Microsatellite typing of *Aspergillus flavus* from clinical and environmental avian isolates

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Aspergillus is one of the most common causes of death in captive birds. *Aspergillus fumigatus* accounts for approximately 95 % of aspergillosis cases and *Aspergillus flavus* is the second most frequent organism associated with avian infections. In the present study, the fungi were grown from avian clinical samples (post-mortem lung material) and environmental samples (eggs, food and litter). Microsatellite markers were used to type seven clinical avian isolates and 22 environmental isolates of *A. flavus*. *A. flavus* was the only species (28 % prevalence) detected in the avian clinical isolates, whereas this species ranked third (19 %) after members of the genera *Penicillium* (39 %) and *Cladosporium* (21 %) in the environmental samples. Upon microsatellite analysis, five to eight distinct alleles were detected for each marker. The marker with the highest discriminatory power had eight alleles and a 0.852 *D* value. The combination of all six markers yielded a 0.991 *D* value with 25 distinct genotypes. One clinical avian isolate (lung biopsy) and one environmental isolate (egg) shared the same genotype. Microsatellite typing of *A. flavus* grown from avian and environmental samples displayed an excellent discriminatory power and 100 % reproducibility. This study showed a clustering of clinical and environmental isolates, which were clearly separated. Based upon these results, aspergillosis in birds may be induced by a great diversity of isolates.

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INTRODUCTION

Fungal infections due to members of the genus *Aspergillus* are a major cause of morbidity and mortality among certain captive or free-ranging bird species, independent of age or immune system status (Beernaert *et al.*, 2008; Tell, 2005). The airborne spores of *Aspergillus* species are ubiquitous, and avian aspergillosis occurs wherever environmental conditions are favourable to fungal growth. Increased relative humidity in poultry houses with a water supply and poor ventilation, as well as mouldy straw bedding and feeding with mouldy grain, expose birds to numerous fungal spores, which is the key risk factor for aspergillosis. In veterinary hospitals, this disease is a frequent cause of secondary infection in already stressed or debilitated birds. Although this fungus is not contagious by the horizontal transmission route, aspergillosis epidemics occur in birds exposed to the same stressors or other environmental risk factors. In comparison with mammals, birds are highly susceptible to respiratory infection with *Aspergillus fumigatus* (Tell, 2005), and pulmonary invasive aspergillosis is a

common disease in birds, causing significant economic losses in the poultry industry, with up to 30 % fatality (Zafra *et al.*, 2008). While outbreaks of human invasive aspergillosis in hospital settings have been studied by several groups (Bertout *et al.*, 2001; Chazalet *et al.*, 1998; Rosehart *et al.*, 2002), only one study focused on the epidemiology of aspergillosis in poultry (Lair-Fullerger *et al.*, 2003), and another recently in captive penguins (Alvarez-Perez *et al.*, 2010). In these reports, *Aspergillus flavus* was second only to *A. fumigatus*, which accounted for approximately 95 % of avian infections.

The descriptions of aspergillosis in penguins focused chiefly on clinical, histopathological and serological findings. Only three studies reported on the molecular typing of the *A. fumigatus* isolates obtained from diseased animals (Alvarez-Perez *et al.*, 2010; Olias *et al.*, 2011; Van Waeyenberghe *et al.*, 2011). This lack of molecular data precludes epidemiological analyses, in particular, it is impossible to determine whether multiple genotypes of members of the genus *Aspergillus* (a polyclonal infection)

or a single fungal genotype (a monoclonal infection) are involved in each infection.

Distinguishing between these possibilities has substantial diagnostic and therapeutic implications. Therefore, typing methods with high discriminatory power, an objective interpretation of the typing results and inter-laboratory reproducibility should be used (de Valk *et al.*, 2007). Typing methods based on short tandem repeats, such as microsatellite length polymorphisms, have these characteristics. The present study aimed to analyse the fungal flora in environmental and clinical samples from birds and to type *A. flavus* isolates using microsatellite markers.

