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Differentiation between Isolates of *Aspergillus fumigatus* from Breeding Turkeys and Their Environment by Genotyping with Microsatellite Markers

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To elucidate the epidemiology of the different forms of avian aspergillosis, 114 *Aspergillus fumigatus* isolates from sacrificed turkeys and 134 *A. fumigatus* isolates from air samples were collected and genotyped by microsatellite polymorphism marker analysis. Air sampling confirmed the huge diversity of *A. fumigatus* populations. Whereas older animals harbored several combinations of genotypes, 1-day-old chicks carried a unique genotype, suggesting a unique source of contamination.

Infection with *Aspergillus fumigatus* is a common respiratory disease in birds. Any avian species, captive or free, domesticated or wild, is susceptible to this fungal infection (11). With turkeys, aspergillosis leads to consequential economic losses related to low productivity, mortality, and carcass condemnations at slaughter inspection. Two forms of the disease are regularly reported for turkeys. The first form is an acute aspergillosis leading to severe outbreaks in very young birds. Dramatic mortality is then observed in chicks a few days old. Clinical signs usually include dyspnea, gasping, and inappetence. Lesions are first located in the air sacs and the lungs, but dissemination rapidly occurs and accounts for additional clinical signs, such as diarrhea and encephalitis (11, 12). The second form is a chronic aspergillosis occurring in adult turkeys. Birds usually survive, but the resulting low productivity is a source a considerable monetary loss (6, 11). Molecular typing studies should help to elucidate the epidemiology of the different forms of avian aspergillosis.

In the present study, a 16-week surveillance program was set in a turkey confinement house where several outbreaks of aspergillosis had previously occurred. Our purpose was to collect and genotype *A. fumigatus* isolates to characterize environmental sources in the confinement house and to assess whether pathogenic genotypes could be identified in turkeys. We used the polymorphic microsatellite marker (PMM) analysis because this technique has a very high level of discriminatory power, produces reproducible results, and is easy to use (1–3, 7).

Animals. A flock comprising 4,500 breeding turkeys (2,250 males and 2,250 females) was subjected to analysis. At the age of 1 day, the animals were placed in a 600-m^2 confinement building near Orléans in the center of France. The birds originated from a hatchery located in Britain. Female turkeys were slaughtered at the age of 12 weeks, and males were slaughtered at the age of 16 weeks.

Mycological samples. Thirty-two animals were subjected to a necropsic examination during the breeding period. Ten 1-day-old chicks, five males and five females, were sacrificed when they arrived in the confinement house. Twenty healthy animals, from 25 to 87 days old, were sacrificed during the study, and two additional birds were retrieved after carcass condemnations at slaughter inspection, on week 16. The lungs were aseptically collected and cut into small pieces. Ten lung tissue sections were applied onto Sabouraud dextrose agar with 0.5% chloramphenicol in petri dishes. The plates were incubated for 4 days at 40°C to enhance the growth of the thermophilic species *A. fumigatus* and limit the contamination by other molds. When macroscopic lesions compatible with aspergillosis were observed at necropsic examination, samples of lungs, kidneys, abdominal air sac, and brain were collected. Tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin and periodic acid-Schiff.

Environmental surveillance was conducted throughout the breeding period during 16 weeks from December 2000 to March 2001. Air samplings were performed once a week in the confinement house with a bioimpactor (Air Strategie Bio-impactor 100-08) loaded with Sabouraud dextrose agar plates. Air samples of 100 liters were taken on each occasion. The plates were incubated at 40°C. Recovered organisms were identified by colonial and conidial morphology. All the colonies identified as *A. fumigatus* were subcultured and stored at 4°C until molecular typing.

