Epidemiology and Antifungal Resistance in Invasive Aspergillosis According to Primary Disease: Review of the Literature

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Abstract
Aspergilli, less susceptible to antifungals emerge and resistance to azoles have been found mainly in Aspergillus fumigatus; this has launched a new phase in handling aspergillosis. Resistant strains have currently been reported from Belgium, Canada, China, Denmark, France, Norway, Spain, Sweden, The Netherlands, UK and the USA. Centres in the UK (Manchester) and The Netherlands (Nijmegen) have described particularly high frequencies (15 and 10% respectively), and a significant increase in azole resistance in recent years. The reason of this high incidence may be due to long term azole therapy in patients with chronic aspergillosis in Manchester, and due to high use of agricultural azoles in Nijmegen. The primary underlying mechanism of resistance is as a result of alterations in the cyp51A target gene, with a variety of mutations found in clinical isolates and one genotype identified in the environmental (LH98). Reports on well documented in vitro and in vivo resistance to echinocandins are rare for Aspergillus species and resistance may be under-diagnosed as susceptibility testing is less frequently performed due to technical reasons.

Key words: Epidemiology, antifungal resistance, Aspergillus, azoles, candins

Introduction
Invasive fungal infections (IFD) are increasingly recognized and represent a primary cause of morbidity and mortality in critically ill patients [1-4]. A variety of factors, including immunosuppressive agents, broad-spectrum antibiotics, and antineoplastic agents influence the incidence and severity of IFDs [1]. Transplant and haematopoietic stem cell transplant recipients, intensive care unit and surgical patients display the population at risk [1, 4-7]. Aspergillus species have become the most important pathogens [8]. The introduction of voriconazole, posaconazole and echinocandins (caspofungin, micafungin and anidulafungin) improved the therapeutic option for treatment of invasive aspergillosis (IA) [9]. Although the outcome of IA is largely influenced by the state of 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. Aspergilli, less susceptible to antifungals emerged and acquired resistance to azoles have been found mainly in Aspergillus fumigatus [10]; this has launched a new era in handling aspergillosis. This article reviews the epidemiology and antifungal resistance against azoles and candins with particular emphasis on Aspergillus species.

Epidemiology of Invasive Aspergillosis
In a 4-year-study Pagano et al. [11] showed that 64% of IFDs in patients with haematological malignancies were caused by moulds and among them 90% were due to Aspergillus species. Overall, the incidence of IA varies according to underlying diseases, pathogen [1f] and geographic location [10, 12, 13]; rates of up to 7% are reported in Europe [11]. Mortality rates for IA are high and vary according to patient population, ranging from 38% in patients with acute myelogenous leukaemia, from 50-60% in patients with organ transplantation and from 70-85% in other immunosuppressed patients [11-16]. Although A. fumigatus still represents the leading cause of IA, species like Aspergillus terreus and Aspergillus flavus become more frequent [15, 17]. These non-A. fumigatus may be intrinsically resistant to antifungal agents (eg A. ustus) [18] and the clinical presentation and evolution of IA may differ from commonly observed A. fumigatus infections [18-23]. Maximizing the efforts to culture the causative pathogen appears necessary to allow identification and susceptibility testing.

Epidemiology of Azole and Candine Resistance in Clinical A. Fumigatus
Antifungal drug resistance is normally quantified using the minimum inhibitory concentration (MIC). The MIC represents the lowest drug concentration that results in a notable reduction or complete lack of fungal growth. In vitro resistance can be primary (intrinsic) or secondary (acquired). Primary resistance occurs
the prevalence is about 2% among clinical

tus

overall, azole-resistance differs from country to coun-

france, sweden, spain and norway [33-39]. In spain,

is an issue due to the limited number of antifungals.

Manchester chronic aspergillosis

Previous azole

Multi azole resistance

Itraconazole, voriconazole or posaconazole resistant

nijmegen

Invasive pulmonary aspergillosis

Mainly azole naive

dominant resistance

TR/L98H

ABPA=allergic bronchopulmonary aspergillosis

table 2.

Itraconazole, voriconazole or posaconazole resistant MIcs are in the resistant range for a single azole

Pan-azole resistant MIcs are in the resistant range for all available active azoles

Multi azole resistance MIcs are in the resistant range for more than one, but not all azoles

MIcs= minimal inhibitory concentrations

naturally, without prior exposure to the drug. Sec-

nary resistance is generated following exposure to

an antifungal and may be associated with an altered

gene expression [10, 24]. Clinical resistance is defined

as the failure to eradicate an infection despite the ad-

ministration of an adequate antifungal [24]. Such fail-

ures can be attributed to a combination of the host,

the pathogen and the drug [24].

