Evolution of the Environmental Contamination by Thermophilic Fungi in a Turkey Confinement House in France

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ABSTRACT Fungal species constitute a major part of environmental contaminants in facilities where animals are housed. The present investigation was aimed at describing the relative abundances of fungal species and their concentrations in a turkey confinement house in France. Fungal cultures from poultry feed, litter, and air were undertaken every week throughout the 16-wk period of breeding. The incubation temperature of 40°C was selected to isolate thermophilic fungal species (especially *Aspergillus* spp. and *Candida albicans*) that are potentially pathogenic for birds. The 2 species *Aspergillus fumigatus* and *Aspergillus flavus* were recovered at a mean of 10.5 and 37.0 cfu/m^3 of air sampled, respectively. Individual samplings yielded concentrations of up to 150.0 cfu/m^3 for *A. flavus* in the first weeks of the investigation. Other fungal species were recovered at a mean of 18.9 cfu/m^3 (maximum 36.3 cfu/m^3) in the air. The yeast *C. albicans* was first detected at wk 4 from litter samples and at wk 7 from poultry feed. Densities of *C. albicans* remained very high in litter samples (63.2 cfu/g) even after new litter was added at wk 10. To analyze the genetic polymorphism of *A. fumigatus*, the most pathogenic mold in birds, a total number of 198 isolates (134 from air, 34 from litter, and 30 from feed samples) were genotyped using 2 polymorphic microsatellite markers. More than half (42 out of 73, 57.5%) of the genotypes were detected only once. This finding suggests that the contamination of the breeding environment is not due to a single source and confirms the very high genetic diversity of environmental *A. fumigatus* isolates. As during the study period, no outbreak of fungal infections occurred; the levels of fungal contaminations reported here do not seem sufficient, at least alone, to trigger fungal infections.

Key words: fungi, contamination, *Aspergillus*, *Candida*, environment

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INTRODUCTION

To increase poultry production, birds are extensively fed in buildings that are mainly confinement structures densely stocked with birds. A mechanical ventilation system is usually set up to maintain the health status of birds indoors. However, microorganisms resulting from litter, droppings, and feed are easily accumulated and aerosolized in such densely populated and enclosed buildings. These microorganisms include parasite, bacterial, and fungal species. In breeding turkeys, airsacculitis caused by molds of the genus *Aspergillus* is of the highest importance (Morris and Fletcher, 1988; Tell, 2005). Two forms of aspergillosis have been found in turkeys. The first one is acute aspergillosis, leading to severe outbreaks in very young birds (brooder aspergillosis). The second form is chronic aspergillosis occurring in adult turkeys. Adult birds may survive, but the resulting low productivity is a source of considerable monetary loss (Richard, 1997). The epidemiology of these 2 forms of aspergillosis in turkeys remains poorly understood. It has been hypothesized that acute aspergillosis occurs after exposure to overwhelming numbers of *Aspergillus* conidia just after hatching. Chronic aspergillosis would be related to contaminated poultry litter or feed during breeding (Richard et al., 1984). Intestinal candidosis is another mycosis that may be observed in avian confinement houses. Guinea fowls are much more sensitive than other birds. However, intestinal candidosis has also been described in other birds such as turkeys and chickens. The responsible yeast, *Candida albicans*, is part of the normal intestinal microflora and does not proliferate outside its hosts. Nevertheless, environmental contamination occurs and may constitute a source for *Candida* exposure for birds.

