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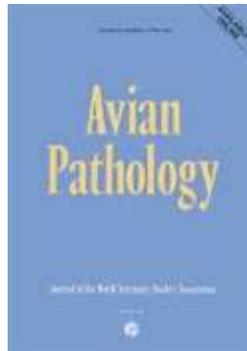
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Microsatellite Typing of Avian Clinical and Environmental Isolates of
Aspergillus fumigatus

Lieven Van Waeyenberghe^{1*}, Frank Pasmans¹, Lies A. Beernaert¹, Freddy Haesebrouck¹, Francis Vercammen², Frank Verstappen³, Gerry M. Dorrestein⁴, Corné H. W. Klaassen⁵, An Martel¹

¹The Department of Pathology, Bacteriology and Avian diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium; ²Royal Zoological Society of Antwerp, Koningin Astridplein 26, Antwerpen, Belgium; ³Veterinary Clinic Hoofdstraat, Hoofdstraat 121, Driebergen, the Netherlands; ⁴The Diagnostic Pathology Laboratory, Dutch Research Institute for Birds and Exotic Animals (NOIVBD), The Netherlands; ⁵Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands.

Short Title: Genotyping *Aspergillus fumigatus* isolates

*Correspondence: Lieven Van Waeyenberghe, Tel.: +32 9 264 7442; Fax: +32 9 264 7490;

E-mail: Lieven.vanwaeyenberghe@ugent.be

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Abstract

Aspergillosis is one of the most common causes of death in captive birds. Aspergillosis in birds is mainly caused by *Aspergillus fumigatus*, a ubiquitous and opportunistic saprophyte. Currently it is not known if there is a link between the environmental isolates and/or human isolates of *A. fumigatus* and those responsible for aspergillosis in birds. Microsatellite typing was used to analyze 65 clinical avian isolates and 23 environmental isolates of *Aspergillus fumigatus*. The 78 genotypes that were obtained were compared to a database containing genotypes of 2514 isolates from human clinical samples and from the environment. There appeared to be no specific association between the observed genotypes and the origin of the isolates (environment, human or bird). Eight genotypes obtained from isolates of diseased birds were also found in human clinical samples. These results indicate that avian isolates of *Aspergillus fumigatus* may cause infection in humans.

Introduction

Fungal infections due to *Aspergillus* species are a major cause of morbidity and mortality among certain species of birds, captive as well as free-ranging, independent of age or status of the immune system (Tell 2005; Beernaert *et al.*, 2008). *Aspergillus fumigatus*, a ubiquitous and saprophytic fungus, is the major etiological agent responsible for aspergillosis (Tell 2005).

To investigate the genetic and the epidemiological relationship between environmental and clinical isolates, fingerprinting methods with high discriminatory power must be applied. Also interlaboratory reproducibility and objective interpretation of the fingerprinting data are highly recommendable (de Valk *et al.*, 2007). All of these characteristics can be found in typing methods based on short tandem repeats (STRs), such as microsatellite length polymorphism (MLP) and STRA*f* typing (Bart-Delabesse *et al.*, 1998; de Valk *et al.*, 2005; de Valk *et al.*, 2007; Vanhee *et al.*, 2008a). While the discriminatory power is high in pattern-based techniques, such as random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis, and amplified fragment length polymorphism (AFLP), STRA*f* typing proved to be more simple and reproducible (Bart-Delabesse *et al.*, 1998; de Valk *et al.*, 2005; de Valk *et al.*, 2007a; Vanhee *et al.*, 2008a,b). However, any typing method is associated with its specific advantages and disadvantages (de Valk *et al.*, 2007 b) and any technical complications associated with microsatellite typing (Pasqualotto *et al.*, 2007; Alvarez-Perez *et al.*, 2009) can be properly addressed (de Valk *et al.*, 2009; Klaassen, 2010).

Previous molecular typing studies showed that there was a high variability among avian isolates and multiple genotypes were recovered from healthy and diseased birds (Lair-Fuller *et al.*, 2003; Olias *et al.*, 2009; Alvarez-Perez *et al.*, 2010). However, currently it is not known if there is a link between environmental isolates and/or human isolates of *A. fumigatus* and those responsible for aspergillosis in birds.

Therefore, in this study STRA f typing was performed on environmental and avian clinical isolates of *A. fumigatus* and the results were compared with a database containing genotypes from *A. fumigatus* isolated from clinical human samples and from the environment.

