

Review Article

Aspergillus species intrinsically resistant to antifungal agents

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Polyphasic taxonomy has had a major impact on the species concept of the genus *Aspergillus*. New sibling species have been described that exhibit *in vitro* susceptibility profiles that differ significantly from that of *Aspergillus fumigatus*. While acquired resistance is an emerging problem in *A. fumigatus*, non-*A. fumigatus* *Aspergillus* species may be intrinsically resistant to specific classes of antifungal agents. Minimum inhibitory concentrations of amphotericin B and azoles for some of the non-*A. fumigatus* *Aspergillus* species are elevated compared to *A. fumigatus*. Furthermore, the clinical presentation and evolution of invasive infections caused by these species may differ from that commonly observed for *A. fumigatus*. As the role of the newly identified *Aspergillus* species in causing invasive aspergillosis remains unclear, surveillance networks that incorporate sequence-based identification of clinical isolates are needed to determine the species distribution, the clinical disease and outcome of patients with invasive aspergillosis. Preclinical and clinical studies are needed to further improve the methods for *in vitro* susceptibility testing and to investigate the impact of elevated MICs on antifungal drug efficacy.

Keywords *Aspergillus* species, resistance, invasive aspergillosis, antifungal agents, susceptibility testing

Introduction

Invasive aspergillosis (IA) remains a difficult to manage infectious disease. Early diagnosis is often difficult and in up to 50% of cases the etiologic agent is not recovered in culture [1]. Furthermore, treatment may be complicated by drug toxicity or interactions [2]. Although the outcome of IA is largely determined by the persistence of the underlying disease or immunosuppression, factors related to the fungus also play a role. Until recently, species identification was sufficient to guide antifungal therapy, but the emergence of acquired resistance limits the use of species identification for predicting activity of antifungal agents [3–6]. Acquired

resistance to azoles has been found mainly in *A. fumigatus* and appears to develop through treatment of patients or through exposure of isolates to azole fungicides in the environment [4,7,8]. Several substitutions have been identified in the *cyp51A* gene that are associated with azole resistance although in some centers the number of resistant isolates without *cyp51A*-mutations is increasing (Harrison E, Howard SJ, Buied A, Bowyer P, Denning DW. The changing prevalence of azole resistance mechanisms in *Aspergillus fumigatus*. 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, September 2009, M-1720). However, resistance to antifungal agents may be intrinsically present and the recent changes in the taxonomy of *Aspergillus* species have had major implications on our understanding of drug susceptibility profiles. In this contribution we aim to review the relationship between *Aspergillus* species and drug susceptibility profiles. The focus of this review will therefore be on *Aspergillus* species other than *A. fumigatus* sensu stricto.

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Taxonomy

The wild type distribution of minimum inhibitory concentrations (MICs) may vary between different *Aspergillus* species. Multilocus sequence-based phylogenetic analyses have emerged as the primary tool for inferring phylogenetic species boundaries and relationships within subgenera and sections. At present, partial β -tubulin or calmodulin sequences are the most promising loci for *Aspergillus* identification, as ITS-sequencing is not specific enough for identification to the species level. Numerous new species have been proposed for the most prevalent *Aspergillus* sections including *Fumigati*, *Flavi*, *Terrei*, *Nidulanti* and *Nigri*. For instance, *Aspergillus* section *Fumigati*, previously identified as *A. fumigatus* based on macroscopic and microscopic morphology, now contains as many as 25 different species, 8 anamorphs and 17 teleomorphs, based on sequence-based identification [9]. Misidentification was common when only macroscopic and microscopic morphology were used [10,11]. The recent taxonomic changes require us to reestablish the MIC distributions of the species complexes and their newly defined sibling species. In addition to investigating the wild type MIC distributions, the role of the new sibling species in invasive fungal infections needs to be established.