METHODS

Mycological analysis. Fungi were cultured from the bird's environment, i.e. from eggs, food and litter samples. The egg shells were swabbed. One gram of each litter or food sample was placed in a tube containing 10 ml sterile water. After vigorous shaking, 1 ml suspension was inoculated onto Sabouraud-chloramphenicol agar and Czapek medium. Culture plates were incubated at 25 °C or at 37 °C for 4 days. Avian clinical isolates were isolated post-mortem by plating lung material from deceased chicks (bird number=25) obtained during aseptic necropsy.

Identification of the isolates and DNA isolation. These *A. flavus* strains were identified on the basis of macroscopic and microscopic morphological characteristics. The morphological identification of *A. flavus* isolates was confirmed by the rRNA ITS1–5.8S–ITS2 region sequence analysis, as previously described by De Hoog *et al.* (2007). DNA was extracted by using a QIAmp kit (QIAGEN), following the manufacturer's instructions, and eluted with 50 µl sterile water.

Microsatellite typing. *A. flavus* isolates were genotyped by using six polymorphic microsatellite markers (AFLA1, AFLA2, AFLA3, AFPM3, AFPM7 and AFPM1), as described previously (Hadrich *et al.*, 2010a). In each multiplex PCR assay, different fluorescent labels (FAM, PET, NED, VIC) were used to distinguish the amplification products from distinct markers. PCR assays were performed in a final volume of 25 µl, containing 1 ng genomic DNA, 1 µM all amplification primers (Table 1), 0.2 mM each dNTP, 3 mM MgCl₂ and 1 U AmpliTaq DNA polymerase (Applied Biosystems) in 1 × reaction buffer (Applied Biosystems). Thermocycling was performed in a T1 thermocycler (Bio-Rad) using the following protocol:

5 min initial denaturation at 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 54 °C and 30 s extension at 72 °C, finally followed by 30 min at 72 °C. PCR products were diluted tenfold with formamide. One microlitre of diluted PCR products was combined with 15 µl formamide and 0.5 µl LIZ 500 marker (Applied Biosystems). Following denaturation, the PCR products were resolved by capillary electrophoresis with polymer POP-4 in an ABI Prism 3130 genetic analyser (Applied Biosystems). Injection and running parameters followed the manufacturer's recommendations (Applied Biosystems). Analyses were performed with Gene Scan software (Applied Biosystems). The reproducibility of microsatellite typing was evaluated by using five different DNA preparations of the same isolate and by performing ten repeated analyses of the same DNA preparation.

Statistical analyses. The Simpson index of diversity, *D*, (Hunter & Gaston, 1988) was computed for each marker and each possible marker's combination, aiming at determining the most parsimonious combination yielding a *D* value >0.95, a sufficiently high discriminatory power, as recommended for typing experiments. The similarity between genotypes was estimated with the Dice coefficient using NTSYSPc numerical taxonomy and multivariate analysis system, version 2.1 (Exeter Software), and an UPGMA dendrogram was generated. Isolates possessing alleles with the same number of repeat units in all loci were defined as a clonal cluster.

RESULTS

The mycological analysis of 150 environmental (eggs, food and litter) samples revealed that members of the genus *Penicillium* (39%) were the most frequent fungal species, followed by those of the genus *Cladosporium* (21%), *A. flavus* (19%), members of the genus *Alternaria* (12%), *A. niger* (3%), members of the genera *Rhizopus* (3%) and *Scopulariopsis* (2%), and *A. ochraceus* (1%). From 25 avian clinical samples, *A. flavus* was the unique species isolated from seven (28%) lung biopsy samples.

