were identified,
Table 1  Ketoconazole (KTZ), itraconazole (ITZ), voriconazole (VOR), posaconazole (POS) and fluconazole (FLZ) E-test and CLSI BMD minimum inhibitory concentration (MIC) data of the Malassezia pachydermatis strains here examined. Essential agreement (EA) among MIC obtained by E-test compared with CLSI BD method for *in vitro* susceptibility testing of *Malassezia pachydermatis* strains is also reported.

<table>
<thead>
<tr>
<th>Antif. agents</th>
<th>Method</th>
<th>Range</th>
<th>MIC 50</th>
<th>MIC 90</th>
<th>EA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>KTZ</td>
<td>CLSI</td>
<td>&lt;0.008–0.064</td>
<td>0.016</td>
<td>0.032</td>
<td>90.3</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>0.002–0.0094</td>
<td>0.006</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>ITZ</td>
<td>CLSI</td>
<td>&lt;0.008–0.016</td>
<td>&lt;0.008</td>
<td>&lt;0.008</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>&lt;0.002–0.016</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td>CLSI</td>
<td>0.032–0.500</td>
<td>0.064</td>
<td>0.125</td>
<td>87.1</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>&lt;0.002–1.5</td>
<td>0.032</td>
<td>0.0190</td>
<td></td>
</tr>
<tr>
<td>POS</td>
<td>CLSI</td>
<td>&lt;0.008–0.064</td>
<td>0.016</td>
<td>0.032</td>
<td>93.5</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>&lt;0.002–0.064</td>
<td>0.008</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>FLZ</td>
<td>CLSI</td>
<td>4–&gt;64</td>
<td>8</td>
<td>16</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>1–256</td>
<td>4</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

MIC<sub>50</sub> and MIC<sub>90</sub>: MICs at which 50% and 90% of isolates tested, respectively, were inhibited.

Table 2  Interpretive antifungal susceptibility classification of 62 isolates of *Malassezia pachydermatis* determined by E-test and CLSI method.

<table>
<thead>
<tr>
<th>Antif. agent</th>
<th>Method</th>
<th>Isolates by category (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Discrepancy (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>KTZ</td>
<td>CLSI</td>
<td>51</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>44</td>
<td>24.2</td>
</tr>
<tr>
<td>ITZ</td>
<td>CLSI</td>
<td>58</td>
<td>9 (3.5)</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>45</td>
<td>10 (16.1)</td>
</tr>
<tr>
<td>VOR</td>
<td>CLSI</td>
<td>53</td>
<td>8 (55.5)</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>43</td>
<td>24.2</td>
</tr>
<tr>
<td>POS</td>
<td>CLSI</td>
<td>54</td>
<td>8 (71.1)</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>47</td>
<td>21.0</td>
</tr>
<tr>
<td>FLZ</td>
<td>CLSI</td>
<td>37</td>
<td>59.7</td>
</tr>
<tr>
<td></td>
<td>E-test</td>
<td>34</td>
<td>33.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percentage of isolates classified as susceptible (S); intermediary susceptible (I); and resistant (R).  
<sup>b</sup>Percentage of results representing minor (isolates classified as ‘S’ or ‘R’ by one of the two tests), major (isolate classified as ‘S’ by the CLSI BMD and ‘R’ by the E-test), or very major (isolate classified as ‘R’ by the CLSI BMD and ‘S’ by the E-test) discrepancies with respect to those of the reference (CLSI M27-A2) method. CA reflects the percentage of isolates classified in the same category by the reference (CLSI M27-A2) method and E-test method.

except for KTZ (77.4%) and FLZ (74.2%). In particular, when discrepancies between the two methods were observed, they were minor errors, with only three very major errors found with KTZ. This finding is likely due to the limitations of the reference method employed [18]. Indeed, trailing endpoints, a well-described phenomenon with CLSI BMD method in tests with azoles, can lead to over interpretation of the MICs [18]. Furthermore, it has been proposed that, in order for a susceptibility test to be considered specific, less than 5% very major and major errors should occur [33]. Therefore, the results from our study show that the E-test method represents an alternative to test the susceptibility of *M. pachydermatis* to all azole compounds used in our studies, with the exception of KTZ.

To our knowledge, a limited number of studies have compared the E-test and CLSI BMD methods in *in vitro* antifungal susceptibility of *Malassezia* yeasts. Velegraki *et al.* [18] reported concordance between the E-test and CLSI BMD methods, whereas the results from studies by Nascente *et al.* [23,24] emphasized the need for standardization of the tests. In particular, it has been suggested that the same media should be employed to achieve concordant microdilution and E-test results [18]. In this study, we used SDA medium with 1% of Tween 80, an inoculum suspension equivalent to $1 \times 10^6$ CFU/ml and 48-h incubation time, which revealed a very good agreement between the E-test and CLSI BMD methods in susceptibility tests of *M. pachydermatis* to different azole compounds. Additionally, the statistical analysis of the results shows that limits of agreement were small enough to confirm that E-test method can be used in place of the CLSI BMD for clinical purpose [34]. In particular, the E-test method is simple to perform, does not require any specialized laboratory equipment and can be used for the routine testing of this species. In this study, an association of *M. pachydermatis*...
to antifungal compounds and the different genotypes was observed. In particular, genotype B was found to have a higher FLZ MIC value than observed in genotypes A and C, irrespective of the presence of skin lesions. Conversely, genotype B isolates recovered from lesions on the dog’s showed a higher susceptibility to KTZ, VOR, and POS. It has been suggested that host skin may be responsible for the selection of specific genetic populations of Malassezia yeasts having an indirect effect on their drugs susceptibility [14,35]. The results of this study support this hypothesis and suggest a direct correlation between specific genotypes of Malassezia and in vitro drug susceptibility. In addition, other phenetic differences exist between M. pachydermatis genotype B and genotypes A/C [15,16], i.e., more frequent lipid-dependent isolates and higher recovery from dogs without skin lesions [15,16]. The lipid dependence is usually associated with a deficiency of genes encoding fatty acid synthase and to an abundance of membrane sterol [37] and/or the composition of skin lipids differs in animals with lesions, thus affecting drug susceptibility. Indeed, resistance to antifungal compounds has been observed in cases of alteration of the composition of membrane sterol [37] and/or the up-regulation of CDR genes encoding the CDR efflux pumps [38].

In conclusion, the results of this study confirm that M. pachydermatis is highly susceptible to ITZ and KTZ, while susceptibility to FLZ is limited. The data reported herein also provide evidence that posaconazole could be used in therapeutics of generalized M. pachydermatis infections. Finally, the good categorical agreement between the E-test and the reference CLSI broth microdilution method provides further confirmation of the reliability of the former method for routine use in clinical mycology laboratories. Future studies should focus on testing the correlation between the susceptibility to antifungal agents in vitro and in vivo.

Acknowledgements

Funding for this work was provided by the University of Bari, Fondi di Ateneo 2010. The authors thank Cinzia Cantacessi for revising the English.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References


This paper was first published online on Early Online on 5 April 2012.


