Mutations in the Cyp51A gene and susceptibility to itraconazole in Aspergillus fumigatus isolated from avian farms in France and China

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ABSTRACT Azole resistance in the fungal pathogen Aspergillus fumigatus is an emerging problem and may develop during azole therapy in humans and animals or exposure to azole fungicides in the environment. To assess the potential risk of azole-resistance emergence in avian farms where azole compounds are used for the control of avian mycoses, we conducted a drug susceptibility study including A. fumigatus isolates from birds and avian farms in France and Southern China. A total number of 175 isolates were analyzed: 57 isolates were collected in France in avian farms where chemoprophylaxis with parconazole was performed; 51 isolates were collected in southern China in avian farms where no chemoprophylaxis was performed; and 67 additional isolates came from the collection of a mycology laboratory. No resistant isolate was detected, and the distribution of minimum inhibitory concentrations was similar for isolates collected in farms with or without azole chemoprophylaxis. For 61 randomly selected isolates, the full coding sequence of the Cyp51A gene was determined to detect mutations. Nine amino acid alterations were found in the target enzyme, 3 of which were new.

Key words: Aspergillus fumigatus, azole drug, susceptibility, France, China

INTRODUCTION Azole resistance in the opportunistic fungal pathogen Aspergillus fumigatus is an emerging problem and may develop during azole therapy in humans and animals or exposure to azole fungicides in the environment (Verweij et al., 2009; van der Linden et al., 2011). The most common mechanism of resistance is a modification of the target enzyme encoded by the Cyp51A gene leading to reduced binding of azole drugs. Howard et al. (2009) demonstrated that the position and type of amino acid substitution within the Cyp51A protein determine the pattern of azole cross-resistance, which is consistent with predicted structural properties of the enzyme. Azole resistance in Aspergillus spp. was first reported in 1997 in 3 clinical isolates obtained from humans in California in the late 1980s (Denning et al., 1997). Since then, clinical failures involving A. fumigatus isolates with acquired triazole resistance have been increasingly reported in humans (Snelders et al., 2008; Verweij et al., 2009). Azole susceptibility of A. fumigatus isolates collected in birds has been only sporadically investigated. Silvanose et al. (2006) examined fungal isolates from the air sacs of falcons before and during antifungal treatment with oral itraconazole or voriconazole. Before treatment, 95% of the isolates, including A. fumigatus, were susceptible to voriconazole. After treatment, 21% of the isolates, including A. fumigatus, were less susceptible or resistant to itraconazole. Beer-naert et al. (2009) examined 59 A. fumigatus isolates from domestic and wild birds. Two of the 4 resistant isolates were collected from birds that received itraconazole.

Aspergillus fumigatus is frequently isolated in hatcheries or avian farms where it may be exposed to azole compounds (Arné et al., 2011; Nieguitsila et al., 2011). Enilconazole and thiabendazole are frequently used for disinfection of hatcheries. Parconazole is orally administered to guinea fowls for the prevention of candidosis, and enilconazole may be used by nebulization in avian farms to reduce the mortality rate due to aspergillosis. The hypothesis that the use of antifungal drugs in avian farms may contribute to the emergence of azole resistance has never been investigated.

In the present study, we assessed the azole susceptibility of a large number of A. fumigatus isolates collect-
ed from birds or from the air of avian farms in France and southern China.

**MATERIALS AND METHODS**

**Collection of Isolates**

A total number of 175 *A. fumigatus* isolates was examined. The first set of 57 isolates was collected in 2 avian farms in France (Loiret) in 2010. In these farms, the buildings sheltered guinea fowls and turkeys, and oral chemoprophylaxis with paraconazole was performed at the beginning of the breeding process. Paraconazole was used at the beginning of the breeding process as medicated feed at a concentration of 30 mg/kg of feed for 4 wk. The second set of 51 isolates was collected in 4 avian farms in Guangxi, China, in 2009 and 2010. In these farms, the buildings sheltered laying hens and broilers, but noazole chemoprophylaxis was performed. In French and Chinese farms, *A. fumigatus* isolates were collected by pharyngeal swabs in living birds, which were handled for regular sanitary control. The animal protocol followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). The protocol was examined by the ethics committee of the veterinary college of Alfort (Ecole nationale vétérinaire d’Alfort, ENVA), France. The committee considered that the sampling technique (with pharyngeal swabs) was not invasive. As a consequence, the committee decided that no specific permits were required for the described field studies in France. The same procedure (with the same conclusion) was followed in Guangxi Province, China.

