Avian *Aspergillus fumigatus* Strains Resistant to both Itraconazole and Voriconazole

L. A. Beernaert, 1* F. Pasmans, 1 L. Van Waeyenberghe, 1 G. M. Dorrestein, 2 F. Verstappen, 3 F. Vercammen, 3 F. Haesebrouck, 1 and A. Martel 1

Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, Merelbeke, Belgium; 2 Diagnostic Pathology Laboratory, Dutch Research Institute for Birds and Exotic Animals (NOIVBD), Wintersegedijk 51, Veldhoven, The Netherlands; 3 Veterinary Clinic Hoofdstraat, Hoofdstraat 121, Driebergen, the Netherlands; and Antwerp Zoo, Koningin Astridplein 26, Antwerp, Belgium

Received 7 November 2008/Returned for modification 12 December 2008/Accepted 19 February 2009

Aspergillosis is the most common fungal disease of the avian respiratory tract and is caused primarily by *Aspergillus fumigatus*. Prognosis of avian aspergillosis is often poor due to delayed diagnostics and treatment failure. Acquired resistance to antifungals has been reported (6, 12). However, Dannaoui et al. (5) and Verweij et al. (22) found that the prevalences of high MICs of amphotericin B (>2 μg/ml), itraconazole (>16 μg/ml), and voriconazole (>1 μg/ml) among clinical isolates of *A. fumigatus* in France and The Netherlands were low (0%, ~2%, and 0%, respectively). A 2003 survey of isolates from human patients hospitalized in North America, Europe, and Latin America showed that *A. fumigatus* was generally less susceptible to amphotericin B and voriconazole than did a 2000 survey (18). *Aspergillus* isolates collected from medical centers worldwide between 2000 and 2006 with MICs of voriconazole and itraconazole of >2 μg/ml were found to be rare (<1% and 0% of the isolates, respectively) (19). Recently, 1,912 clinical *A. fumigatus* isolates from a university medical center in The Netherlands (1994 to 2007) were investigated and indicated that *Aspergillus* isolates were acquired resistance to amphotericin B, itraconazole, and voriconazole. Isolates were determined using the standard microdilution broth method (CLSI M38-A2). Four isolates showed acquired resistance to itraconazole and voriconazole, harboring implications for the treatment of aspergillosis in both birds and humans.

The in vitro susceptibilities of 59 avian *Aspergillus fumigatus* strains to amphotericin B, itraconazole, and voriconazole were determined using the standard microdilution broth method (CLSI M38-A2). Four isolates showed acquired resistance to itraconazole and voriconazole, and the ability to grow at 48°C was used to confirm species identity. To exclude repeated testing of identical strains, microsatellite length polymorphism was performed (4). The MICs of amphotericin B, itraconazole, and voriconazole were determined using the broth microdilution method as described in the Clinical and Laboratory Standards Institute (CLSI) document M38-A2, with *Candida krusei* IHEM 9560 (ATCC 6258) as a quality control.

The results of the in vitro susceptibility testing of amphotericin B, itraconazole, and voriconazole for the 59 avian *A. fumigatus* isolates are summarized in Table 1 and Fig. 1. For amphotericin B, a monomodal MIC distribution was seen, indicating the absence of acquired resistance. Figure 1, however, shows a bimodal MIC distribution for itraconazole and voriconazole, indicating acquired resistance in the four isolates in the higher range of MICs. These isolates were genetically different and were obtained from different avian species and environments. The main goal of in vitro susceptibility testing of antifungal agents is to predict the clinical outcome of therapy. However, interpretive breakpoints for any mold-drug combination have been described (18) and are included in Table 1.

### Table 1. MIC ranges, MIC₅₀₉₀, and MIC₉₀₉₀ of amphotericin B, itraconazole, and voriconazole for 59 avian *A. fumigatus* isolates

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>MIC range (μg/ml)</th>
<th>MIC₅₀ (μg/ml)</th>
<th>MIC₉₀ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.5–2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.25–16</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.13–8</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* MICs were determined using the standard microdilution broth method (CLSI M38-A2).
not been established yet, although high itraconazole and amphotericin B MICs have been associated with treatment failure in humans and/or mice (6, 8, 12, 15). Data regarding association between MICs and clinical outcome are not available for voriconazole. In this study, the MIC of itraconazole was at least 32 times higher and that of voriconazole at least 8 to 16 times higher in the four isolates with acquired resistance than in the other isolates. The likelihood that birds or humans infected with these isolates will respond well to treatment with these agents should be considered to be low.

Various mechanisms of azole resistance have been described (2, 7, 13, 17). To date, the most prevalent mechanism of azole resistance appears to be the modification of the cyp51A gene, encoding the target enzyme 14α-sterol demethylase (16). These mutations, reducing the binding of azoles to the enzymatic site, have been associated with different antifungal susceptibility profiles, depending on the azole structure and concomitant binding affinities (14). Hence, mutations resulting in resistance to itraconazole appear to differ from those providing resistance to voriconazole (20, 23). In this study, the four isolates having itraconazole MICs of >16 μg/ml were also less susceptible to voriconazole (MICs of 4 to 8 μg/ml), which might indicate cross-resistance. When elevated MICs for itraconazole and voriconazole arise, resistance mechanisms resulting in elevations of all azole MICs seem to be most likely. Possible mechanisms are amino acid substitutions of methionine at position 220 in the cyp51A gene product and substitution of leucine at position 98 for histidine in the cyp51A gene product, together with two copies of a 34-bp sequence in tandem in the cyp51A gene promoter (16, 17, 20, 21).

The source of the resistant isolates found in these birds is unclear. In human medicine, acquisition of itraconazole resistance after prolonged therapy has been documented (6). In this study, two of the four resistant strains were isolated from birds that received itraconazole. The possible effect of antimycotic treatment in birds is unknown, and because isolates were not available before treatment, this could not be further investigated. Determination of resistant isolates in birds can also be considered an indication of the presence of acquired resistance in the surrounding environment. Acquisition of resistance in the environment might be induced by the extensive use of azoles for plant protection (11), which consequently gain access to susceptible birds and humans. Hence, further investigation of this phenomenon is necessary, as it harbors implications for the treatment of aspergillosis in both birds and humans.

To our knowledge, this is the first report of birds infected with A. fumigatus strains that harbor acquired resistance to both itraconazole and voriconazole.

This work was supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen), Brussels, Belgium.

We have no conflicts of interest regarding the present work.

The skillful technical assistance of S. De Bruycker is greatly appreciated.

REFERENCES
Vol. 53, 2009
SUSCEPTIBILITIES OF AVIAN A. FUMIGATUS STRAINS 2201


