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Direct antifungal susceptibility testing of positive *Candida* blood cultures by Sensititre YeastOne

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SUMMARY

Invasive candidiasis is associated with high morbidity and mortality. Differences in the virulence and susceptibility of the various *Candida* spp. to antifungal drugs make the identification and rapid MIC determination very important for clinical management.

The aim of this study was to improve the turnaround time (TAT) for antimicrobial test generation by susceptibility testing directly from the bottle of blood culture positive for yeasts, circumventing the isolation process and thereby generating an accurate antifungal MIC determination as quickly as possible.

Sensititre YeastOne was used by direct inoculation from positive blood culture bottles in 40 cases of candidaemia. All the results were compared with those obtained using standard laboratory procedures after subculturing from a positive bottle onto solid media.

The results obtained from direct inoculation of Sensititre YeastOne compared with tests carried out using standard procedures show that out of a total of 40 strains tested no *very major errors* or *major errors* and only 4 *minor errors* occurred (98% agreement rate out of a total of 240 drug/bug combinations tested), thus generating an accurate antifungal MIC determination and saving an average time of 24 hours compared with the time required for the standard procedures traditionally used.

KEY WORDS: Invasive candidiasis, Direct antifungal MIC determination, Sensititre YeastOne

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INTRODUCTION

Candida spp. are the fourth commonly isolated organisms from nosocomial bloodstream infections (BSIs) (9%), with the highest mortality rate (39,2%) (Pfaller *et al.*, 2001; Wisplinghoff *et al.*, 2004). Although *C. albicans* is the most common cause of mycoses in hospitalized patients, the rate of candidaemia caused by *C.* non *albicans* spp. is increasing (Tortorano *et al.*, 2006). Among these candidiases, *C. glabrata* and *C. krusei* are common (15-25% of bloodstream *Candida* isolates worldwide) and difficult to treat because of their reduced susceptibility to common antifungal agents (Pfaller *et al.*, 2004). Thus, the rate of fungal infections and the different susceptibility to antifungal drugs among different species of *Candida* highlight the importance of an accurate species identification and above all a rapid antifungal MIC determination (Pfaller *et al.*, 1998; Sandven *et al.*, 1998; St-Germain *et al.*, 2001).

It has recently been established that 48 hours of incubation for blood cultures may be sufficient to detect 90% of *Candida* spp. (Bourbeau and Pohlman, 2001). Moreover, as is well known, traditional antifungal susceptibility testing of blood cultures positive by microdilution method requires 24 hours more incubation to obtain isolated colonies, and an additional 24 hours to generate MICs for *Candida* spp. (about 4 days from the time of sample are needed to obtain an antifungal MIC determination).

With the aim of reducing the turnaround time

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(TAT) several authors emphasise the importance of generating an antimicrobial susceptibility test (AST) directly from blood cultures (Bouza *et al.*, 2004; Ling *et al.*, 2003, Morrel *et al.*, 2005; Funke and Funke-Kissling., 2004, Bruins *et al.*, 2004; De Cueto *et al.*, 2004). Nevertheless, although the importance of AST directly from positive blood cultures is largely considered for bacterial isolates, poorly documented data exist for yeasts.
This study shows for the first time that the performance time for antifungal susceptibility can be improved using the Sensititre YeastOne directly from *Candida* spp. positive blood cultures, bypassing the isolation process and accelerating the reporting of antifungal susceptibility with the explicit aim of intervening to reduce the TAT to generate susceptibility results.

MATERIALS AND METHODS

Specimens
A total of 40 blood cultures submitted to our laboratory from different clinical units within S. Maria degli Angeli Hospital (Pordenone, Italy) positive for yeasts were evaluated. In short, when the standard aerobic and/or standard anaerobic blood culture bottles, incubated using the BacT/ALERT 3D system (bioMérieux, Marcy l'Etoile, France) signalled positive, a microscopic examination with Gram stain was carried out to confirm the isolate and the presence of yeasts only. Blood cultures yielding more than one organism were excluded from data analysis.

Direct species identification
Subcultures of positive blood cultures were carried out on solid medium CHROMagar (KIMA, Padova, Italy) and incubated for 24 hours. All yeast isolates observed on CHROMagar were identified by colony morphology and pigmentation (Horvath *et al.*, 2003).

