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1 **Microsatellite Typing of Avian Clinical and Environmental Isolates of**
2 ***Aspergillus fumigatus***
3

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20 Genotyping *Aspergillus fumigatus* isolates
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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~~9~~ genotypes obtained were compared to a database containing genotypes of approximately 2500 strains isolated 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.

51 **Introduction**

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

57 To investigate the genetic and the epidemiological relationship between
58 environmental and clinical isolates, fingerprinting methods with high discriminatory
59 power must be applied. Also interlaboratory reproducibility and objective
60 interpretation of the fingerprinting data are highly recommendable (de Valk *et al.*,
61 2007). All of these characteristics can be found in typing methods based on short
62 tandem repeats (STRs), such as microsatellite length polymorphism (MLP) and
63 STR*Af* typing (Bart-Delabesse *et al.*, 1998; de Valk *et al.*, 2005; de Valk *et al.*, 2007;
64 Vanhee *et al.*, 2008a). While the discriminatory power is high in pattern-based
65 techniques, such as random amplified polymorphic DNA (RAPD) analysis, restriction
66 fragment length polymorphism (RFLP) analysis, and amplified fragment length
67 polymorphism (AFLP), STR*Af* typing proved to be more simple and reproducible
68 (Bart-Delabesse *et al.*, 1998; de Valk *et al.*, 2005; de Valk *et al.*, 2007; Vanhee *et al.*,
69 2008a,b).

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

75 Therefore, in this study STR*Af* typing was performed on environmental and avian
76 clinical isolates of *A. fumigatus* and the results were compared with a database
77 containing genotypes from *A. fumigatus* isolated from clinical human samples and
78 from the environment.
79
80 **Material and Methods**
81 **Isolates.** Sixty-five clinical avian and 23 environmental isolates of *A. fumigatus* were
82 subjected to STR*Af* typing. The clinical isolates were collected at 6 different
83 institutes, 4 located in Belgium and 2 in The Netherlands and were obtained from
84 birds, belonging to 13 orders, 18 families and 35 species (Table 1), that died from an
85 *A. fumigatus* infection. To collect the environmental isolates, Sabouraud dextrose agar
86 plates were placed at 20 different locations in the vicinity of Ghent, Belgium. After
87 incubation for three days at room temperature, the plates were placed in an incubator
88 at 37°C. Fast-growing greenish colonies were purified on Sabouraud dextrose agar.
89 The isolates were identified based on the macro- and micro-morphology of the
90 fungus. Determination of partial DNA sequences of the beta-tubulin and rodletA
91 genes (Alcazar-Fuoli *et al*., 2008) and the ability to grow at 48°C were used to
92 confirm species identity.
93
94 **STR*Af* assay**. Fungal DNA was prepared from all isolates as described by Beernaert
95 *et al*. (2008). PCR primers for the STR*Af*2, -3 and -4 panels and amplification
96 conditions were as described by de Valk *et al*. (2005), except that FAM labelling was
97 replaced by VIC labelling, HEX labelling by NED labelling and TET labelling by
98 PET labelling. Allelic ladders were used with the same fluorescent labels as above for
99 each of the three trinucleotide markers in the STR*Af*3 panel as described by de Valk *et*

100 *al.* (2009). The obtained PCR products were diluted 10-fold with distilled water. Two
101 microliter of the diluted PCR products were added to 12 µl of formamide (Amresco
102 Inc, Ohio, USA) and 1 µl of GS 500 LIZ size standard (Applied Biosystems, Halle,
103 Belgium). Following denaturation of the samples for 2 min at 95°C and rapid cooling
104 to 0°C for 30 min, they were injected onto an ABI Prism 3100 Genetic Analyzer
105 (Applied Biosystems, Halle, Belgium) equipped with a 16 capillary array.
106 Genemapper v3.5 (Applied Biosystems, Halle, Belgium) was used to determine the
107 size of each amplified fragment. All results are reported as repeat numbers. Repeat
108 numbers for the markers in the STR*Af*3 panel were determined by comparison to the
109 allelic ladders. The repeat numbers of the markers in the STR*Af*2 and -4 panels were
110 determined with the reference size values taken from the original publication and
111 validated using a set of reference isolates with known genotypes (de Valk *et al.*,
112 2005).

113

114 **Data analysis**. Typing data was imported into BioNumerics version 6 software
115 (Applied Maths, St-Martens-Latem, Belgium) and analyzed using the categorical
116 multistate similarity coefficient with UPGMA clustering. The obtained genotypes
117 were then compared to a database containing genotypes from approximately 2500
118 human and environmental *A. fumigatus* isolates from Europe.

