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Short communication

Assessment of the antifungal susceptibility of *Malassezia pachydermatis* in various media using a CLSI protocol

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ABSTRACT

The microdilution antifungal method (CLSI BMD, M27-A3) was used for testing the antifungal susceptibility of *Malassezia* species. However, optimal broth media that allow sufficient growth of *M. pachydermatitis* and produce reliable and reproducible MICs using the CLSI BMD protocol are yet to be established. In this study, the susceptibility of *M. pachydermatis* isolates to ketoconazole (KTZ), itraconazole (ITZ) and fluconazole (FLZ) was evaluated *in vitro* by the CLSI BMD test using Christensen's urea broth (CUB) and mRPMI 1640 containing lipid supplementation, Sabouraud dextrose broth with 1% tween 80 (SDB), and Dixon broth (DXB). A FLZ-resistant *M. pachydermatis* was generated *in vitro* and tested under the same conditions. A good growth of *M. pachydermatis* incubated for 48 and 72 h, respectively, was observed in CUB, SDB and DXB and not in mRPMI 1640 ($p < 0.001$). No statistically significant differences were detected between the MIC values registered after 48 h and 72 h incubation. ITZ displayed lower MIC values than KTZ and FLZ regardless of the media employed. A large number of FLZ-resistant *Malassezia* strains (86.6%) was observed using DXB. A MIC > 64 mg/L was observed only when the FLZ-resistant *M. pachydermatis* isolate was tested in SDB. Based on the results obtained herein, culture in SDB, stock inoculum suspensions of $1–5 \times 10^6$ CFU/ml, and an incubation time of 48 h are proposed as optimal conditions for the evaluation of the *in vitro* antifungal susceptibility of *M. pachydermatis* using a modified CLSI BMD protocol.

1. Introduction

Malassezia pachydermatis is a common commensal yeast of the skin of animals, that may become pathogenic under the influence of predisposing factors (Cafarchia and Otranto, 2004; Cafarchia et al., 2007; Miceli et al., 2011). The clinical manifestations include otitis externa and various forms of dermatitis in domestic animals and nosocomial infections in humans (Chryssanthou et al., 2001; Batra et al., 2005). Dermatitis by *Malassezia* is one of the most common diseases of dog skin, and treatment of severe infections may involve systemic therapy with high doses of antifungal agents (*e.g.*, ketoconazole [KTZ] and itraconazole [ITZ]) for prolonged time periods (Negre et al., 2009). The increasing number of case reports of human and animal skin infections by *Malassezia* (Batra et al., 2005; Miceli et al., 2011) has stimulated research towards the establishment of a standardized method for the assessment of susceptibility of *M. pachydermatis* to various antifungal agents *in vitro*. The reference method (CLSI, M27-A2 document, to date M27-A3 document – CLSI, 2008) for testing the susceptibility of yeasts (*i.e.*, *Candida* spp. and *Cryptococcus neoformans*) is inapplicable to yeasts of the genus *Malassezia*, because of their lipo-dependent

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properties (Velegraki et al., 2004; Cantón et al., 2009; Nijima et al., 2011; Cafarchia et al., 2012). Christensen's urea broth (CUB) and RPMI 1640 broth (mRPMI) containing lipid supplementation, Sabouraud dextrose broth with 1% tween 80 (SDB), and Dixon broth (DXB) (Velegraki et al., 2004; Rincón et al., 2006; Cantón et al., 2009; Nijima et al., 2011; Cafarchia et al., 2012) are generally employed to replace the RPMI1640 in the CLSI BMD protocol. However, optimal broth media that allow sufficient growth of *M. pachydermatitis* and produce reliable and reproducible MICs using the CLSI BMD protocol are yet to be established. The present study aims to (i) investigate the *in vitro* susceptibility of clinical isolates of *M. pachydermatis* to fluconazole (FLZ), KTZ and ITZ, as measured by CLSI BMD protocol, using CUB, mRPMI 1640, SDB and DXB broths as media, (ii) to estimate the agreement for the CLSI BMD method between different media (iii) to evaluate the incubation time for testing *M. pachydermatis* to these azoles, and (iv) to establish the optimal medium and time of incubation for testing the susceptibility of *M. pachydermatis* to antifungal agents *in vitro*.