Standard species identification
In order to confirm the species identification obtained by direct chromogen method, yeasts isolated on Sabouraud dextrose agar (SDA) (KIMA) were also evaluated for germ tube production in serum and their biochemical patterns were determined by ID 32 C panels (bioMérieux) according to the manufacturer's instructions after an additional 48 hours on the mini API autoreader (bioMérieux).

Standard antimicrobial susceptibility testing (SAST)
Sensititre YeastOne (TREK Diagnostic Systems, Cleveland, Ohio) was used in this study as the reference method. The panels, containing dried (dilution range, µg/ml) amphotericin B (0.008-16), fluconazole (0.125-256), itraconazole (0.008-16), ketoconazole (0.008-16), 5-fluorocytosine (0.03-64), voriconazole (0.008-16) and alamar blue as an indicator dye were inoculated and read according to the manufacturer's instructions. In short, yeast isolates from a 24 h culture on SDA were suspended in water to the equivalent of a 0.5 McFarland turbidity standard. After inoculation and incubation at 35°C in air, panels were

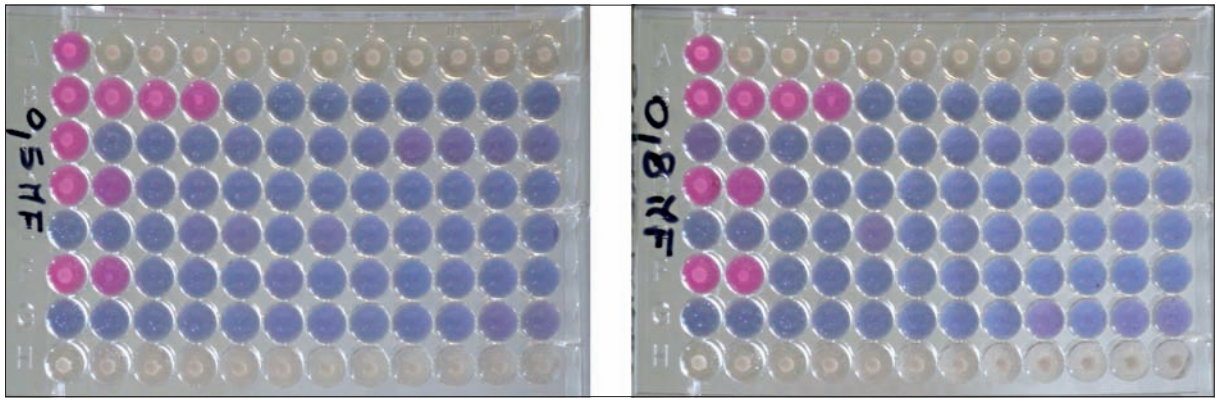

FIGURE 1 - *Comparison of two different sizes of inoculum preparations (0.5 and 0.8 Mc Farland) used for DAST assay procedures. (the figure shows that one of each comparison made either for C. albicans, or for C. non-albicans obtained similar results).*

read and colorimetric minimum inhibitory concentrations (MICs) were interpreted as the lowest concentration of antifungal that substantially inhibited the growth of the yeast as detected by observing a colour change from blue (no growth) to red (growth) (Figure 1).
Interpretation of the MIC was determined by the values given in the NCCLS document M27-A2 (2002). Quality control was performed as stated by the manufacturer using *C. parapsilosis* (ATCC 22019).

Direct antimicrobial susceptibility testing (DAST)
The Sensititre YeastOne method was used to perform DAST. In short, after shaking the positive blood culture bottle, the sample was aspirated by vacuum principle into Sarstedt S-Monovette serum gel 4.9 ml tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 1,525 g for 15 minutes, to obtain separation from the red cells.
After the serum discharge, the microbial pellet, in the upper layer of gel, was gradually re-suspended in physiological solution to obtain a turbidity of 0.5 and 0.8 Mc Farland.
Sensititre YeastOne testing was carried by adding 20 µl of inoculum suspension in RPMI 1640 broth to obtain a working suspension of which 100 µl was added in each well. The panels were then sealed in accordance with the manufacturer's instructions and as described in the previous section.
The results obtained for each single specimen with DAST and SAST were compared.
Discrepancies between the two methods were defined as very major, major, and minor. Very major errors were those determined as sensitive by DAST but resistant by SAST. Major errors were those reported as resistant by DAST but sensitive by SAST. Minor errors were those determined to be susceptible or resistant by DAST and intermediate by SAST or vice versa.