A total of 29 *A. flavus* isolates (22 from eggs, food and litter, and seven from lung biopsies) were typed using microsatellite markers. There were five to eight distinct alleles in each microsatellite marker (Fig. 1). The highest discriminatory power for a single locus was obtained with the AFPM7 marker, which had eight distinct alleles and a *D* value of 0.852 (Table 1). The combination of all six

Table 1. Features of the six polymorphic microsatellite sequences of *A. flavus* upon analysis of 29 avian isolates

Primer name	Primer sequence (5'–3')	Repeat unit	Fragment size (bp)	No. of alleles	<i>D</i>
AFLA1	CGTTGGCATGTTATCGTCACCTACTGAATGGCGGGACCTA	AC	192–270	7	0.818
AFLA3	CTGAAAGGGTAAGGGGAAGGCACGCGAACTTATGGGACTT	TAGG	182–262	7	0.784
AFLA7	GCGGACACTGGATGAATAGCAACAAATCGGTGGTTGCTTC	TAG	190–244	6	0.695
AFPM3	CCTTTCGCACTCCGAGACCACCACCACTGATGAGGG	(AT)6AAGGGCG(GA)	188–254	5	0.738
AFPM4	AGCGATACAGTTTAAACACCTCTTGCTATACATATCTTCA-CC	CA	198–272	8	0.848
AFPM7	TTGAGGCTGCTGTGGAACGCCAAATACCAATTACGTCCAAC-AAGGG	AC	212–252	8	0.852

Fig. 1. Allele size distribution of *A. flavus* at six microsatellites upon analysis of 29 avian isolates. Samples: solid bars, lung biopsy; striped bars, litter samples; grey bars, eggs; white bars, feed.

markers yielded 25 different genotypes with a *D* value 0.991. The dendrogram (Fig. 2) shows eight isolates that differ at two loci and 19 isolates that differ at four loci. One genotype (BP02_EG18) was identical in a clinical avian and an environmental (egg) isolate.

DISCUSSION

Typing *A. flavus* isolates may help to improve the understanding of the distribution of this major pathogen in different situations and environments, including susceptible

Fig. 2. Dendrogram generated from genotyping seven clinical and 22 environmental *A. flavus* avian isolates using UPGMA methods.

birds in poultry farms. This molecular approach may also lead to a better understanding of the colonization pattern of this fungus. To date, whether genetic virulence factors are associated with *A. flavus* infection is still an open question that, to our knowledge, has not been addressed in *A. flavus* avian clinical isolates. While many different studies have focused on *A. fumigatus* typing (Bart-Delabesse *et al.*, 1998; Lair-Fullerenger *et al.*, 2003; Van Waeyenberghe *et al.*, 2011), *A. fumigatus* was not detected in the present study. In contrast, *A. flavus* was isolated from eggs, food and litter samples, and was the only species isolated from clinical samples.

Although *A. fumigatus* is the most common aetiological agent of infection caused by members of the genus *Aspergillus*, it is not a unique pathogenic species in this genus. *A. flavus* can also cause avian infections (Barton *et al.*, 1992; Knudtson & Meinecke, 1972; Okoye *et al.*, 1989; Richard & Thurston, 1983). In fact, *A. flavus* is the predominant aetiological agent of human invasive aspergillosis in Sfax, Tunisia (Hadrich *et al.*, 2010b). However, the potential health problems in turkeys should not be underestimated, because *A. flavus* is a toxigenic species. The presence of mycotoxins within, as well as on, the surface of *A. flavus* spores has been shown (Hendry & Cole, 1993). Furthermore, a higher toxicity has been associated with the respiratory route, compared with that of the oral route. The dose of mycotoxin required to cause particular effects when administered by inhalation is one order of magnitude lower than that required by ingestion (Hendry & Cole, 1993).

This study demonstrated an extremely high genetic diversity among avian clinical isolates. In contrast, Alvarez-Perez *et al.* (2010) reported 13 distinct genotypes in 33 isolates from five diseased birds, and Lair-Fullerenger *et al.* (2003) reported 23 distinct genotypes in 114 isolates from 30 healthy and two diseased turkeys. The relatively low number of distinct genotypes compared with the number of isolates in those studies might be explained by, firstly, the limited number of animals with aspergillosis that were studied; secondly, the confined space in which the birds were raised and the presence of multiple isolates from each animal; and finally, the genotyping with only four microsatellite markers, as described by Bart-Delabesse *et al.* (1998), which is probably less discriminating than the six microsatellite marker panel used herein.