Susceptibility testing

The term resistance infers that interpretative breakpoints are available that distinguish between susceptible and resistant isolates. While interpretative breakpoints for *Aspergillus* species have not been established, breakpoints were recently proposed for itraconazole, voriconazole and posaconazole, in the setting of acquired resistance in *A. fumigatus* [12–14]. These breakpoints await confirmation by the CLSI and EUCAST and apply to *A. fumigatus* only. For non-*A. fumigatus* *Aspergillus* isolates interpretative breakpoints are not available and it is not possible to classify specific species or isolates based on the MIC. The *in-vitro* activity of antifungal agents on non-*A. fumigatus* *Aspergillus* isolates is often compared with *A. fumigatus*. For some species experimental animal or clinical data support the interpretation of the MIC of specific antifungal agents. However, for some drugs such as amphotericin B (AmB) a correlation between *in vitro* susceptibility and *in vivo* efficacy is lacking [15,16]. Furthermore, the experience with MIC-testing of non-*A. fumigatus* *Aspergillus* isolates is limited and the growth characteristics or production of foliates may have an impact on the MIC results or its reproducibility.

In this review we assume that intrinsic attenuated susceptibility is present if elevated MIC values are found as compared to *A. fumigatus* and if these MICs are consistently found for the vast majority of the members of

a species. Of course the latter criterion does not rule out the possibility that a resistance mechanism may have been acquired some time ago and has subsequently spread to the majority of the species.

Aspergillus section *Fumigati*

Although *A. fumigatus* is generally susceptible to the major classes of antifungal agents with anti-mold activity, several sibling species have recently been identified that exhibit reduced susceptibility to one or more antifungal drug classes (Table 1). *A. udagawae* shows a reduced susceptibility to AmB and voriconazole [9,17–19], while other species show reduced susceptibility to AmB and all mold-active azoles (*N. pseudofisheri*, *A. fumigati*affinis and *A. viridinutans*) [10,17,20,21]. *A. lentulus* shows a reduced susceptibility to AmB, all mold-active azoles and caspofungin [9,17,20,22–24]. The differences in susceptibility profiles may be clinically relevant, but currently very limited data exists concerning the role and prevalence of sibling species of *A. fumigatus* as causative agents of IA and other diseases associated with *Aspergillus* spp. [10,17,19, 21–25]. Recently, a collection of 218 *Aspergillus* isolates obtained from the transplant-associated infection surveillance network was analyzed [17]. The surveillance network had collected the *Aspergillus* isolates from patients with proven and probable IA over a 6-year period [17,18]. Sequence-based identification of the isolates showed that some of the sibling species caused invasive disease although the prevalence was low (Table 2) [17]. Nevertheless, the clinical impact of their reduced *in vitro* susceptibility remains to be determined, as clinical experience is limited and experimental models are lacking.

Aspergillus section *Flavi*

A. flavus is the second most common cause of IA in many centers, but is reported to be the predominant *Aspergillus* species isolated in countries with arid dry conditions, including most of the Middle East, Africa and Southeast Asia, possibly due to its ability to survive at higher temperatures [26]. The TRANSNET surveillance study showed that 13.2% of proven and probable IA cases were due to *A. flavus*, which was confirmed by sequence-based identification (Table 2) [17,18]. Clinical *A. flavus* isolates are generally highly susceptible to commonly used antifungal drugs such as polyenes, triazoles and echinocandins [18]. However, slightly higher MICs to itraconazole and voriconazole are sporadically reported [27–29], though these *in vitro* findings need to be confirmed in larger studies. The MICs of *A. flavus* clinical isolates to AmB are consistently two-fold dilution steps higher than those of *A. fumigatus* (Table 1). Clinical cases of treatment failure have been

Table 1 Characteristics and intrinsic resistance profiles of *Aspergillus* species.