The present study was aimed at describing the relative abundances of fungal species and their concentrations in

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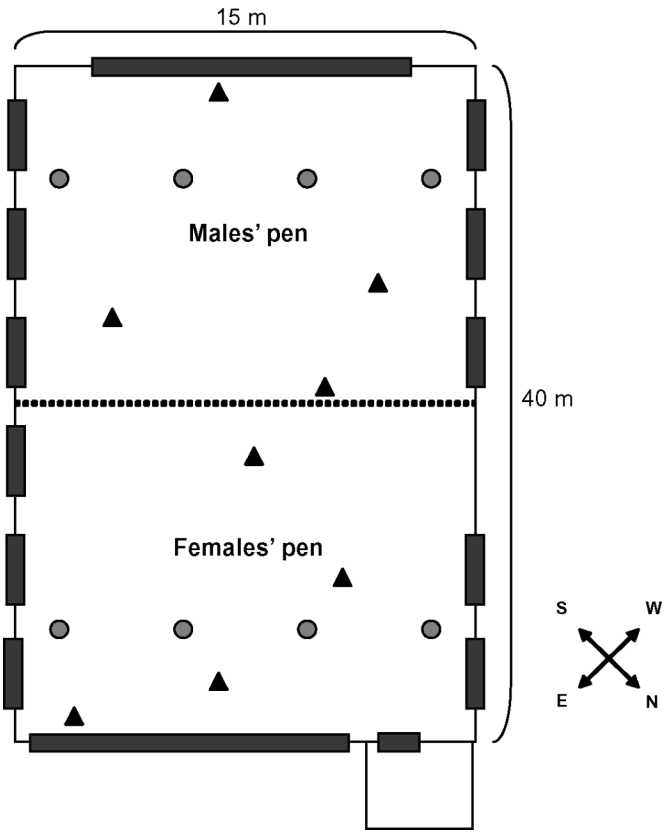

Figure 1. Schematic diagram of the confinement house where the turkeys were bred. Doors and windows are indicated by black parallelepipeds. Males and females turkeys were maintained in 2 distinct pens. Every week, air samples were taken at 8 sites (circles). Litter was collected at 8 different locations (triangles).

a facility where turkeys were housed. A 16-wk surveillance program was undertaken for the detection of fungal species in the air, food, and litter. The incubation temperature of 40°C was selected to isolate only thermophilic fungal species (especially *Aspergillus* spp. and *C. albicans*) that are potentially pathogenic for birds. The *Aspergillus fumigatus* isolates collected during the study period were genotyped and compared by use of 2 polymorphic microsatellite markers (**PMM**; Bart-Delabesse et al., 1998).

MATERIALS AND METHODS

Confinement House and Birds

The study was made in a 600-m^2 grower-type confinement house, a basic pole barn 15 m wide and 40 m long. The building held about 4,500 breeding turkeys (2,250 males and 2,250 females) of the British United Turkeys 9 strain (Figure 1). The birds were placed in the facility when they were 1 d of age. The breeding period lasted 12 wk for the females and 16 wk for the males.

Mechanical ventilation was provided by 10 wall fans along the barn. The fans blew air into the barn. A large door at each end and smaller windows along the sides could be opened to provide additional ventilation. Gas make-up air heaters were disposed in the building during the first 7 wk to maintain a desired indoor temperature for the chicks. The temperature decreased from 34°C (the first wk) to 20°C (at wk 7). Litter in the barn consisted of fresh straw spread on the floor a few days before the arrival of the chicks. Additional fresh straw was spread at the beginning of wk 10.

Sampling and Fungal Isolation

Over the 16-wk period of breeding, the environmental contamination by thermophilic fungal species was assessed in the confinement house. Every week, serial air samples of 100 L were made with a bioimpactor (Air Strategie Bioimpactor 100-08 Laboratoire de Chimie et Biologie, Lasalle, France) loaded with Sabouraud-chloramphenicol (**SC**; 0.5%, Sigma, Saint-Quentin Fallavier, France) dextrose agar plates. Air samples were taken from 8 locations inside the confinement house (Figure 1). The air sampler was placed 1 m from the soil. Samples of litter and food were also collected weekly. One gram of each litter or food sample was introduced in a tube containing 10 mL of sterile water. After vigorous shaking, 1 mL was inoculated onto a SC dextrose agar plate. Culture plates were incubated at 40°C for 4 d and examined daily. The temperature of 40°C was chosen to select the growth of the thermophilic species, which may behave as opportunistic pathogens for birds. Molds were identified by their macroscopic and microscopic appearance after lactophenol cotton blue staining (de Hoog et al., 2000). Yeasts were subcultured and identified with the Api32C system (BioMerieux, Marcy l'Etoile France). For each positive sample, fungal contamination was estimated by counting the number of colony-forming units (per m^3 for air samples and per g for litter and feed samples).

