that differentiates between \textit{S. aureus} and CoNS, as \textit{S. aureus} produces a nuclease that is uniquely and consistently thermostable.\(^1\) TSN testing involves removing 2–3 mL of blood broth from a blood culture and heating the blood broth in a boiling water bath for 15 min. Once cooled, two to three drops are placed in a 6 mm well cut in the media (Southern Group Laboratory, Corby, UK) and the plate is incubated for 2–4 h at 37°C. A positive reaction, indicating thermonuclease activity, shows an area of clearing at the edge of the well.\(^1\)

Addenbrooke’s Hospital is a tertiary referral hospital with 1100 beds. The impact of the TSN test on the immediate management of positive blood cultures was assessed for the calendar month of August 2007. Blood cultures were processed using BacT/Alert 3D (bioMérieux, Basingstoke, UK), and TSN testing was performed when Gram-positive cocci in clumps were seen on microscopy. Blood cultures growing the same organism within a 2 week period were counted as one episode. Patients were identified prospectively and assessed to determine the reliability of the test (in terms of sensitivity, specificity and positive- and negative-predictive values), when compared with tube coagulase, and the impact of the TSN result following a positive TSN result had no effect on therapy. A negative TSN did not result in treatment cessation when patients were already on antimicrobial agents at the time of the positive blood culture. The TSN made no immediate clinical impact in 78 of 88 (9.1%) cases pending the TSN result and was not commenced when a negative result was obtained. Antimicrobial therapy was withheld in 8 of 88 (9.1%) cases. The performance of TSN has been described previously.\(^1\)

The apparent single true false-negative TSN result is of concern due to potential delays in treatment. This has been described previously with the BacT/Alert blood culture system.\(^3\)

TSN is simple and cheap and does not require complex and expensive equipment or expertise, enabling it to be used in any clinical laboratory. Although this study is small, with only 90 episodes, it is prospective in nature. We believe that TSN is a useful adjunct to routine staphylococcal identification methods and leads to a reduction in unnecessary antimicrobial use.

**Acknowledgements**

We acknowledge Rachael Smith for laboratory and computer support.

**Funding**

No specific funding was received.

**Transparency declarations**

None to declare.

**References**


**In vitro activities of combinations of amphotericin B, posaconazole and four other agents against \textit{Rhizopus}**

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Keywords: zygomycosis, itraconazole, \textit{Rhizopus oryzae}, \textit{Rhizopus microsporus var. rhizopodiformis}

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**Table 1. Performance table comparing TSN and coagulase results**

<table>
<thead>
<tr>
<th></th>
<th>Coagulase positive</th>
<th>Coagulase negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSN positive</td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>TSN negative</td>
<td>2</td>
<td>77</td>
<td>79</td>
</tr>
<tr>
<td>No TSN result</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>11</td>
<td>79</td>
<td>90</td>
</tr>
</tbody>
</table>
Sir,

Zygomycosis is a severe, life-threatening fungal infection that occurs mainly in patients with diabetes mellitus, or with haematological malignancies, after organ transplantation, in conditions of iron overload or those treated with immunosuppressive drugs. *Rhizopus oryzae*, followed by *Rhizopus microsporus* var. *rhizopodiformis*, are the most frequently involved organisms, accounting for ~75% of all cases. Amphotericin B is the drug of choice for the treatment of zygomycosis, but overall mortality rate is high. Recently, posaconazole has shown to be active as salvage therapy for disseminated zygomycosis, but few studies still exist and the search of novel therapeutic strategies is still necessary. Combination of antifungal agents with different active mechanisms could be an interesting therapeutic option for improving clinical outcomes. Few data exist on the *in vitro* effect of combinations of antifungal drugs against zygomycetes. Only associations of amphotericin B with flucytosine and of terbinafine with either amphotericin B or triazoles have been evaluated. The interaction between amphotericin B and azoles against zygomycetes has not been investigated. There is recent evidence for synergistic interaction between the echinocandin drug caspofungin and amphotericin B lipid complex in a murine model of disseminated *R. oryzae* infection. However, *in vitro* studies on interactions of echinocandins with other antifungals against *Rhizopus* spp. have not been performed. The aim of this study was to investigate the *in vitro* interaction of amphotericin B and micafungin, a recently developed echinocandin, and of each with posaconazole, itraconazole, voriconazole or ravuconazole against *Rhizopus*.