| Species | Characteristics | Resistance profile | Comments | References |
|---|---|---|---|---------------|
| <i>A. lentulus</i> | Newly recognized to cause IA | Reduced susceptibility to amphotericin B, azoles and variable susceptibility to caspofungin | Sibling species of <i>A. fumigatus</i> | 9,17,20,22–24 |
| <i>A. udagawae</i> | Uncommon cause of IA | Reduced susceptibility to amphotericin B and voriconazole | Sibling species of <i>A. fumigatus</i> | 9,17–19,25 |
| <i>N. pseudofisherii</i> | Uncommon cause of IA | Variable susceptibility to amphotericin B and reduced susceptibility to azoles | Sibling species of <i>A. fumigatus</i> | 10,17,20 |
| <i>A. fumigatiaffinis</i> | No cases of IA reported | Reduced susceptibility to amphotericin B and azoles | Sibling species of <i>A. fumigatus</i> | 20 |
| <i>A. viridinutans</i> | Newly recognized to cause IA in patients with primary immunodeficiencies | Reduced susceptibility to amphotericin B and azoles | Sibling species of <i>A. fumigatus</i> | 20,21 |
| <i>A. flavus</i> | Common in dry climates | Reduced susceptibility to amphotericin B | | 17,18,26–31 |
| <i>A. nidulans</i> | Primarily causes IA in patients with CGD | Reduced susceptibility to amphotericin B | | 16,17,32–34 |
| <i>A. tetrazonus</i> (<i>E. quadrilineata</i>) | Newly recognized to cause IA in CGD | Susceptible to amphotericin B but reduced susceptibility to caspofungin | Sibling species of <i>A. nidulans</i> | 17,34 |
| <i>A. terreus</i> | Propensity to disseminate with positive blood cultures | Reduced susceptibility to amphotericin B | | 18,41–46 |
| <i>A. alabamensis</i> | No cases of IA reported | Reduced susceptibility to amphotericin B | Sibling species of <i>A. terreus</i> | 55 |
| <i>A. niger</i> | Uncommon cause of IA; Common cause of otomycosis | Variable susceptibility patterns with reduced activity of azoles | | 27,52–54,56 |
| <i>A. tubingensis</i> | Newly recognized to cause keratitis and IA | Variable susceptibility patterns with reduced activity of azoles | Sibling species of <i>A. niger</i> | 17,53,57 |
| <i>A. calidoustus</i> | Uncommon cause of IA; Propensity to disseminate | Resistant to the triazoles and variable susceptibility to caspofungin | Previously reported as <i>A. ustus</i> | 17,35–40 |
| <i>A. versicolor</i> | Uncommon cause of IA; Common cause of onychomycosis | Reduced susceptibility to amphotericin B and variable susceptibility to azoles | | 18,56,58 |
| <i>A. sydowii</i> | Newly recognized to cause onychomycosis and peritonitis in patients undergoing dialysis; Uncommon cause of IA. | Reduced susceptibility to amphotericin B and variable susceptibility to azoles | Sibling species of <i>A. versicolor</i> | 17,54,59,60 |
| <i>A. persii</i> | Newly recognized to cause onychomycosis | Reduced susceptibility to amphotericin B and caspofungin | Recently recognized as being a new species in section <i>Circumdati</i> | 61 |

reported and might be associated with the higher MIC of AmB [27,30]. Reduced efficacy of AmB was confirmed in a non-neutropenic model of disseminated *A. flavus* infection as the drug failed to prolong survival at a dose of 1 mg per kg/day [31]. However, in this model the full dose-response curve of AmB was not determined and therefore, it remains unclear if infected animals would have responded to higher dosages.

Aspergillus* section *Nidulanti

The identification and reporting of infections due to *A. nidulans* is confusing due to the common isolation of

its teleomorph *Emericella* in clinical microbiology laboratories. Although reporting of the sexual state that is present in the culture of the clinical specimen seems logical, it can be confusing for the physician. As it is the anamorph that causes the infection in humans, reporting the anamorphic state may be more appropriate.

In general, invasive infections due to *A. nidulans* are relatively uncommon, with the vast majority of cases being reported in patients with chronic granulomatous disease (CGD). Apparently a specific phagocyte defect in this patient group renders them more prone to invasive *A. nidulans* infection. In CGD patients *A. nidulans* infections have a propensity to spread readily from the lung to adjacent

Table 2 Distribution of *Aspergillus* species according to sequence-based identification in the transplant associated infection surveillance network [17,18].

| Species complex | Frequency (%) | Sequence-based identification | Frequency (%) |
|----------------------|----------------|-------------------------------|----------------|
| <i>A. fumigatus</i> | 147/218 (67.4) | <i>A. fumigatus</i> | 139/147 (93.9) |
| | | <i>A. lentulus</i> | 4/147 (2.7) |
| | | <i>A. udagawae</i> | 3/147 (2.0) |
| | | <i>N. pseudofischeri</i> | 1/147 (0.8) |
| <i>A. flavus</i> | 29/218 (13.2) | <i>A. flavus</i> | 29/29 (100) |
| <i>A. niger</i> | 19/218 (8.7) | <i>A. niger</i> | 13/19 (68) |
| | | <i>A. tubingensis</i> | 6/19 (32) |
| | | <i>A. terreus</i> | 11/11 (100) |
| <i>A. terreus</i> | 11/218 (7.4) | <i>A. terreus</i> | 11/11 (100) |
| <i>A. ustus</i> | 6/218 (2.7) | <i>A. calidoustus</i> | 6/6 (100) |
| <i>A. versicolor</i> | 5/218 (2.3) | <i>A. versicolor</i> | 3/5 (60) |
| | | <i>A. sydowii</i> | 2/5 (40) |
| <i>A. nidulans</i> | 1/218 (0.5) | <i>A. tetrazonus</i> | 1/1 (100) |