RESULTS

In agreement with Bourbeau and Pohlman (2001), under our conditions more than 95% of blood culture bottles positive for yeasts signalled in the first 48 hours of culture.
A total of 40 blood cultures positive for *Candida* were evaluated by DAST and SAST. Of the 40 isolates, 20 were *C. albicans*, 10 *C. glabrata*, 6 *C. tropicalis*, 4 *C. krusei*. CROMagar and DAST results were available 24 hours after the blood culture signalled positive. Standard species identification and SAST results required 72 and 48 hours respectively, because an additional 24 hours of incubation were necessary to obtain isolated colonies. 100% similarity between both identification methods performed was identified (data not shown).
Not significant differences were observed among the results obtained with the two different sizes of inoculum used for DAST (an example is shown in Figure 1). Table 1 shows the MIC ranges, MIC 50 (MICs at which 50% of the isolates tested were inhibited) and MIC 90 (MICs at which 90% of the isolates tested were inhibited) of the results obtained for 40 isolates of *Candida* spp. with DAST and SAST.
As shown in Table 2, compared to the reference method, all 20 strains of *C. albicans* examined by DAST produced completely comparable results (100% similarity between 120 individual antifungal test results). For two of the 10 strains of *C. glabrata* the difference of a dilution obtained for fluconazole led to an overestimated result from S-DD to R.
However, it was considered a *minor error* since it was clinically insignificant (96.6% similarity between the 60 single results).
Two *minor errors* were detected for fluconazole among the results obtained for the six strains of *C. tropicalis*, with an overestimate from S to S-DD; once again they were considered clinically insignificant (94.4% similarity between the 36 single results).
In the case of the four strains of *C. krusei*, a yeast known for its particular criticism under the interpretative profile for the natural resistance expressed, one dilution difference for fluconazole (128 µg/ml with DAST and 256 µg/ml with SAST) was observed, without any impact on the expected interpretive category (i.e., resistant) (100% similarity between the 24 individual results). As summarized in Table 2, on the whole, our results show that out of a total of 40 strains tested no *very major errors* or *major errors* were revealed and only four *minor errors* (98% agreement rate out of a total of 240 drug/bug combinations tested).

TABLE 1 - *In vitro susceptibilities (MIC's range, MIC_{50} and MIC_{90}) of the 40 isolates of Candida spp., as determined by standard (SAST) and direct (DAST) antimicrobial susceptibility testing.*

MIC (μg/ml)						
Candida spp. (no. of isolates) and antifungal agents	*MIC's Range (SAST)*	*MIC's Range (DAST)*	*MIC_{50} (SAST)*	*MIC_{50} (DAST)*	*MIC_{90} (SAST)*	*MIC_{90} (DAST)*
Candida albicans (20)						
amphotericin	0.008-0.06	0.008-0.06	0.03	0.03	0.06	0.06
fluconazole	0.125-0.25	0.125-0.25	0.125	0.125	0.25	0.25
itraconazole	0.016-0.03	0.016-0.03	0.016	0.016	0.03	0.03
ketoconazole	0.008-0.016	0.008-0.016	0.008	0.008	0.016	0.016
5-fluorocytosine	0.03-0.06	0.03-0.06	0.03	0.03	0.06	0.06
voriconazole	0.008-0.016	0.008-0.016	0.008	0.008	0.016	0.016
Candida glabrata (10)						
amphotericin	0.03-0.25	0.03-0.25	0.06	0.06	0.125	0.125
fluconazole	16-32	16-64	32	32	64	64
itraconazole	0.5-4	0.5-4	1	1	2	2
ketoconazole	0.016-2	0.016-2	1	1	2	2
5-fluorocytosine	0.03-2	0.03-2	0.06	0.06	0.5	0.5
voriconazole	0.25-2	0.25-2	0.5	0.5	1	1
Candida tropicalis (6)						
amphotericin	0.25-1	0.25-1	0.25	0.25	0.5	0.5
fluconazole	4-8	4-16	4	4	8	8
itraconazole	0.25-2	0.25-2	0.5	0.5	1	1
ketoconazole	0.125-1	0.125-1	0.5	0.5	1	1
5-fluorocytosine	0.25-4	0.25-4	0.5	0.5	2	2
voriconazole	0.008-1	0.008-1	0.06	0.06	0.5	0.5
Candida krusei (4)						
amphotericin	0.25-1	0.25-1	0.25	0.25	0.5	0.5
fluconazole	256	128	256	128	256	128
itraconazole	0.5-2	0.5-2	1	1	2	2
ketoconazole	0.5-2	0.5-2	1	1	2	2
5-fluorocytosine	0.125-16	0.125-16	0.5	0.5	4	4
voriconazole	0.125-2	0.125-2	0.25	0.25	1	1