Sixty-seven additional isolates came from the collection of the mycology laboratory of ENVA. These isolates were collected during 2007 to 2011 from different species of birds (mostly poultry with lesions of aspergillosis) in France. The birds were not exposed to azole compounds.

**Antifungal Susceptibility Testing**

Etest gradient strips were used in accordance with the manufacturer’s instructions (BioMérieux, Lyon, France). *Aspergillus fumigatus* isolates were grown on malt extract agar at 37°C for 7 d. Conidia suspensions were prepared in sterile saline and adjusted to a concentration of 10⁶ conidia/mL. Petri dishes were filled with RPMI 1640 medium, supplemented with glucose (2%) with final pH 7.0 (AES, Combourg, France). Plates were inoculated with 200 μL of the appropriate cell suspension, dried at room temperature for 15 min before the Etest gradient strips of itraconazole were applied. The plates were incubated at 37°C and read at 48 or 72 h. The Etest minimum inhibitory concentrations (MIC) were estimated as the drug concentrations at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip. When the test yielded a MIC value ≥2 μg/mL, the isolate was subcultured and susceptibilities to itraconazole, voriconazole, and posaconazole were determined by the Clinical Laboratory Standards Institute M38-A2 reference method (CLSI, 2008). The final drug concentrations ranged from 0.015 to 8 μg/L.

**Genotypic Analyses**

For all isolates (n = 175) molecular identification was performed by sequencing a part of the highly conserved β-tubulin gene, as described previously (Balajee et al., 2005), to rule out any species within the *Aspergillus* section *Fumigati*, which are closely related to *A. fumigatus*. For 32 (out of 57) randomly selected isolates from avian farms in France and 29 (out of 51) randomly selected isolates from avian farms in China, the full coding sequence of both strands of the *Cyp51A* gene was determined by PCR amplification. Extraction of DNA was performed with QIAamp DNA miniKit (Qiagen). Two primer sets were used: P450A1 ([5′-ATG ATG CGG ATG CTA TGG-3′]/P450A2 (5′-CTG TCT CAC TTG GAT GTG-3′); Diaz-Guerra et al., 2003) and A7 ([5′-TCA TAT GTT GCT CAG CGG-3′]/A2 (GGG GTC GTC AAT GGA CTA-3′); Mellado et al., 2001]. The 2 sequences were assembled and edited with SeqMan II and EditSeq software packages (Lasergene, DNASTar Inc., Madison, WI). Sequences were compared with that of the reference strain CM-237 (GenBank accession number AF338659).

Multiple-locus variable-number tandem-repeat (VNTR) analysis was used to determine the genetic distances between *A. fumigatus* isolates, as described previously (Thierry et al., 2010). Polymerase chain reactions were performed in a total volume of 15 μL containing 1 to 5 ng of DNA, 1× PCR reaction buffer, 0.5 U of Taq polymerase (Takara Bio Inc., Shiga, Japan), 250 μM of each deoxynucleotide triphosphate, and 0.5 μM of each flanking primer. All isolates from avian farms in China (n = 51) and 30 (out of 57) isolates from avian farms in France (including isolates F78 and F82) were tested using the 10 VNTR markers selected by Thierry et al. (2010). The VNTR profiles were analyzed with BioNumerics software package version 4.6 (Applied-Maths, Bruxelles, Belgium) as a character data set. A graphing algorithm termed minimum spanning tree (MST) was used. The priority rule for constructing MST was set in order that the type that had the highest number of single-locus variants would be linked first. A cutoff value of maximum differences of 1 VNTR out of 10 was applied to define clusters.

**RESULTS**

All the isolates from avian farms in France and China had itraconazole MIC values between 0.19 and 3 μg/mL. Furthermore, the distribution of MIC of isolates collected in French farms with antifungal chemoprophylaxis was similar to that of isolates collected from China where no chemoprophylaxis was made. Elevated
MIC values (≥2 μg/mL) were detected in only 4 isolates: 2 isolates [C3 and C15 (3 μg/mL)] from avian farms in Guangxi, China, and 2 isolates [F78 (2 μg/mL) and F82 (3 μg/mL)] from avian farms in France. Antifungal susceptibility testing by the reference CLSI method revealed that these 4 isolates had MIC ≤0.5 mg/L for itraconazole, voriconazole, and posaconazole. These MIC were lower than the corresponding epidemiological cutoff values indicating that these isolates were wild-type. As a consequence, no resistant isolates were detected in the present investigation.