structures and to disseminate. One clinical study indicated that the total dose of AmB used in patients with *A. nidulans* infection was higher than for those with *A. fumigatus* infections (231 mg versus 56 mg, respectively). In addition, the duration of AmB therapy was longer, 220 days in *A. nidulans* infections compared with 65 days in *A. fumigatus* infections [32]. Although the outcome of patients with *A. nidulans* infections is determined by the specific pathogenesis and persistent underlying immune defect, resistance to the polyene AmB may play a role. Using Clinical Laboratory Standards Institute methodology, *A. nidulans* was shown to have MIC values of 1–2 mg/l, which is higher than commonly observed with *A. fumigatus* [33]. In another study, the mean MIC of *A. nidulans* isolates, of which the identification was confirmed by sequence-based methods, showed a mean MIC of 2.5 mg/l [34]. In a neutropenic model of disseminated aspergillosis due to *A. nidulans*, AmB at 1 mg/kg per day was not effective in improving survival, as opposed to caspofungin which improved survival in a dose-dependent manner [31]. As multiple dosing regimens of AmB were not investigated it remains unclear if animals would respond to higher dosages. The MIC of the *A. nidulans* isolate used in this experiment to AmB was 0.25 mg/l [31], indicating a poor correlation between *in vitro* susceptibility and *in vivo* efficacy. Lack of correlation between *in vitro* susceptibility and *in vivo* efficacy for AmB was also observed in another murine model of IA caused by *A. fumigatus* [16]. In that model a correlation was found with respect to an *A. terreus* isolate [16].

The new taxonomy of *Aspergillus* section *Nidulanti* has shown that *A. nidulans* is not the only *Aspergillus* species within this section to cause IA in patients with CGD. Sequence-based analysis of an international collection of *Aspergillus* species belonging to the section *Nidulanti* showed that *Emericella quadrilineata*

(anamorph *Aspergillus tetrazonus*) is also a common cause of IA [34]. Six of 12 *A. tetrazonus* isolates (50%) had been identified incorrectly as *A. nidulans* based on the macroscopic and microscopic morphology (Fig. 1) [34]. *A. tetrazonus* and *A. nidulans* are very closely related and are able to mate with each other. Interestingly, despite the close relatedness between these species, significant differences were found in their *in vitro* susceptibility. The mean MIC of AmB was 0.5 mg/l for *A. tetrazonus* compared to 2.5 mg/l for *A. nidulans*. [34] In contrast, *A. nidulans* was more susceptible to caspofungin than *A. tetrazonus* (median MIC 0.3 mg/l and 1.8 mg/l, respectively) (Table 1) [17,34].

Aspergillus section *Usti*

A. ustus is commonly found in food, soil and indoor environments worldwide, but infections in humans are relatively infrequent. Invasive infections have been described in immunocompromised hosts. A recent overview reported 22 cases of IA due to *A. ustus*, and remarkably, 50% of the patients had a primary cutaneous infections (Table 1) [35]. Using the polyphasic taxonomic approach the section *Usti* was found to include eight species, *A. ustus*, *A. puniceus*, *A. granulatus*, *A. pseudodefectus*, *A. calidoustus*, *A. insuetus* and *A. keveii* sp. nov. [36]. Sequence analysis of 34 clinical isolates that had been previously identified as *A. ustus* on the basis of morphologic examinations showed that clinical isolates almost exclusively belonged to the new species *A. calidoustus*. The exceptions included two clinical isolates that were classified as *A. puniceus* and as *A. ustus*. The *A. ustus* isolate was recovered from a brain biopsy of a patient with central nervous system aspergillosis. The phenotype of *A. ustus sensu stricto*, however, shows that this species is unable to grow at 37°C, which calls into question the clinical relevance of the culture

