Concomitant occurrence of itraconazole-resistant and -susceptible strains of Aspergillus fumigatus in routine cultures

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Introduction

Invasive aspergillosis (IA) is a life-threatening infection in severely immunocompromised patients.1,2 Aspergillus fumigatus, a common airborne fungal pathogen, is the principal aetiological agent in IA.1,2 Therapeutic options are limited due to variable efficacy of antifungal drugs against clinical A. fumigatus isolates. Oral triazoles (itraconazole, posaconazole and voriconazole) are highly effective against A. fumigatus isolates in vitro and are currently used as first-line therapy in the management and prophylaxis of IA.3,4 Triazole resistance in A. fumigatus is an emerging public health problem with serious therapeutic implications.5–7 While patients may acquire resistant strains from the environment, triazole resistance may also develop during extended prophylaxis or therapy.6,8,10 Recently, in vitro and/or in vivo resistance to triazoles has also been described in Aspergillus flavus and Aspergillus terreus.11,12

Triazole resistance in environmental isolates and clinical A. fumigatus isolates from treatment-naïve patients is principally mediated by a 34 or 46 bp tandem repeat in the promoter region (TR34/TR46) together with an L98H or a Y121F/T289A substitution, respectively, in the cyp51A gene, encoding 14α-sterol demethylase.6,8,10 However, non-synonymous mutations at several codon positions in cyp51A are found in clinical isolates from triazole-treated patients.5,10,13 Overexpression of efflux pumps has also been documented as a mechanism conferring resistance to triazoles in A. fumigatus isolates lacking cyp51A mutations.6 Continuous and dynamic interaction between A. fumigatus, triazole antifungal agents, azole fungicides and human hosts exists in nature. In this scenario, is it possible that triazole resistance can be cryptic or may go undetected in a mixed (susceptible and resistant) population even when culture/susceptibility testing is performed? A recent study of azole resistance in A. fumigatus from bronchoalveolar lavage samples and cultures from patients

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with aspergillosis seems to support such possibilities. The minimum number of conidia required to form a colony during culture of A. fumigatus from environmental and clinical samples is presently unknown. Assuming that more than one conidia are required for a positive culture, both environmental and clinical A. fumigatus isolates could potentially represent a mixture (of two or more strains), including, in some cases, a drug-susceptible and a drug-resistant strain. A major impediment in this regard is the inability of currently available diagnostic methods to rapidly identify mixed cultures. This study investigated the presence of mixed cultures by analysis of A. fumigatus strains recently isolated from environmental and clinical samples in Kuwait.

Methods

A. fumigatus isolates, antifungal susceptibility testing and typing

Reference A. fumigatus strains (CBS 113.26 and E-76) carrying wild-type and mutant (TR34/L98H) sequences in the promoter region and at codon 98 of cyp51A (cy51A98) were used as controls. A total of 50 environmental and 16 clinical A. fumigatus isolates were initially tested (listed in Table S1, available as Supplementary Data at JAC Online). Isolation of A. fumigatus and in vitro antifungal susceptibility testing by Etest was carried out as described previously. Isolates with itraconazole MICs of ≥2 mg/L were considered as resistant. When the presence of mixed cultures was indicated by molecular testing, serial dilution and plating was carried out to separate itraconazole-susceptible and -resistant phenotypes among A. fumigatus cultures. Selected single colonies of subcultures were also tested for susceptibility to itraconazole, posaconazole and voriconazole by the reference broth microdilution (M38-A2) method (BMD). Isolates with MICs of ≥2, ≥0.5 and ≥2 mg/L were considered as resistant to itraconazole, posaconazole and voriconazole, respectively. The phylogenetic relationships of selected A. fumigatus isolates were determined by nine-locus microsatellite analysis and data were compared with seven previously characterized triazole-resistant A. fumigatus isolates from Kuwait containing TR34/L98H mutations.

Molecular analysis of cyp51A

DNA from the isolates was prepared and the presence of tandem repeats in the cyp51A promoter region was detected by a simple PCR assay described recently. Other triazole resistance-conferring mutations within the structural gene were detected by PCR amplification followed by direct DNA sequencing of cyp51A, as described in detail previously. The study was approved by the Ethics Committee, Faculty of Medicine, Kuwait University.

Results and discussion

One environmental A. fumigatus isolate (R-43-1) exhibited a high level of resistance (MIC ≥32 mg/L) while the remaining 49 isolates were susceptible to itraconazole. A PCR amplification assay for the promoter region indicated the presence of both wild-type and tandem-repeat (TR34) sequences in isolate R-43-1 while the other 49 isolates contained only wild-type sequence (Figure S1). PCR sequencing of cyp51A from isolate R-43-1 also indicated the presence of both wild-type (CTC) and mutant (CAC) sequences at cyp51A98 while only the wild-type (CTC) sequence was found in the remaining 49 isolates. Assuming that more than one conidia are required for a positive culture, we performed serial dilutions and plating of A. fumigatus R-43-1 and analysed 10 well-separated colonies for antifungal susceptibility testing and molecular genetic characterization of resistance-conferring mutations in cyp51A. Five colonies (R-43-1-coln1, R-43-1-coln7, R-43-1-coln9, R-43-1-coln10) contained TR34/L98H mutations, four colonies (R-43-1-coln2, R-43-1-coln3, R-43-1-coln4 and R-43-1-coln6) still exhibited the presence of wild-type and tandem-repeat (TR34) sequences in the promoter region of various proportions (perhaps due to contribution of different proportions of conidia representing wild-type and mutant alleles for cyp51A) and one colony (R-43-1-coln5) had only wild-type sequences in the promoter region (Figure S2) and at cyp51A98. The MIC values for R-43-1-coln5 and R-43-1-coln8 of itraconazole by Etest were 0.75 and ≥32 mg/L, respectively (Table 1). Isolate R-43-1-coln8 was also resistant to itraconazole, posaconazole and voriconazole by BMD (Table 1). More importantly, R-43-1-coln8 (taken as representative of the five colonies) and R-43-1-coln5 were distinctly different strains as revealed by microsatellite typing (Figure 1). These data strongly suggest that conidia sequester, possibly due to hydrophobic interactions, leading to