Using an RFLP technique, Debeaupuis *et al.* (1997) found 424 different genotypes among 879 isolates. By using microsatellite analysis, Bart-Delabesse *et al.* (1998) detected 43 genotypes represented only once among 62 isolates. Thierry *et al.* (2010) used multiple-locus variable-number tandem repeat analysis to type 57 *A. fumigatus* unrelated isolates collected from avian species and their environment, and they found a clear separation between isolates according to their geographical origin rather than their respective hosts. In the present study, both environmental and avian isolates were scattered throughout the dendrogram (Fig. 2), suggesting that any environmental isolate of *A. flavus* might

be infectious to birds. This is in line with Peden & Rhoades (1992), who showed that any isolate, irrespective of its origin (environmental, mammalian or avian), could cause aspergillosis when they were inoculated into the air sacs of turkeys. In our study, one *A. flavus* avian clinical (lung biopsy) and one environmental (egg) isolate shared an identical genotype. Interestingly, the contamination might be acquired inside the egg by the passage of spores through a dirty and stained shell, especially if it has micro-lesions. Recently, Van Waeyenberghe *et al.* (2011) compared the genotypes of avian isolates with those of 2514 human clinical and environmental isolates. They found that the same *A. fumigatus* genotype may infect both humans and birds.

In conclusion, microsatellite typing of avian and environmental *A. flavus* isolates displayed excellent discriminatory power. This study showed an interesting clustering with a clear separation between clinical and environmental isolates, and pointed out the great diversity of *A. flavus* genotypes involved in bird infections.

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REFERENCES

- Alvarez-Perez, S., Mateos, A., Dominguez, L., Martinez-Nevado, E., Blanco, J. L. & Garcia, M. E. (2010). Polyclonal *Aspergillus fumigatus* infection in captive penguins. *Vet Microbiol* **144**, 444–449.
- Bart-Delabesse, E., Humbert, J. F., Delabesse, E. & Bretagne, S. (1998). Microsatellite markers for typing *Aspergillus fumigatus* isolates. *J Clin Microbiol* **36**, 2413–2418.
- Barton, J. T., Daft, B. M., Read, D. H., Kinde, H. & Bickford, A. A. (1992). Tracheal aspergillosis in 6 1/2-week-old chickens caused by *Aspergillus flavus*. *Avian Dis* **36**, 1081–1085.
- Beernaert, L. A., Pasmans, F., Haesebrouck, F. & Martel, A. (2008). Modelling *Aspergillus fumigatus* infections in racing pigeons (*Columba livia domestica*). *Avian Pathol* **37**, 545–549.
- Bertout, S., Renaud, F., Barton, R., Symoens, F., Burnod, J., Piens, M. A., Lebeau, B., Viviani, M. A., Chapuis, F. & other authors (2001). Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: investigation using multiple typing methods. *J Clin Microbiol* **39**, 1731–1737.
- Chazalet, V., Debeaupuis, J. P., Sarfati, J., Lortholary, J., Ribaud, P., Shah, P., Cornet, M., Vu Thien, H., Gluckman, E. & other authors (1998). Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. *J Clin Microbiol* **36**, 1494–1500.
- De Hoog, G. S., Nishikaku, A. S., Fernandez-Zeppenfeldt, G., Padin-González, C., Burger, E., Badali, H., Richard-Yegres, N. & van den Ende, A. H. (2007). Molecular analysis and pathogenicity of the *Cladophialophora carrionii* complex, with the description of a novel species. *Stud Mycol* **58**, 219–234.
- De Valk, H. A., Meis, J. F. & Klaassen, C. H. (2007). Microsatellite based typing of *Aspergillus fumigatus*: strengths, pitfalls and solutions. *J Microbiol Methods* **69**, 268–272.
- Debeaupuis, J. P., Sarfati, J., Chazalet, V. & Latgé, J. P. (1997). Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. *Infect Immun* **65**, 3080–3085.