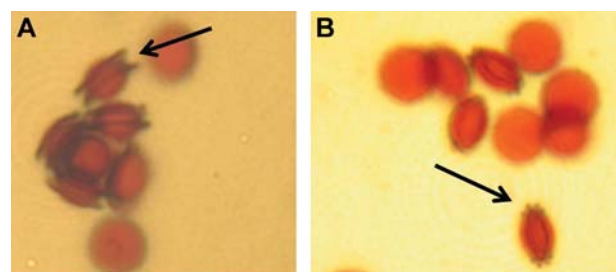


Fig. 1 Light microscopic appearance of ascospores of *Emericella nidulans* (A) and *Emericella quadrilineata* (B). Differentiation of these species on the basis of morphology of the anamorphic state is virtually impossible. Only subtle differences between the ascospores may be seen using light microscopy. The arrow indicates the presence of two longitudinal crests in *E. nidulans* (A) and 4 short equatorial crests in *E. quadrilineata* (B).

result obtained from the brain biopsy [35]. *In-vitro* susceptibility testing showed that the azoles are not active against *A. calidoustus* with MICs of ≥ 8 mg/l [35]. This is consistent with clinical experience as cases have been reported that fail azole therapy or breakthrough infections in patients receiving itraconazole or voriconazole [37–39]. However, other classes of drugs also appear less active compared to their results against *A. fumigatus* [35]. The MICs of AmB were 1–2 mg/l, which is relatively high [35]. In a review of eight cases of IA due to *A. calidoustus* in allogeneic HSCT recipients, patient outcome was dismal despite antifungal treatment with lipid formulations of AmB [39]. In the above mentioned case series, IA was believed to be the cause of death in 6 of 7 patients. This suggests that AmB may not be very effective, although host factors may have played a role. One fungus-related factor may be the fact that *A. calidoustus* rapidly disseminates as six of the eight patients developed disseminated infection [39]. The MICs of caspofungin were variable ranging between 0.25 and 4 mg/l (Table 1) [35]. One patient developed a breakthrough infection during secondary prophylaxis with caspofungin, with a MIC of the causative isolate of >4 mg/l [39]. The consistent reporting of lack of activity of azoles against *A. calidoustus* and of treatment failures indicate that *A. calidoustus* is intrinsically resistant to the triazoles and that azole therapy should be avoided in cases where this species is recovered [40].

Aspergillus section Terrei

Resistance of *A. terreus* to AmB is well recognized. *A. terreus* isolates show *in vitro* higher MICs to AmB than *A. fumigatus* (Table 1) [18,41,42]. Most clinical studies support a lack of activity of AmB against *A. terreus* [43–46]. Two investigations showed an association of invasive infections due to *A. terreus* and the prophylactic administration of AmB aerosols [29,44]. Another study found significant lower response rates of AmB in patients with *A. terreus* infection, compared to those treated for non-*A. terreus* *Aspergillus* infections [45]. Dissemination occurred in 63% of the patients, indicating that the course of disease is different to that observed for *A. fumigatus*. This may have an impact on treatment and outcome as well [45]. It was found in a fourth study comparing the response rate in 32 patients with invasive *A. terreus* infection to 33 with invasive *A. fumigatus* infections that there was no difference in outcomes among those patients treated with AmB [46]. The overall rate of response to antifungal therapy, including the lipid-formulations of AmB, was similar for both patient groups. However, the number of patients in each group was limited and the number of patients showing a clinical response was low in both groups [46]. It was suggested in this study that IA caused by *A. terreus* was

more often nosocomial in origin compared with IA due to *A. fumigatus* ($P < 0.03$) [46].