Material and Methods

Isolates. Sixty-five clinical avian and 23 environmental isolates of *A. fumigatus* were subjected to STRA f typing. The clinical isolates were collected at 6 different institutes, 4 located in Belgium and 2 in The Netherlands and were obtained from birds, belonging to 13 orders, 18 families and 35 species (Table 1), that died from an *A. fumigatus* infection. To collect the environmental isolates, Sabouraud dextrose agar plates were placed at 20 different locations in the vicinity of Ghent, Belgium. After incubation for three days at room temperature, the plates were placed in an incubator at 37°C. Fast-growing greenish colonies were purified on Sabouraud dextrose agar.

The isolates were identified based on the macro- and micro-morphology of the fungus. Determination of partial DNA sequences of the beta-tubulin and rodletA genes (Alcazar-Fuoli *et al.*, 2008) and the ability to grow at 48°C were used to confirm species identity.

STRA f assay. Fungal DNA was prepared from all isolates as described by Beernaert *et al.* (2008). PCR primers for the STRA f 2, -3 and -4 panels and amplification conditions were as described by de Valk *et al.* (2005), except that FAM labelling was replaced by VIC labelling, HEX labelling by NED labelling and TET labelling by PET labelling. Allelic ladders were used with the same fluorescent labels as above for each of the three trinucleotide markers in the STRA f 3 panel as described by de Valk *et al.* (2009). The obtained PCR products were diluted 10-fold with distilled water. Two microliters of the diluted PCR products were added

to 12 µl of formamide (Amresco Inc, Ohio, USA) and 1 µl of GS 500 LIZ size standard (Applied Biosystems, Halle, Belgium). Following denaturation of the samples for 2 min at 95°C and rapid cooling to 0°C for 30 min, they were injected onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Halle, Belgium) equipped with a 16 capillary array. Genemapper v3.5 (Applied Biosystems, Halle, Belgium) was used to determine the size of each amplified fragment. All results are reported as repeat numbers. Repeat numbers for the markers in the STRA_{f3} panel were determined by comparison to the allelic ladders. The repeat numbers of the markers in the STRA_{f2} and -4 panels were determined with the reference size values taken from the original publication and validated using a set of reference isolates with known genotypes (de Valk *et al.*, 2005).

Data analysis. Typing data was imported into BioNumerics version 6 software (Applied Maths, St-Martens-Latem, Belgium) and analyzed using the categorical multistate similarity coefficient with UPGMA clustering. The obtained genotypes were then compared to a database containing 2514 genotypes from human and environmental *A. fumigatus* isolates from Europe and the USA.

Results

A total of 88 *A. fumigatus* isolates were analyzed using STRA_f typing and a total of 78 genotypes were obtained. Seventy genotypes were found once, 6 genotypes were found twice and 2 genotypes were found three times. From the genotypes observed more than once, no relation could be observed between the species of bird in the clinical isolates and the area of isolation in environmental isolates. There was also no relation between the geographic area of

the clinical isolate K7 and the environmental isolate O7. The Simpsons index of diversity (D) for the clinical avian isolates was calculated to be 0.995.

In the dendrogram (Figure 1), 8 clusters can be identified of related genotypes differing only at a single locus. In one case, an identical genotype was found in a clinical avian isolate and an environmental isolate. Two clusters of genotypes contained clinical and environmental isolates, four clusters only clinical isolates and two clusters only environmental isolates.

Comparing the results of our analysis to a database containing 2514 genotypes from human and environmental isolates revealed that 58 genotypes in our collection of avian and environmental isolates were not observed before. Twenty genotypes were observed before either in human clinical samples or in environmental samples. Twelve genotypes from avian clinical isolates were identical to genotypes found before in human (n=3) or environmental (n=4) isolates, or both (n=5) from the Netherlands, Switzerland, Germany and Spain. Eight environmental genotypes from Belgium were identical to genotypes found in human and/or environmental isolates from the Netherlands, Spain, Germany, the United States of America and Norway.

Discussion

Previous epidemiological studies have investigated the origin of avian aspergillosis in limited geographical areas and in one species of birds (Lair-Fulleriger *et al.*, 2003; Olias *et al.*, 2009; Alvarez-Perez *et al.*, 2010). This is the first study determining the genetic diversity among *A. fumigatus* isolates obtained from 35 avian species collected at 6 different institutes and environmental isolates collected at 20 different locations in Belgium.