Microsatellite typing. Each isolate was subcultured on Sabouraud slants for 3 days at 37°C. A conidial suspension in sterile distilled water was obtained from each subculture, frozen in liquid nitrogen, and centrifuged. DNA extraction from 180 μl of supernatant was performed by use of the Dneasy tissue kit (Qiagen, Courtabœuf, France). Since upon a previous analysis of 102 isolates, two microsatellite markers, referred to as C and D, were shown to have a discriminatory

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TABLE 1. Genotypes of *A. fumigatus* isolates collected from breeding turkeys

Age of bird, days (animal identification)	No. of isolates	No. of genotypes	Microsatellite genotype reference(s)[a]
1-day-old chicks			
1 (I)	4	1	A108B106C173D108
1 (II)	4	1	A108B106C173D108
1 (III)	4	1	A108B106C173D108
1 (IV)	4	1	A108B106C173D108
1 (V)	3	1	A108B106C173D108
1 (VI)	4	1	A108B106C173D108
Healthy turkeys (>1 day old)			
25 (VII)	9	6	C171D110, C173D76, C175D76, C177D104, C181D82, C181D84
25 (VIII)	2	2	C175D76, C179D86
37 (IX)	5	2	C173D108, C173D110
44 (X)	4	3	C173D108, C175D76, C179D84
65 (XI)	14	2	C167D98, C173D118
65 (XII)	2	1	C173D76
65 (XIII)	16	3	C169D102, C173D76, C177D120
79 (XIV)	2	2	C171D98, C173D116
87 (XV)	1	1	C169D102
Turkeys with aspergillosis			
106 (XVI)	27	1	C173D112
106 (XVII)	9	1	C169D102

[a] Genotype reference comprises microsatellite loci (A, B, C, and D) and corresponding molecular sizes (in base pairs) of PCR products.

power of 0.980 (2), these two markers were retained in a first step to analyze all the isolates. One primer of each set specific for markers C and D was labeled with a fluorescent dye, either 6-carboxyfluorescein or 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein) (Oligo-Express, Paris, France), for detection with an automated DNA sequencer. Reaction conditions were as described by Bart-Delabesse et al. (2). Each PCR product consisted of a single band, which was considered to be an allelic type because *A. fumigatus* is haploid. The coding system for each genotype included the name of the PMMs (C and D) followed by allele sizes in base pairs. For isolates of the same genotype, the two other PMMs previously described, i.e., A and B (2), were used to confirm the identity of the genotype.

Globally, 248 *A. fumigatus* isolates were typed by PMM analysis. This led to the resolution of 70 distinct genotypes. More than half (37 of 70; 52.9%) of these genotypes were detected only once. For a specific PMM, only one band was observed after amplification. This finding confirms that there was no mixture of several isolates in our study.

The presence of *A. fumigatus* was detected in 17 turkeys (6 chicks and 11 birds older than 1 day) out of the 32 sacrificed animals. A total number of 114 isolates were collected, which represented 17 distinct genotypes (Table 1). Eight (8 of 17; 47.0%) genotypes were observed only once.

Twenty-three isolates were obtained from six healthy chicks that were sacrificed upon their arrival in the confinement house. These isolates corresponded to the unique genotype C173D108 (Table 1). This genotype was also detected from two healthy turkeys, which were sacrificed at days 37 and 44. The identity of the 23 isolates from chicks was confirmed using two additional PMMs (A and B). Unique allelic types were found (A108 and B106). Due to the very high discriminatory power of microsatellite markers, it is highly probable that the chicks had been simultaneously contaminated by the same fungal source during or after hatchering. This would mean that a given isolate could heavily contaminate hatcheries and subsequently also contaminate confinement houses. We planned to collect and genotype environmental isolates from several hatcheries to confirm this hypothesis. However, no case of aspergillosis was observed in the first week of the breeding period. This finding indicates that the chicks were probably able to eliminate the isolates they acquired in the hatchery. The susceptibility of the particular genotype C173D108 to itraconazole and amphotericin B was determined by an agar diffusion-dilution method (the Etest method) (8). MICs (1.0 and 0.25 μg/ml for the two antifungal drugs, respectively) were within usual values and did not indicate resistance, which could have been the consequence of the use of azole derivatives for decontamination of the hatchery.

A total number of 55 *A. fumigatus* isolates were obtained from 9 healthy turkeys, which were sacrificed during the breeding period. Lesions compatible with aspergillosis were never observed, and each animal carried from one to six distinct *A. fumigatus* genotypes (Table 1). A similar situation is described for cystic fibrosis patients who are supposed to be colonized rather than infected by *A. fumigatus* isolates (9, 14).