Animal studies support the lack of efficacy of AmB against *A. terreus*, while itraconazole, posaconazole and caspofungin were shown to be effective [47,48]. Though, one unpublished study indicates that high-dose liposomal amphotericin B might be an effective strategy, as higher-dosed liposomal amphotericin B regimens prolonged survival and reduced fungal burden in neutropenic mice with pulmonary aspergillosis caused by *A. terreus* (Lewis R, Albert N, Liao G, Wang W, Prince R, Kontoyiannis D. Evaluation of liposomal amphotericin B dose-escalation and de-escalation strategies in a neutropenic murine model of *Aspergillus terreus* pneumonia. Advances Against Aspergillosis 2010, Rome, February 2010, poster 83). Clinically, voriconazole has also been shown to be effective, but animal studies are lacking. The reduced susceptibility to AmB has been associated with a low membrane ergosterol content in *A. terreus* [48,49]. Others have suggested that a significant higher catalase production in *A. terreus* compared with *A. fumigatus* may be an important factor [50]. In addition, one animal study suggested that *A. terreus* was less virulent than *A. fumigatus*, but the number of isolates that were investigated were too low to draw general conclusions [48].

A special feature of *A. terreus* is the display of aleurioconidia or accessory conidia directly on hyphae that have been suggested to enhance virulence by facilitating dissemination [48,49]. The accessory conidia contain less membrane ergosterol and have high MICs to AmB, similar to phialidic conidia [49].

Aspergillus section Nigri

The black aspergilli, formerly generally identified as *A. niger*, are now known to show a high biodiversity of species, when sequence-based information is used in their identification [51]. Species belonging to *Aspergillus* section *Nigri* are distributed worldwide and mainly cause otomycoses. Furthermore, they are considered to be the third leading cause of invasive pulmonary aspergillosis [52].

Based on susceptibility to the azoles three different susceptibility patterns were distinguished in the *Aspergillus* section *Nigri*, i.e., low versus high MICs and a third group showing an uncommon paradoxical effect of the azoles. However, these groups did not coincide with species boundaries, making it difficult to interpret as an intrinsic or acquired property of this mold [27,52–54]. However, subtle differences of *in vitro* susceptibility have been observed for some of the new sibling species in that *A. tubingensis* and *A. foetidus* show slightly higher MICs of azoles compared to *A. fumigatus* and *A. niger sensu stricto* (Table 1) [53]. A paradoxical or ‘Eagle effect’ was seen

in the itraconazole susceptibility testing and is also not related to the species identity [52–54]. The clinical impact of this paradoxical effect needs to be studied further. Resistance to AmB and agents of the echinocandin class has not been reported in members of the *A. niger* complex.

Other *Aspergillus* species

Intrinsic resistance has been reported sporadically in less common *Aspergillus* species, or in those that do not commonly cause IA [17,18,54–56,58–61]. These species are listed in Table 1, but will not be discussed in detail.

Conclusion

In conclusion, the wild type susceptibility pattern of non-*A. fumigatus* *Aspergillus* species may be different from that of *A. fumigatus*. Non-*A. fumigatus* *Aspergillus* species may be intrinsically resistant to specific classes of antifungal agents, although many have elevated MICs of AmB. However, little is known with respect to the efficacy of antifungal agents against non-*A. fumigatus* *Aspergillus* isolates and with respect to factors that contribute to outcome such as immunopathogenesis, fitness, and virulence characteristics. These factors have been poorly studied in animal models, and we currently rely primarily on *in vitro* data on which we base treatment decisions. But even MIC-distributions have included a limited number of clinical isolates for most non-*A. fumigatus* *Aspergillus* species compared to *A. fumigatus*, which hampers our ability to assign differences *in vitro* susceptibility to the different species. Therefore, molecular identification remains important to gain more insight into the efficacy of antifungal agents. Furthermore, the clinical presentation and evolution of invasive infections caused by these species may differ from that commonly observed for *A. fumigatus*. Early dissemination or positive blood culture are properties observed in some species that clearly differs from *A. fumigatus*. Maximizing the efforts to culture the causative isolate in patients suspected of IA [62] appears more than ever necessary to allow identification to the species level and susceptibility testing. To increase a correct identification, clinical microbiology laboratories should routinely perform sequence-based identification on clinically relevant *Aspergillus* isolates. As the role of the newly identified *Aspergillus* species in causing IA remains unclear, surveillance networks, similar to TRANSNET, that incorporate sequence-based identification of clinical isolates are needed to determine the species distribution, the clinical disease pattern and outcome of patients with IA. Preclinical and clinical studies are needed to further improve the methods for

in vitro susceptibility testing and to investigate the impact of elevated MICs on drug efficacy.

Declaration of interest: JL: none. AW: Pfizer, Gilead. PV: Biorad, Pfizer, Gilead, Schering-Plough, Merck and Basilea.

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