Using the European Committee for Antibiotic Sus-

ceptibility Testing (EUCAST) methodology break-

points were recently proposed for *A. fumigatus* and

itraconazole, voriconazole and posaconazole [25]; for

itraconazole and voriconazole, <2 mg/L (susceptible),

2 mg/L (intermediate) and >2 mg/L (resistant); for

posaconazole, <0.25, 0.5 and >0.5 mg/L respectively.

It is suggested to differentiate between single-azole,

pan-azole and multi-azole resistance (see Table 1), the

majority of infections is associated with clinical failure

treated with the ascertainment agent [26].

It was thought that acquired resistance of *As-

pergillus* species to triazoles is unfrequently [27, 28].

Yet reports from the Netherlands and Manchester dis-

play an alarming increase of azole resistance in *A. fu-

migatus* since 1998 [26, 29, 30]. The first published

case of itraconazole-resistance in *A. fumigatus* appeared

in 1997 [31]; in 2000, a survey testing over 900
isolates showed a 2% prevalence of ITC resistance in

Manchester [32]. In 2007 the percentage of patients

with an azole-resistant *A. fumigatus* increased up to

15% [25, 26]. In the Netherlands azole resistance in-

creased dramatically from 2.5% in 2000, to 4.9% in

2002, to 6.6% in 2004 and to 10% in 2009 [25]. This

represents an increasing frequency of 6% per year and

is an issue due to the limited number of antifungals.

Overall, azole-resistance differs from country to coun-

try and occurred sporadically in Belgium, Denmark,

France, Sweden, Spain and Norway [33-39]. In Spain,

the prevalence is about 2% among clinical *A. fu-

migatus* and in Austria about 0%. In the USA, species with

MICs of voriconazole and posaconazole > 2 mg/L re-

main rare, less than 1% [27].

The clinical presentation and disease evolution may be

related to the underlying genotypes in *A. fumigatus* (Table 2). Isolates have been collected from patients

suffering from chronic aspergillosis [29] and invasive
diseases [26, 33].

Azole drug resistance in *A. fumigatus* has been re-

ported both, before and after drug exposure; acquired

resistance appears to develop through treatment of

patients or through exposure of isolates toazole fumigides in the environment [40].

These findings have major implications for clinical

practice especially as fungal drug resistance is an acute

issue due to the limited number of antifungal com-
pounds. Alternative strategies utilising combination

therapy will become more attractive. Experts expect that triazole resistance in this haploid, sparingly sexual

worldwide airborne fungus will increase [8]. Key ele-

ments in the management of patients will be an accu-

rate speication of *Aspergillus* species and the perfor-

mance of in vitro susceptibility testing for an approxi-

mate antifungal treatment. Presently we do not have ex-

act data on the prevalence of azole-resistance *As-

pergillus* in Germany, but seems to be rather low than

high. Anyway, a shift has occurred in the epidemiology of

invasive infections in Europe [8]. Where invasive can-
didiasis was once the predominant type of invasive

fungal infections, invasive mould infections have be-

come increasingly important, including those caused

by unusual pathogens. Moulds have become the lead-

ing cause of IFD in some populations. *Aspergillus*

species are the most frequent mould pathogens, but

the number of infections caused by previously rare

pathogens, such as the Zygomycetes and Fusarium

species, is increasing. The reasons for the shift in the

epidemiology are multifactorial, but are a result, at

least in part, of the increased use of extensive voriconazole and echinocandins as prophylaxis/treat-
MECHANISM OF AZOLE-RESISTANCE IN \textit{A. FUMIGATUS}

The triazoles block the ergosterol biosynthetic pathway at the C14-α-demethylation stage [44]. These antifungals bind to lanosterol 14-α-demethylase (14-α-DM, or \textit{Cyp51b}) which is encoded by the \textit{Erg11} gene. Such step leads to depletion of ergosterol and an accumulation of lanosterol and other toxic 14-α-methylated sterols.

Several pathomechanisms account for azole resistance in \textit{A. fumigatus}; these include a modification of target enzymes, an increased expression of drug efflux mechanisms, an overexpression of target enzymes, an incorporation of exogenous cholesterol, an overexpression of \textit{Hsp90} and of a sterole-regulatory element binding protein [45, 46]. The resistance phenotype depends on the amino-acid substitution and more than one azole can be affected. Azole-resistant isolates have been reported as multidrug resistant [47], multi-azole resistant [48], azole cross-resistant [27] and multiple-triazole resistant [26, 33] isolates. In most cases azole resistance has been associated with point mutations in \textit{cyp51A}, which represents the target enzyme of the azoles [25]; hot spots at codons 54, 98 and 220 are most frequently characterized [26, 33, 35, 48-50]. Interestingly, other mutations have been found in azole susceptible strains and so are unlikely to be associated with resistance [51].