Nineteen clinical isolates were tested, comprising 9 isolates of *R. oryzae* and 10 of *R. microsporus* var. *rhizopodiformis*. The isolates were grown on potato dextrose agar plates and incubated at 30°C for 4–7 days. Inocula were prepared following the CLSI (formerly NCCLS) M38-A document. The spore suspensions were adjusted to a final concentration of 4 × 10^3–5 × 10^5 cfu/mL with a haemocytometer. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included as quality controls.

The following drugs were tested: amphotericin B (USP, Rockville, MD, USA), posaconazole (Schering-Plough Europe, Brussels, Belgium), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), voriconazole (Pfizer Inc., Madrid, Spain), ravuconazole (Bristol-Myers Squibb Company, New Brunswick, NJ, USA) and micafungin (Astellas Pharma Inc., Tokyo, Japan). All were obtained as pure powders from their respective manufacturers and dissolved in DMSO, apart from micafungin, which was dissolved in water.

The MIC of all drugs was defined as the lowest concentration that produced a 100% growth inhibition after 24 h of incubation at 35°C. Drug interactions were assessed by a checkerboard microdilution method that also included the determination of the MIC of each drug alone on the same plate, by using the parameters outlined in CLSI. Antifungal agents were placed in rows or in columns of the trays to test the different combinations. The fractional inhibitory concentration index (FICI) was used to classify drug interaction. Nearly 80% of the tests were repeated, and interactions demonstrated mainly the same tendencies.

Of the 171 tests performed, synergistic interaction was observed in 25 (14.6%) cases, and for the rest, we obtained an indifferent effect. No antagonistic interactions were observed in any case. Amphotericin B plus posaconazole or itraconazole were the combinations that presented the highest percentage of synergistic interaction, 47.4% and 42.1%, respectively, being more frequent against *R. microsporus* var. *rhizopodiformis* (6/10 and 5/10, respectively) than against *R. oryzae* (3/9 for both combinations) (Table 1). Amphotericin B combined with ravuconazole showed synergism for 21.1% of the isolates. Interactions of amphotericin B with voriconazole or micafungin and of micafungin with itraconazole were mainly indifferent; they were synergistic in only 5.3% to 10.6% of the tests. Combinations of micafungin with posaconazole, voriconazole or ravuconazole showed indifferent interactions.

In murine models of disseminated zygomycosis, posaconazole has demonstrated only limited efficacy. However, Spellberg et al. have recently benefited by using the combination of amphotericin B lipid complex and caspofungin with diabetic mice infected with *R. oryzae*. Over the last few years, posaconazole has shown efficacy as salvage therapy for invasive zygomycosis. Eventually, the activity of posaconazole alone, added to its synergistic effect when combined with amphotericin B, could render this antifungal association particularly attractive.

In contrast to previous *in vitro* studies, in which the combination of micafungin with amphotericin B produced synergistic effects against *Scedosporium* spp.

In summary, our results demonstrated that the combination of amphotericin B with posaconazole or itraconazole showed a

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**Table 1. Interactions of amphotericin B with posaconazole, itraconazole, or ravuconazole against Rhizopus spp.**

<table>
<thead>
<tr>
<th>Rhizopus species (number of isolates)</th>
<th>AMB/PSC GM MIC (mg/L)</th>
<th>AMB/ITC GM MIC (mg/L)</th>
<th>AMB/RVC GM MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMB</td>
<td>PSC</td>
<td>AMB/PSC %Syn</td>
</tr>
<tr>
<td><em>R. oryzae</em> (9)</td>
<td>0.73</td>
<td>1.47</td>
<td>0.15/0.50</td>
</tr>
<tr>
<td><em>R. microsporus</em> (10)</td>
<td>1.00</td>
<td>1.74</td>
<td>0.23/0.50</td>
</tr>
<tr>
<td>Total (19)</td>
<td>0.86</td>
<td>1.60</td>
<td>0.19/0.50</td>
</tr>
</tbody>
</table>

AMB, amphotericin B; PSC, posaconazole; ITC, itraconazole; RVC, ravuconazole; GM, geometric mean; %Syn, percentage of isolates showing synergistic interaction.
remarkable degree of synergism against Rhizopus isolates. Further in vivo studies are warranted to explore the potential role of these combinations for the treatment of zygomycosis.