<table>
<thead>
<tr>
<th>A. fumigatus sample</th>
<th>MIC (mg/L) by Etest of itraconazole</th>
<th>MIC (mg/L) by the broth microdilution method</th>
<th>TR34/L98H in the cyp51A gene</th>
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<tbody>
<tr>
<td>R-43-1-coln5</td>
<td>0.75</td>
<td>0.125</td>
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<td>≥32</td>
<td>&gt;16</td>
<td>yes/yes</td>
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<td>1</td>
<td>0.25</td>
<td>no/no</td>
</tr>
<tr>
<td>Kw2772-coln5</td>
<td>1.5</td>
<td>0.25</td>
<td>no/no</td>
</tr>
<tr>
<td>Kw2772-coln6</td>
<td>8</td>
<td>&gt;16</td>
<td>yes/yes</td>
</tr>
<tr>
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<td>8</td>
<td>&gt;16</td>
<td>yes/yes</td>
</tr>
<tr>
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<td>&gt;16</td>
<td>yes/yes</td>
</tr>
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<td>&gt;16</td>
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<tr>
<td>Kw1431-coln9</td>
<td>≥32</td>
<td>&gt;16</td>
<td>yes/yes</td>
</tr>
</tbody>
</table>
more than one (more likely at least four) conidia yielding an in vitro-grown A. fumigatus colony.

Only 1 clinical A. fumigatus isolate (Kw1431) was resistant to itraconazole by Etest while the remaining 15 isolates were susceptible (Table 1). A PCR amplification assay for the promoter region indicated the presence of TR34 in isolate Kw1431, both wild-type and TR34 sequences in two isolates (Kw2772 and Kw3328) and only wild-type sequence in the remaining 13 clinical isolates. Seven isolates (Kw30, Kw147, Kw373, Kw1431, Kw2772, Kw1842 and Kw3328) were chosen for serial dilution and plating for subculture and isolation of (at least five) single colonies for further analyses. All 10 colonies from A. fumigatus isolate Kw1431, resistant to itraconazole by Etest and to itraconazole, posaconazole and voriconazole by BMD (representative data from 4 colonies are shown in Table 1), contained TR34/L98H mutations in cyp51A and all 4 colonies exhibited the same fingerprinting pattern in microsatellite analysis (Figure 1). Similarly, all individual (5–10) colonies from Kw30, Kw147, Kw373 and Kw1842 were susceptible to itraconazole (MIC values of 0.5–1 mg/L) and contained wild-type sequences in cyp51A. Of eight colonies screened for A. fumigatus Kw2772, five colonies (Kw2772-coln1 to Kw2772-coln5) were susceptible while three colonies (Kw2772-coln6 to Kw2772-coln8) were resistant to itraconazole by Etest and BMD (representative data are shown in Table 1). Isolates Kw2772-coln6 and Kw2772-coln8 were also resistant to posaconazole and voriconazole by BMD (Table 1). Interestingly, all five itraconazole-susceptible colonies contained wild-type sequence in cyp51A (both the promoter and the structural gene) while the three itraconazole-resistant colonies contained TR34/L98H mutations in cyp51A. Furthermore, two selected itraconazole-susceptible colonies (Kw2772-coln2 and Kw2772-coln5) exhibited identical fingerprinting patterns, which were different from the fingerprinting patterns of the two selected itraconazole-resistant colonies (Kw2772-coln6 and Kw2772-coln8) (Figure 1), confirming the presence of a mixed culture in the original A. fumigatus isolate Kw2772. Microsatellite typing data also showed that isolates Kw2772-coln6 and Kw2772-coln8 belonged to the same genotype as another clinical isolate (Kw1431) and this pattern was closely related to two environmental triazole-resistant isolates (E-454 and E-76) containing TR34/L98H mutations in cyp51A, which were recently isolated from the hospital environment in Kuwait.15 Serial dilution and plating of A. fumigatus isolate Kw3328 also identified the presence of a mixed culture exhibiting different susceptibility patterns. This was confirmed by the presence/absence of TR34/L98H mutations in cyp51A and different fingerprinting patterns by microsatellite typing, as described above for A. fumigatus isolate Kw2772. None of the clinical isolates analysed in this study was recovered from patients with IA.

It is important to emphasize here that only some molecular tests such as PCR amplification assays yielding different amplifications from drug-susceptible and -resistant strains or line probe...
assays are most suitable for detecting mixed cultures, while other
genotypic tests including direct DNA sequencing as well as pheno-
typic susceptibility tests often fail to detect mixed cultures.7,14
Since the major mechanism conferring resistance to triazoles in
Aspergillus fumigatus isolates from environmental sources and treatment-
naive patients involves a tandem repeat in the promoter region
together with an L98H or a Y121F/T289A substitution in cyp51A,
mixed cultures containing these mutations can be readily
detected by PCR amplification of the promoter region as carried
out in this study. However, detection of mixed cultures will not
be feasible in pulmonary specimens from patients with IA who
have been previously treated with triazoles.

In conclusion, the concomitant presence of triazole-susceptible
and -resistant strains in single colonies of routine A. fumigatus
cultures obtained from environmental and clinical samples has
been demonstrated.

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Supplementary data
Table S1, Figure S1 and Figure S2 are available as Supplementary data at
JAC Online (http://jac.oxfordjournals.org/).

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