119

120 **Results**

121 A total of 88 *A. fumigatus* isolates were analyzed using STR*Af* typing and a total of
122 78~~9~~ genotypes were obtained. Seventy genotypes were found once, 6 genotypes were
123 found twice and 2 genotypes were found three times. The Simpsons index of diversity
124 (D) for the clinical avian isolates was calculated to be 0.995.

125 In the dendrogram (Figure 1), 8 clusters can be identified, in which the genotype of
126 the isolates only differed at a single locus. In one cluster, identical genotypes were
127 found for a clinical avian isolate and an environmental isolate. Two clusters contained
128 clinical and environmental isolates, four clusters only clinical isolates and two clusters
129 only environmental isolates.
130 Comparing the results of our analysis to a database containing genotypes from
131 approximately 2500 human and environmental isolates revealed that 59 genotypes in
132 our collection of avian and environmental isolates were unique. Eight genotypes
133 (clinical as well as environmental) belonging to 4 of the 8 clusters mentioned above
134 were also present in the collection of 2500 isolates. Eight genotypes from clinical
135 avian isolates were found in human isolates from the Netherlands, Switzerland and
136 Germany and 9 genotypes were also found in environmental isolates from the
137 Netherlands and Spain. Eight environmental genotypes from Belgium were identical
138 to genotypes found in human or environmental isolates from the Netherlands, Spain,
139 Germany, the United States of America and Norway.
140

141 **Discussion**

142 Previous epidemiological studies have investigated the origin of avian aspergillosis in
143 limited geographical areas and in one species of birds (Lair-Fulleringer *et al*., 2003;
144 Olias *et al*., 2009; Alvarez-Perez *et al*., 2010). This is the first study determining the
145 genetic diversity among *A. fumigatus* isolates obtained from 35 avian species
146 collected at 6 different institutes and environmental isolates collected at 20 different
147 locations in Belgium.
148 The results of this study demonstrate that the genetic diversity among avian clinical
149 isolates is extremely high. The 65 isolates, collected from 65 birds affected with

150 aspergillosis, belonged to 57 distinct genotypes. In contrast, Alvarez-Perez *et al.*
151 (2010) reported 13 distinct genotypes in 33 isolates from 5 diseased birds and Lair-
152 Fullinger *et al.* (2003) 23 distinct genotypes in 114 isolates from 30 healthy and 2
153 diseased turkeys. The low number of distinct genotypes in relation to the number of
154 isolates in those studies could be explained firstly by the fact that a limited number of
155 animals with aspergillosis were used. Secondly, the animals belonged to closed
156 collections and multiple isolates from each animal were examined. Finally, the
157 genotyping was performed with a method described by Bart-Delabesse *et al.* (1998)
158 including only four microsatellite markers being less discriminatory than the panel of
159 9 microsatellite markers described here. Alternatively, these animals may have been
160 exposed to a common source of material(s) that may have been contaminated with a
161 limited diversity of *Aspergillus fumigatus* spores such as bird feed.

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

167 High reproducibility of the STR*Af* assay and the ease of interlaboratory exchange of
168 the results allowed comparing the dataset of 108 clinical avian and environmental
169 isolates to a dataset of 2500 genotypes from human and environmental isolates
170 (~~Pasqualotto~~ de Valk *et al.*, 2009~~7~~). There appeared to be no specific association
171 between the observed genotypes and the origin of the isolates (environment, human or
172 bird). This was expected because of the high degree of genetic diversity among *A.*
173 *fumigatus* isolates, independent of the species or geographical region (Debeaupuis *et*
174 *al.*, 1997; Chazalet *et al.*, 1998; Rosehart *et al.*, 2002; Menotti *et al.*, 2005; Klaassen

175 *et al*., 2009). Moreover, 8 genotypes derived from diseased birds were also isolated
176 from humans with aspergillosis indicating that avian isolates of *A. fumigatus* could be
177 considered infectious to humans.
178
179 **Acknowledgements**

180 This work was supported by the Institute for the Promotion of Innovation through
181 Science and Technology in Flanders (IWT Vlaanderen), Brussels, Belgium.
182
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274 **Figure legends**

275 Figure 1: Dendrogram generated from genotyping 65 clinical and 23 environmental *A.*
276 *fumigatus* isolates. Isolates recovered from clinical samples are denoted as K and
277 strains isolated from the environment are denoted as O. Framed are the genotypes that
278 show microvariation. The scale bars above the dendrogram indicates the percentage
279 identity between the genotypes.

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

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

		Enicognathus leptorhynchus	1	K33
		Lathamus discolor	1	K39
Sphenisciformes	Spheniscidae	*Spheniscus sp*	1	K32
Strigiformes	Strigidae	*Athene cunicularia*	2	K17; K31

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