The results of this study demonstrate that the genetic diversity among avian clinical isolates is extremely high. The 65 isolates, collected from 65 birds affected with aspergillosis,

belonged to 57 distinct genotypes. In contrast, Alvarez-Perez *et al.* (2010) reported 13 distinct genotypes in 33 isolates from 5 diseased birds and Lair-Fullinger *et al.* (2003) 23 distinct genotypes in 114 isolates from 30 healthy and 2 diseased turkeys. The low number of distinct genotypes in relation to the number of isolates in those studies could be explained firstly by the fact that a limited number of animals with aspergillosis were used. Secondly, the animals belonged to closed collections and multiple isolates from each animal were examined. Finally, the genotyping was performed with a method described by Bart-Delabesse *et al.* (1998) including only four microsatellite markers being less discriminatory than the panel of 9 microsatellite markers described here. Alternatively, these animals may have been exposed to a common source of material(s) that may have been contaminated with a limited genotypic diversity of *A. fumigatus* spores such as bird feed.

The environmental and avian isolates are widespread throughout the dendrogram, suggesting that any environmental isolate of *A. fumigatus* is possibly infectious to birds. This finding is supported by the study of Peden and Rhoades (1992), who inoculated isolates from diverse origins (environmental, mammalian, and avian) in air sacs of turkeys. All isolates were able to induce aspergillosis in these birds.

High reproducibility of the STRAf assay and the ease of interlaboratory exchange of the results allowed comparing the dataset of 88 clinical avian and environmental isolates to a dataset of 2514 genotypes from human and environmental isolates (de Valk *et al.*, 2009). There appeared to be no specific association between the observed genotypes and the origin of the isolates (environment, human or bird). This was expected because of the high degree of genetic diversity among *A. fumigatus* isolates, independent of the species or geographical region (Debeaupuis *et al.*, 1997; Chazalet *et al.*, 1998; Rosehart *et al.*, 2002; Menotti *et al.*, 2005; Klaassen *et al.*, 2009). Moreover, 8 genotypes derived from diseased birds were also isolated from human clinical samples indicating that avian isolates of *A. fumigatus* could be considered infectious to humans.

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colonised patients with cystic fibrosis. *European Journal of Clinical Microbiology and Infectious Diseases*, 27, 1005-1007.

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Figure legends

Figure 1. *Dendrogram generated from genotyping 65 clinical and 23 environmental *A. fumigatus* isolates. Isolates recovered from clinical samples are denoted as K and strains isolated from the environment are denoted as O. Framed are the genotypes that show microvariation. The scale bars above the dendrogram indicates the percentage identity between the genotypes. The abbreviated countries are CH: Switzerland; D: Germany; N: Norway; NL: Netherlands; SP: Spain; USA: United States of America.*

Table 1. Order, Family and Species of domestic and wild birds from which *A. fumigatus* isolates were obtained.

| Order | Family | Species | N ^o of isolates | Designation of isolates |
|-----------------|--------------|---------------------------------|----------------------------|---|
| Accipitriformes | Accipitridae | <i>Geranoaetes melanoleucus</i> | 2 | K30; K57 |
| | | <i>Accipiter gentilis</i> | 2 | K51; K58 |
| Anseriformes | Anatidae | <i>Netta rufina</i> | 2 | K66; K76 |
| | | <i>Melanitta nigra</i> | 2 | K61; K82 |
| | | <i>Mergus serrator</i> | 1 | K67 |
| | | <i>Anas platyrhynchos</i> | 1 | K43 |
| | | <i>Uria aalge</i> | 2 | K10; K11 |
| Charadriiformes | Alcidae | <i>Fratercula artica</i> | 1 | K64 |
| | | <i>Larus marinus</i> | 1 | K37 |
| Ciconiiformes | Ciconidae | <i>Ciconia ciconia</i> | 1 | K20 |
| Columbiformes | Columbidae | <i>Columba livia</i> | 6 | K12; K24; K25; K36; K42; K73 |
| | | <i>Falco rusticolus</i> | 4 | K8; K22; K34; K52 |
| Falconiformes | Falconidae | <i>Falco peregrinus</i> | 1 | K54 |
| | | <i>Gallus gallus domesticus</i> | 4 | K6; K15; K16; K72 |
| | | <i>Polyplectron emphanum</i> | 1 | K81 |
| | | <i>Pavo cristatus</i> | 1 | K4 |
| | | <i>Rollulus rouloul</i> | 1 | K65 |
| Galliformes | Phasianidae | <i>Anthropoides paradiseus</i> | 1 | K75 |
| Gruiformes | Gruidae | <i>Pica pica</i> | 1 | K21 |
| | | <i>Corvus monedula</i> | 1 | K23 |
| | | <i>Gymnorhina tibicen</i> | 1 | K18 |
| | | <i>Pyrrhula pyrrhula</i> | 1 | K45 |
| | | <i>Leucopsar rothschildi</i> | 1 | K19 |
| Passeriformes | Corvidae | <i>Rhamphastos toco</i> | 1 | K46 |
| | | <i>Cacatua sp</i> | 1 | K9 |
| Psittaciformes | Psittacidae | <i>Psittacus erythacus</i> | 12 | K7; K48; K55; K59; K60; K62; K68; K69; K70; K78; K79; K80 |
| | | <i>Cyanoramphus spp</i> | 3 | K3; K5; K49 |
| | | <i>Amazona barbadensis</i> | 1 | K63 |
| | | <i>Pionus sp</i> | 1 | K50 |
| | | <i>Poicephalus senegalus</i> | 1 | K56 |
| | | <i>Neophema sp</i> | 1 | K47 |
| | | <i>Enicognathus</i> | 1 | K33 |