During the 16-wk study period, records on daily mortality were kept. Records of carcass condemnation at the slaughterhouse were also analyzed.

Microsatellite Typing of *A. fumigatus* isolates

Representative isolates of *A. fumigatus* were subcultured on SC slants for 4 d at 37°C. The conidia suspension obtained from each subculture was frozen in liquid N and centrifuged. The DNA extraction from 180 μL of supernatant was performed by a Qiagen kit (DNeasy Tissue kit, Qiagen, Courtaboeuf, France). Two sets of primers specific for microsatellites C and D were used to amplify each isolate DNA (Bart-Delabesse et al., 1998). One primer of each set 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. Polymerase chain reaction amplifications were performed in a 20-μL volume containing 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 9.0), and 50 m*M* KCl; forward and reverse primers at concentrations of 100 nM each; the deoxynucleoside triphosphates at concentrations of 100 μM each; and 0.5 U of *Taq* DNA polymerase (Pharmacia Biotech, Orsay, France), 5% (vol/vol) di-

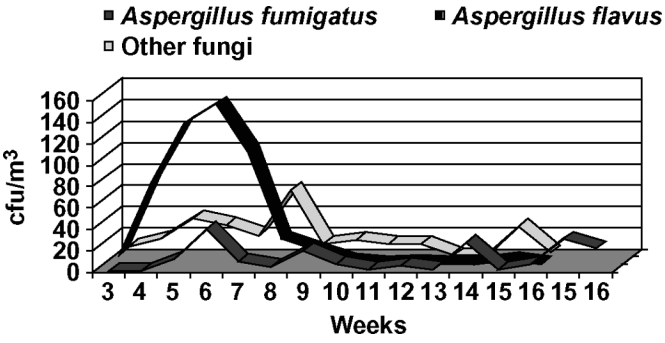

Figure 2. Fungal contamination of air samples.

methyl sulfoxide, and 50 ng of DNA template. Amplification was carried out in a Perkin-Elmer Cetus system 480 thermocycler (Perkin-Elmer, Courtaboeuf, France) with denaturation for 10 min at 95°C, 30 cycles of 20 s at 95°C, 20 s at 55°C, and 20 s at 72°C and a final extension step at 72°C for 5 min. The PCR products were diluted 1:10 in water, and 1 μL of each was run on a 36-cm long acrylamide urea gel (4.25% polyacrylamide, 8.3 M urea, and 1 × Tris-borate-EDTA for 2 h at 3,000V and 51°C). The N,N,N,N'tetramethyl-6-carboxyrhodamine-labeled GeneScan size standard (Perkin-Elmer) was loaded into each well, along with the PCR products. Signals were read with an automatic sequencer (ABI377, Applied Biosystems, Courtaboeuf, France), and the data were stored and analyzed with GeneScan Software (Version 2.0.2, Perkin-Elmer) by the local Southern sizing method.

RESULTS

Fungal Contamination

A total number of 284 environmental samples were collected: 112 from the air, 124 from the litter, and 48 from the feed. After the 4-d incubation at 40°C, fungal colonies were detected in 111 samples from the air (99.1%), 108 samples from the litter (87.1%), and 43 samples from the poultry feed (89.6%). Molds were detected in 196 samples (69%; 94 air samples, 62 litter samples, and 40 feed samples). The 3 species that were most frequently identified were: *Absidia corymbifera* (114 samples; 40.1%), *A. fumigatus* (114 samples; 40.1%), and *Aspergillus flavus* (67 samples; 23.6%). *Scopulariopsis* spp. and *Penicillium* spp. were also regularly encountered, in addition to yeasts of the genus *Candida*. The opportunistic species *C. albicans* was detected from 195 environmental samples (68.7%).