2. Materials and methods

2.1. Malassezia pachydermatis strains

A total of 30 *Malassezia pachydermatis* strains were tested. The isolates were identified phenotypically (macro- and microscopic morphology) and physiologically as previously reported (Guillot et al., 1996; Cafarchia et al.,2011). In order to establish optimal broth media that produces reliable and reproducible MICs, a FLZ-resistant *M. pachydermatis* was generated *in vitro* (Fekete-Forgács et al., 2000; Jesus et al., 2011) and tested under the same conditions. Isolates were maintained in modified-Dixon agar for 7 days at 32 °C and tested for their *in vitro* susceptibility to antifungal agents. All isolates were deposited in the fungal collection at the Faculty of Veterinary Medicine of the University of Bari, Italy.

2.2. In vitro growth of Malassezia yeasts using different media

Growth of *Malassezia* strains in CUB, mRPMI 1640, SDB and DXB broths was tested after 48 and 72 h incubation, respectively. In particular, mRPMI 1640 broth was buffered with MOPS and supplemented with 2% glucose, 0.4% ox bile, 1% Tween 40, 0.2% glycerol and 0.2% oleic acid (mRPMI) and CUB was supplemented with 0.1% Tween 80 and 0.5% Tween 40 (Velegraki et al., 2004; Rincón et al., 2006). The starting inocula of *M. pachydermatis*, obtained using 7 days-old colonies grown in Dixon agar, were suspended in 5 ml of sterile distilled water; turbidity was adjusted spectrophotometrically (Biosan DEN 1) to an optical density of 2.4, corresponding to $1–5 \times 10^6$ CFU/ml. Subsequently, two dilutions (1:100 and 1:20) of the inoculum were performed using each medium and a total of 100 μL of the final dilution was transferred into a 96-well microtiter plate containing 100 μL of each medium. Triplicates of each plate were incubated for 48–72 h at 32 °C together with a negative control (medium only) and growth of *Malassezia* was observed, measured spectrophotometrically and expressed as average of the absorbance values at 595 nm minus the average of absorbance of the negative control (OD).

2.3. In vitro susceptibility testing

Susceptibility of *M. pachydermatis* strains in CUB, SDB and DXB (Velegraki et al., 2004; Rincón et al., 2006; Cantón et al., 2009; Nijima et al., 2011; Cafarchia et al., 2012) to antifungal agents was tested using the reference CLSI BMD protocol. The stock inoculum suspensions were prepared as described above. KTZ, ITZ (Sigma-Aldrich®, Milan, Italy) and FLZ (Pfizer Pharmaceuticals, Groton, CT, USA) were supplied by the manufacturers as pure standard compounds.

KTZ, ITZ and FLZ were prepared as 100× stocks in dimethyl sulfoxide (Merck, Darmstadt, Germany) or in water (FLZ) and stored at −80 °C until they were used. The concentration of each antifungal drug ranged from 0.008 to 16 mg/L, with the exception of FLZ, whose concentration ranged from 0.03 to 64 mg/L. Visual reading of plates was performed after 48 h and 72 h of incubation at 32 °C; the growth of each strain at various drug concentrations, as well as of a positive control cultured in drug-free medium, was recorded. The MIC of each isolate was defined as the lowest concentration of the agent producing a predominant decrease in turbidity (*i.e.*, 90% of inhibition) when compared to the control growth as previously reported (Velegraki et al., 2004). Triplicates of each plate were run and each drug dilution was tested as duplicates in each plate. Quality control strains (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258; American Type Culture Collection, Manassas, VA) were included to assess the accuracy of the drug dilutions and the reproducibility of the results (CLSI, 2008).

2.4. Results interpretation.

Tentative breakpoints to classify strains as susceptible (S), susceptible dependent upon dose (SDD), or resistant (R) have never been established for *M. pachydermatis*. Breakpoints (*i.e.*, KTZ: S ≤8 mg/L, R ≥16 mg/L; ITZ: S ≤0.125 mg/L, SDD 0.25–0.5 mg/L, R ≥1 mg/L; FLZ: S ≤8 mg/L, SDD 16–32 mg/L, R ≥64 mg/L) established for different azole compounds on *Candida* spp. isolates (Rex et al., 1997) were used in the present study and the interpretive categories for each medium (*i.e.*, S, SDD, R) were compared. MIC results of no more than two-fold dilutions between the methods (*i.e.*, culture in different media) were defined as being in essential agreement (EA), whereas results falling within the same interpretive category were considered in categorical agreement (CA) (Iatta et al., 2011).

2.5. Statistical analysis

Both on-scale and off-scale results were included in the analysis. The low and the high off-scale MICs were converted as the lowest MIC or the highest MIC, respectively. MIC values at 48 and 72 h, respectively, were screened with paired Student's *t*-test. Differences in CLSI BMD MIC values at 48 and 72 h, using different media were