The resistance mechanisms differ between the Dutch and British azole-resistant isolates; in the Netherlands, the presence of a single resistance mechanism (denoted by TR/198H, a point mutation at codon 98 accompanied by a tandem repeat in the promoter region), was found in over 90\% of clinical \textit{A. fumigatus} isolates. By contrast, several \textit{CYP51A} mutations are present in the UK strains [25, 33] and no prevalence of any one alteration. The reasons for this might be due to differences in the patient population from which the isolates originate [25]. For the Dutch isolates, an environmental source is very likely. Azole-resistance may develop due to exposure of \textit{A. fumigatus} to azole fungicides for plant protection [30, 33]. Howard et al. suggest that the reasons of the widespread increase of azole-resistance in the UK may be part of long-term azole drug exposures in patients [29].

MECHANISM OF ECHINOCANDIN-RESISTANCE IN \textit{ASPERGILLUS SPEZIES}

The β-(1,3)-D-glucan is an integral part of the fungal cell wall. Echinocandins are a unique class which block the β-(1,3)-D-glucan synthase by inhibiting β-(1,3)-D-glucan synthase [52]. This process leads to abnormal hyphal growth in moulds [24]. Much less is currently known about echinocandin resistance in \textit{Aspergillus} and only few clinical isolates associated with treatment failures have been investigated. In such isolate mutation in the \textit{FKS1} target gene was not detected, but expression of the \textit{FKS1} gene was found to be upregulated [37]. Manipulated or laboratory-selected strains with various degrees of caspofungin resistance have been described [42, 53, 54]. Some of these strains have been found to have mutations in the ECM33 gene (\textit{AfECm33}), encoding cell wall proteins. Strains with mutations in the \textit{FKS1} gene encoding a subunit of the β-1,3-D-glucan synthase enzyme have been generated [42]. In other resistant \textit{Aspergillus} mutants the glucan synthase exhibited a wild-type \textit{AfFKS1} gene sequence, where the function, level, and the enzyme itself were susceptible to caspofungin [42, 45].

Differences in the susceptibility to the echinocandins exist among the \textit{Aspergillus} species. For example, \textit{Aspergillus niger} is much more susceptible to echinocandins than other species probably in charge of its different cell-wall composition [55]. \textit{Aspergillus lentulus} isolates are less susceptible to caspofungin, although they maintain susceptibility to anidulafungin and micafungin. The analysis of the \textit{A. lentulus} \textit{FKS} sequence did not reveal a polymorphism at any of the known hot-spot regions of the gene [56].

CROSS RESISTANCE AMONG AZOLES AND ECHINOCANDINS

Cross-resistance patterns are closely linked with the position of the mutation in the \textit{cyp51A} gene [29, 57]. Isolates with alterations at eg codons 98 demonstrate a pan-azole resistance phenotype. Isolates with mutations at codon 54 remain voriconazole susceptible although cross-resistant to posaconazole.

Cross-resistance patterns in isolates with M220 alterations appear to be unpredictable, particularly with respect to voriconazole. The risk of cross-resistance between the azole compounds is high, in one report 74\% and 65\% of itraconazole resistant isolates were cross-resistant to posaconazole and voriconazole respectively [29].

Between itraconazole, voriconazole and ravucona-zole cross-resistance was demonstrated in 10 clinical isolates of \textit{A. fumigatus} obtained from patients with long-term exposure to itraconazole or voriconazole [50]. Also, broad-spectrum cross-resistance among all the azoles has been shown in \textit{A. fumigatus} in a patient receiving prolonged itraconazole prophylaxis [47]. Overall, there is a limited number of reported cases that help us to understand the clinical impact of azole resistance on clinical outcome. For example, in a small case series of patients with IA and no respond to voriconazole, treatment with posaconazole was successful in 50\% of infections [58]. On the other hand...
in animal model of IA caused by an itraconazole-resistant *A. fumigatus* strain, posaconazole, which is structurally similar to itraconazole, was active in high doses [59].

The potential frequency of cross-resistance amongst echinocandins in *Aspergillus* species is still unclear and has not been investigated in detail [60]. At present, there is no evidence that the activity and efficacy of other antifungal compounds, such as the polyenes and echinocandins, is attenuated in azole-resistant isolates [26,33].

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