We finally examined 36 isolates from two animals (turkeys XVI and XVII) in which lesions of aspergillosis were detected at slaughter inspection. Histological examination confirmed the presence of hyaline fungal hyphae in lungs, abdominal air sacs, and kidneys. Typical conidial heads were also observed in the abdominal air sac. These turkeys were infected by their own distinct genotypes (C173D112 in turkey XVI and C169D102 in turkey XVII) (Table 1). Similarly, several investigations have demonstrated that only one or two genotypes are usually responsible for invasive aspergillosis in humans (1, 5).

A total number of 134 isolates representing 53 distinct genotypes were obtained from air samples (Table 2). Thirty-three (33 of 53; 62.3%) genotypes were observed only once. The remaining 20 genotypes were detected during several weeks,

TABLE 2. Genotypes of *A. fumigatus* isolates collected from air samples[a]

Sampling week	No. of *A. fumigatus* isolates	No. of microsatellite genotypes	Microsatellite genotype reference(s)[b]
3	3	3	C167D102, **C171D110**, **C175D76**
4	1	1	**C171D110**
5	7	5	C171D108, C171D124, **C173D76**, **C173D110**, **C175D76**, C181D106
6	30	18	C167D76, C167D84, C167D92, C167D96, C167D102, C167D104, C171D102, **C171D110**, C173D66, **C173D76**, C173D78, C173D98, **C173D108**, C173D114, C175D66, **C175D76**, C177D100, C183D78
7	15	8	C167D96, C167D104, C171D128, C173D86, **C173D108**, **C175D76**, C177D140, C185D98
8	3	3	C167D104, C173D82, **C173D108**
9	19	16	C167D84, **C167D98**, C167D100, C167D102, C167D104, C169D108, C171D64, C171D104, **C171D110**, **C173D108**, C171D122, **C175D76**, C177D60, C177D84, **C179D86**, C181D94
10	7	4	C167D100, **C173D76**, **C173D108**, **C175D76**
11	2	2	**C173D116**, C177D118
12	7	5	C167D84, C167D86, C173D114, C177D106, C181D126
13	4	4	C167D92, **C169D102**, **C173D108**, C179D114
14	22	9	C167D96, **C167D98**, **C169D102**, C169D112, C171D108, **C171D110**, **C175D76**, C177D110, **C179D84**
15	9	5	C171D108, **C173D108**, **C175D76**, C179D60, C179D152
16	5	5	C171D106, **C171D110**, C171D126, C177D82, C179D78

[a] Environmental genotypes also detected in animals are indicated in bold. Genotypes concomitantly detected in air samples and in animals (the same week) are underlined

[b] Genotype reference comprises microsatellite loci (C and D) and corresponding molecular sizes (in base pairs) of PCR products.

with a maximum persistence of 8 weeks for genotype C175D76. Similar results were reported when the genetic diversity of *A. fumigatus* isolates from a hospital environment was investigated (1, 2, 4, 5, 13). Using a restriction fragment length polymorphism technique, Debeaupuis et al. (5) found 424 different genotypes among 879 isolates. By using PMM analysis, Bart-Delabesse et al. (1) detected 43 genotypes represented only once among 62 isolates. In the present study, 10 genotypes were detected both in animals and in air samples. Isolates corresponding to the unique genotype (C173D108) detected in 1-day-old chicks were frequently recovered in air samples (on weeks 6, 7, 8, 9, 10, 13, and 15), suggesting a possible contamination of the environment by the birds placed in the confinement house. In seven cases, the same genotype was concomitantly observed in turkeys and in air samples. Moreover, the same genotype was detected in both healthy and infected birds, suggesting the absence of particular virulent genotypes for turkeys. Previous investigations have shown that no particular *A. fumigatus* genotype was associated with virulence in humans (1, 2, 5). In birds, the absence of particular virulent *A. fumigatus* isolates was confirmed by Peden and Rhoades, who inoculated isolates from diverse origins (environmental, mammalian, and avian) in air sacs of turkeys (10).

Our study confirms the usefulness of PMM for typing of numerous isolates of *A. fumigatus*. This kind of investigation can detect massive contamination of turkeys at a given time, which could be amenable to specific preventive measures.

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