Funding
This work was supported by a grant from Fondo de Investigaciones Sanitarias from the Ministerio de Sanidad y Consumo of Spain (PI 05003).

Transparency declarations
None to declare.

References

Journal of Antimicrobial Chemotherapy
doi:10.1093/jac/dkm528
Advance Access publication 15 January 2008

Piperacillin/tazobactam-heteroresistant Pseudomonas aeruginosa from urinary infection, successfully treated by piperacillin/tazobactam

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Keywords: population analysis, time–kill, heteroresistance, persisters, β-lactams, heterogeneity

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Sir,
Pseudomonal infections have limited therapeutic options due to the intrinsic resistance of the microorganism and its ability to integrate further resistance mechanisms.1 Although antipseudomonal β-lactams such as carbapenems and piperacillin/tazobactam still remain clinically useful, carbapenem-heteroresistant mutants of Pseudomonas aeruginosa have recently been described.2 We wish to further report the occurrence of an isolate showing heteroresistance to piperacillin/tazobactam.

P. aeruginosa strain 7171 (PA7171) was isolated in November 2006 from a urinary tract infection sample of a 38-year-old male who was hospitalized for 7 days at the University Hospital of Larissa. Species identification and initial antibiotic susceptibility testing were performed by the Vitek 2 automated system (bioMérieux, Marcy l’Étoile, France), according to the manufacturer’s instructions. Susceptibility to several antipseudomonal drugs was also determined by disc diffusion, agar dilution and Etest (AB Biodisk, Solna, Sweden) methods. P. aeruginosa ATCC 27853 was used as a control in all susceptibility testing assays. By the automated system, PA7171 was reported as non-susceptible to amikacin, aztreonam, cefepime, ciprofloxacin and meropenem and susceptible to ceftazidime, piperacillin/tazobactam, imipenem and colistin. By agar dilution, the isolate was non-susceptible to amikacin (MIC > 256 mg/L), cefepime (64 mg/L), ceftazidime (32 mg/L), ciprofloxacin (>256 mg/L), meropenem (32 mg/L) and aztreonam (16 mg/L) and susceptible to imipenem (2 mg/L) and piperacillin/tazobactam (16 mg/L). According to the agar dilution MICs, the isolate was categorized as susceptible to piperacillin/tazobactam by using both CLSI and BSAC breakpoints. However, the isolate exhibited distinct colonies within the inhibition halo around the piperacillin/tazobactam disc (Figure 1a) and Etest strip, implying the presence of heteroresistant subpopulations.

In order to investigate the possibility of heteroresistance to piperacillin/tazobactam, population analysis was undertaken as previously described.3 The analysis was performed in triplicate, and the mean values of resistant cfu were estimated and plotted on a semi-logarithmic graph. Population analysis of PA7171 was compared with that of ATCC 27853. It was shown that PA7171 grew in the presence of piperacillin/tazobactam up to a concentration of 128 mg/L with a frequency of ~10−7 (Figure 1b). Time–kill kinetics utilizing piperacillin/tazobactam were examined in PA7171 and P. aeruginosa ATCC 27853. In this assay, PA7171 exhibited a bactericidal curve similar to that of ATCC 27853 (Figure 1c). Particularly, cell populations gradually decreased and were eliminated within 24 h, whereas no cell regrowth was detected.

When the heteroresistant colonies grown in the highest drug concentration were subcultured weekly in antibiotic-free medium and re-tested by disc diffusion, agar dilution and Etest, the piperacillin/tazobactam agar dilution MIC was the same as that of the native population (16 mg/L). Again, a subpopulation of heteroresistant cells grew within the zone of inhibition around the antibiotic disc or Etest strip, similar to the situation previously described for carbapenem-heteroresistant Acinetobacter baumannii clinical isolates.4 It should be noted that after the subcultures in antibiotic-free medium, considerably less colonies were grown within the inhibition halo.