| | | | | |
|-----------------|--------------|---------------------------|---|----------|
| | | <i>leptorhynchus</i> | | |
| | | <i>Lathamus discolor</i> | 1 | K39 |
| Sphenisciformes | Spheniscidae | <i>Spheniscus sp</i> | 1 | K32 |
| Strigiformes | Strigidae | <i>Athene cunicularia</i> | 2 | K17; K31 |

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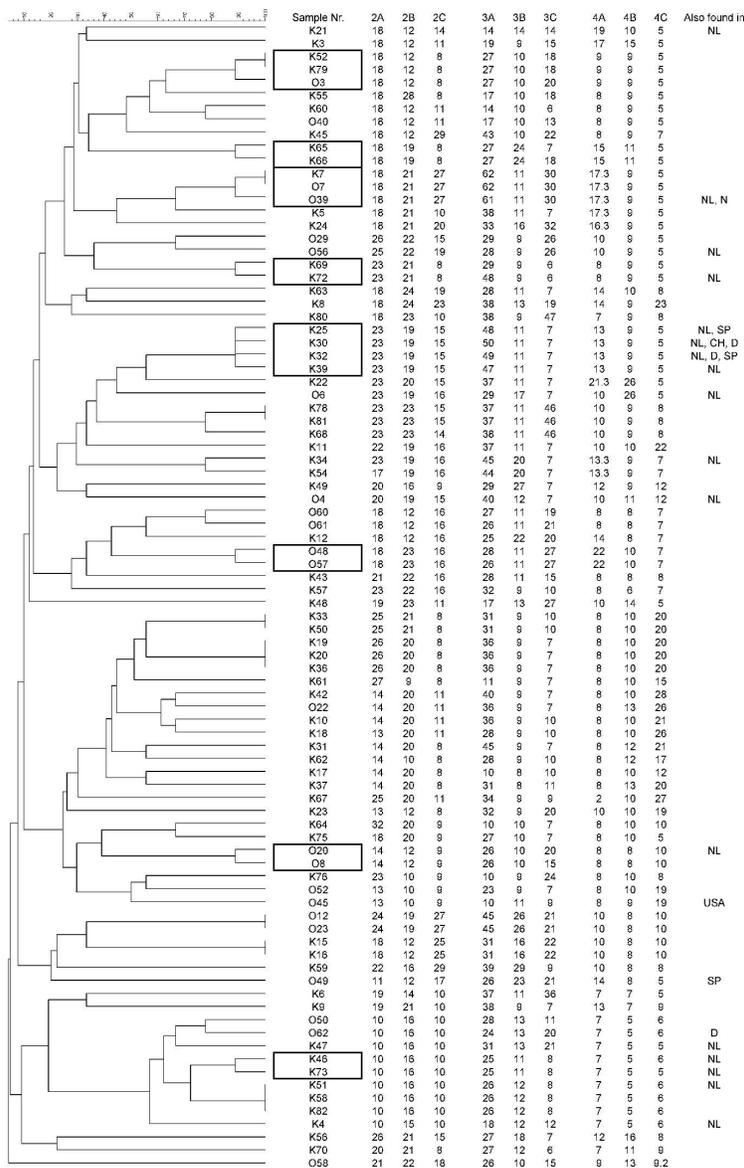


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