Direct sequencing of Cyp51A gene was performed in 61 A. fumigatus isolates. For 50 isolates, the sequence was identical to that of the reference strain CM-237. A modification of the Cyp51A sequence was identified in 11 isolates (3 isolates with MIC ≥2 μg/mL and 8 isolates with MIC <2 μg/mL). A total number of 20 nucleotidic mutations were detected. Overall, the isolates with Etest MIC ≥2 μg/mL had a higher number of point mutations than isolates with Etest MIC <2 μg/mL (data not shown). Eleven of the point mutations were silent and 9 yielded to amino acid substitutions in the protein. Six of these substitutions had already been described (F46Y, M172V, N248T, N248K, D255E, and E427K), whereas 3 substitutions (A116R, E130D, and Q131H) were original. Three amino acid substitutions were specifically detected in isolates with MIC ≥ 2 μg/mL (F46Y, M172V, and E427K). Hot spot mutations (G54, L98+TR, and M220) commonly found in triazole-resistant isolates (collected in hospitals and sometimes in the environment) were never detected in the present study.

A total number of 81 A. fumigatus isolates were typed with the panel of 10 VNTR. This analysis yielded 74 different genotypes. Among all genotypes, 70 were only found once. Two genotypes were shared by 2 isolates, one genotype was shared by 3 isolates and another one was shared by 4 isolates. When the presence of amino acid substitutions was considered as the discriminant parameter, the graphing algorithm MST analysis revealed small clusters of Cyp51A-mutated isolates (Figure 1). Isolate C19, the only one with original substitutions E130D and Q131H, was genetically distinct from all other isolates.

**DISCUSSION**

To our knowledge, the present study is the first one to report the azole susceptibility of a large number of A. fumigatus collected in avian farms. Resistant isolates were not detected. Amino acid substitutions were detected in Cyp51A, but hot spot mutations related to pan-triazole resistance were not present in isolates from French and Chinese avian farms. Parconazole, thiabendazole, and enilconazole are not members of the triazole group, and we hypothesize that exposure to

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**Figure 1.** Minimum spanning tree of 81 Aspergillus fumigatus isolates based on categorical analysis of 10 variable-number tandem-repeats (VNTR) according to Thierry et al. (2010). Each circle represents a unique genotype. The diameter of each circle corresponds to the number of isolates sharing the same genotype. Genotypes connected by a shaded background differ by a maximum of 1 of the 10 VNTR markers and could be considered a clonal complex. Thick connecting lines represent one marker difference; regular connecting lines represent 2 or 3 marker differences; thin interrupted lines represent 4 or more differences. The length of each branch is also proportional to the number of differences. The color of the circle is related to the presence of amino acid substitutions in Cyp51A: black for isolates with 3 to 5 amino acid substitutions, dark gray for isolates with 1 to 2 amino acid substitutions, light gray for isolates without any amino acid substitution, and white for isolates whose Cyp51A was not sequenced.
this type of drug does not have the same effect as that related to itraconazole or voriconazole in hospitals or to triazole fungicides in the environment.

An interesting aspect of the present study is the variety of substitutions in Cyp51A and the genetic distances between the isolates with substitutions. To date, more than 20 substitutions have been identified in *A. fumigatus* azole-resistant or -susceptible isolates (Rodriguez-Tudela et al., 2008; Howard et al., 2009; Escribano et al., 2011). Three new substitutions were described during the present investigation (A116R, E130D, and Q131H). These substitutions were detected in isolates with MIC <2 μg/mL from Chinese farms. Multiple-locus VNTR analysis revealed small clusters of Cyp51A-mutated isolates. Similar results were obtained by Snelders et al. (2008) using microsatellite markers; they demonstrated that 32 itraconazole-resistant isolates had distinct but clustered genotypes. Recently, Lockhart et al. (2011) surveyed 497 *A. fumigatus* isolates collected from 2008 to 2009 as part of the ARTEMIS global surveillance study for elevated MIC values to itraconazole, voriconazole, and posaconazole. Sequencing of the Cyp51A gene revealed that 8/29 isolates with elevated MIC values to one or more triazoles, all originating from China, contained the TR/L98H mutation associated with resistant European isolates of *A. fumigatus*. Using microsatellite markers, Lockhart et al. (2011) demonstrated that all of the isolates with elevated triazole MIC values had distinct genotypes. The 8 isolates with the TR/L98H mutation were distributed throughout the dendrogram, with only 2 sets of 2 isolates clustering as nearest neighbors, ruling out clonal spread of a single isolate between patients within the institutions from which they were cultured.

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**REFERENCES**