- Hadrich, I., Makni, F., Ayadi, A. & Ranque, S. (2010a).** Microsatellite typing to trace *Aspergillus flavus* infections in a hematology unit. *J Clin Microbiol* **48**, 2396–2401.
- Hadrich, I., Makni, F., Sellami, H., Cheikhrouhou, F., Sellami, A., Bouaziz, H., Hdiji, S., Elloumi, M. & Ayadi, A. (2010b).** Invasive aspergillosis: epidemiology and environmental study in haematology patients (Sfax, Tunisia). *Mycoses* **53**, 443–447.
- Hendry, K. M. & Cole, E. C. (1993).** A review of mycotoxins in indoor air. *J Toxicol Environ Health* **38**, 183–198.
- Hunter, P. R. & Gaston, M. A. (1988).** Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* **26**, 2465–2466.
- Knudtson, W. U. & Meinecke, C. F. (1972).** Pneumonic aspergillosis in poult caused by *Aspergillus flavus*. *Avian Dis* **16**, 1130–1132.
- Lair-Fuller, S., Guillot, J., Desterke, C., Seguin, D., Warin, S., Bezille, A., Chermette, R. & Bretagne, S. (2003).** Differentiation between isolates of *Aspergillus fumigatus* from breeding turkeys and their environment by genotyping with microsatellite markers. *J Clin Microbiol* **41**, 1798–1800.
- Okoye, J. O., Gughani, H. C. & Okeke, C. N. (1989).** Pulmonary infections due to *Aspergillus flavus* in turkey poult and goslings. *Mycoses* **32**, 336–339.
- Olias, P., Gruber, A. D., Hafez, H. M., Lierz, M., Slesiona, S., Brock, M. & Jacobsen, I. D. (2011).** Molecular epidemiology and virulence assessment of *Aspergillus fumigatus* isolates from white stork chicks and their environment. *Vet Microbiol* **148**, 348–355.
- Peden, W. M. & Rhoades, K. R. (1992).** Pathogenicity differences of multiple isolates of *Aspergillus fumigatus* in turkeys. *Avian Dis* **36**, 537–542.
- Richard, J. L. & Thurston, J. R. (1983).** Rapid hematogenous dissemination of *Aspergillus fumigatus* and *A. flavus* spores in turkey poult following aerosol exposure. *Avian Dis* **27**, 1025–1033.
- Rosehart, K., Richards, M. H. & Bidochka, M. J. (2002).** Microsatellite analysis of environmental and clinical isolates of the opportunist fungal pathogen *Aspergillus fumigatus*. *J Med Microbiol* **51**, 1128–1134.
- Tell, L. A. (2005).** Aspergillosis in mammals and birds: impact on veterinary medicine. *Med Mycol* **43** (Suppl. 1), S71–S73.
- Thierry, S., Wang, D., Arné, P., Deville, M., De Bruin, B., Niegutsila, A., Pourcel, C., Laroucau, K., Chermette, R. & other authors (2010).** Multiple-locus variable-number tandem repeat analysis for molecular typing of *Aspergillus fumigatus*. *BMC Microbiol* **10**, 315.
- Van Waeyenberghe, L., Pasmans, F., Beernaert, L. A., Haesebrouck, F., Vercammen, F., Verstappen, F., Dorrestein, G. M., Klaassen, C. H. & Martel, A. (2011).** Microsatellite typing of avian clinical and environmental isolates of *Aspergillus fumigatus*. *Avian Pathol* **40**, 73–77.
- Zafra, R., Pérez, J., Pérez-Ecija, R. A., Borge, C., Bustamante, R., Carbonero, A. & Tarradas, C. (2008).** Concurrent aspergillosis and ascites with high mortality in a farm of growing broiler chickens. *Avian Dis* **52**, 711–713.