DISCUSSION

Candidaemia is associated with one of the highest rates of mortality of all bloodstream infections (BSIs) (Harbarth *et al.,* 2002). Crude mortality rates of 67% were reported for BSI due to *Candida* spp, compared to BSI due to *Enterobacter* spp (53%), *Pseudomonas aeruginosa* (47%), or *Staphylococcus aureus* (24%) (Harbarth *et al.,* 2002). Inadequate empirical antifungal treatment (Ibrahim *et al.*, 2000; Bouza *et al.*, 2004), or even a delay of initiation of adequate antifungal therapy in patients with candidaemia (Morrel *et al.,* 2005; Garey *et al.*, 2006) is associated with a high risk of mortality and prolonged hospital stay. The emergence of antifungal resistance mechanisms, unknown until a few years ago, is increasing and widely observed and documented worldwide (Pappas *et al.,* 2004; Pfaller *et al.*, 2007; Pfaller *et al.*, 2001; Pfaller *et al.*, 1998; Pfaller *et al.*, 2004).

On the one hand, this phenomenon emphasized the need to evaluate an antifungal susceptibility test that assures a high reproducibility *in vitro,* according to NCCLS M27-A document (2002), and on the other to ensure that antifungal testing results are available as soon as possible to avoid inadequate empirical treatments being con-

TABLE 2 - *Agreement in antimicrobial susceptibility testing between direct (DAST) and standard (SAST) methods for Candida spp. using Sensititre YeastOne.*

No. of isolates	*Isolates tested*	**Single antifungal consistent results*	*Minor errors*	*Major errors*	*Very major errors*	***Comment (% agreement between DAST and SATS interpretative category)*
20	*Candida albicans*	120	0	0	0	Total agreement (100%)
10	*Candida glabrata*	58	2	0	0	Overestimate (from S-DD to R) of fluconazole in 2 strains (96.5%)
6	*Candida tropicalis*	34	2	0	0	Overestimate (from S to S-DD) of fluconazole in 2 strain (94.4%)
4	*Candida krusei*	24	0	0	0	Difference between a dilution of fluconazole in both strains tested, but always within the range of the resistance (100%)

*This column shows the number of tests performed represented by the total number of antifungal agents tested out of all the strains. **Interpretative criteria for amphotericine B: S ≤1 µg/ml. Interpretative criteria for fluoconazole: S ≤8 µg/ml; S-DD 16-32 µg/ml; R ≥64 µg/ml. Interpretative criteria for itraconazole: S ≤0.125 µg/ml; S-DD 0.25-0.5 µg/ml; R ≥1 µg/ml. Interpretative criteria for 5-fluorocytosine: S ≤4 µg/ml; I 8-16 µg/ml; R ≥32 µg/ml. Interpretative criteria for voriconazole: S ≤1 µg/ml; S-DD 2 µg/ml; R ≥4 µg/ml. Interpretative criteria for ketoconazole: S<0.5 µg/ml; R >1 µg/ml.

tinued. In order to impact on the timely choice of appropriate antifungal therapy, this study is the first to evaluate the performance of a broth microdilution antimicrobial test directly from the blood culture bottles positive for yeasts and offers evidence that this method could really improve the TAT of generating an antifungal susceptibility result. In fact we show that the direct use of Sensititre YeastOne allows the TAT to be reduced very significantly, saving an average time of 48 hours compared with the time required for the standard procedures traditionally used.

Although the low number of samples examined limits our experimental method, it is clear that by using the procedures described it is possible to obtain a preliminary evaluation which is completely consistent for 98% of the antifungal agents tested, only 3 days on average after taking the sample. This means that within this time a concrete indication for managing the antifungal therapy underway can be given or, if necessary, modified (Pappas *et al.,* 2004).

The DAST results we obtained, available 24 hours after the blood cultures signalled positive for yeasts compared to 48 for SAST, show that this method could significantly and positively impact upon the time for earlier administration of appropriate targeted antifungal therapy. In conclusion, in support of the SAST method used by all laboratories as the only method approved by international guidelines to date, our results show that the DAST method could be a very useful instrument to improve and promptly institute therapy by offering clinicians preliminary but specific results of antifungal susceptibility for *C. albicans* and for *C. non-albicans* species.

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