The results of the surveillance program for the detection of fungal conidia in the air inside the confinement house are shown in Figure 2. During the first 9 wk, air samples yielded the growth of many *A. flavus* colonies (from 8.6 to 150.0 cfu/m^3; mean 72.9 cfu/m^3). Samples were also positive for *A. fumigatus* (32 out of 56) or for another fungal species (47 out of 56), but at a low level, from 1.3 to 40.0 cfu/m^3 for *A. fumigatus* and from 10 to 62.5 cfu/m^3 for other fungal species. Seven weeks later, only 3 air samples were positive for *A. flavus*, with a very low level from 1.3 to 3.8 cfu/m^3. Concomitantly, air samples were positive for *A. fumigatus* and for other fungal species with a level from 2.5 to 27.5 cfu/m^3 and from 1.3 to 28.8 cfu/m^3, respectively.

During the surveillance period, the density of *A. fumigatus* in litter and feed samples was low and constant. Samples obtained during the 16-wk-study period yielded *A. fumigatus* at 0.7 cfu/g (from 0.0 to 2.3) in poultry feed and at 0.3 cfu/g (from 0.0 to 1.5) in litter. After new litter was added at wk 10, there was no isolation of fungi for 2 wk. However, during wk 14, the number of *A. fumigatus* colonies increased (1.5 cfu/g). There were no differences among sampling sites inside the facility.

Colonies of *C. albicans* were first detected at wk 4 from litter samples and at wk 7 from poultry feed. Densities of *C. albicans* remained very high in litter samples (63.2 cfu/g), even after new litter was added at wk 10.

A. fumigatus genotyping

Globally, 198 *A. fumigatus* were typed by PMM analysis. This led to the resolution of 73 distinct genotypes. Each of these genotypes was characterized by a specific combination of allele size at microsatellite loci C and D. More than half (42 out of 73, 57.5%) of these genotypes were detected only once. Figure 3 indicates the allele size distributions of *A. fumigatus* isolates at the 2 microsatellite loci C and D. Polymorphic microsatellite marker D was confirmed to be highly polymorphic, with 32 different alleles. Eleven of these alleles (D60, D64, D66, D68, D78, D80, D82, D86, D124, D140, D152) were considered as new ones, because they were not described in the initial study of Bart-Delabesse et al. (1998). Conversely, only 2 alleles initially described by Bart-Delabesse et al. (1998) were not detected in the present study. The use of PMM C yielded 11 alleles. Only 2 of these alleles were considered as new ones (C185 and C189). One allele (C165) described by Bart-Delabesse et al. (1998) was not detected in the present study. For a specific PMM, only 1 band was observed after amplification. This finding confirmed that there was no mixture of several isolates in our study. Indeed, if several isolates had been accidentally mixed, several alleles should have been observed, because *A. fumigatus* is haploid.

A total number of 134 isolates representing 53 distinct genotypes were obtained from air samples. Thirty-three genotypes were observed only once. The remaining 20 genotypes were detected during several weeks, with a maximum persistence of 8 wk for genotype C175D76. Twenty-four genotypes were obtained from 34 litter isolates. Nineteen of these genotypes (79.2%) were detected only once. Twenty-two genotypes were obtained from 30 feed samples. Seventeen of these genotypes (77.3%) were detected only once. Sixteen genotypes were encountered both in air and litter samples, 9 both in air and feed samples and 5 both in air, litter, and feed samples.

Lesions of aspergillosis were detected in only 2 birds whose carcasses were condemned at slaughter inspection (Lair-Fulleringer et al., 2003). Macroscopic lesions were observed in the abdominal air sac, lungs, and kidneys.

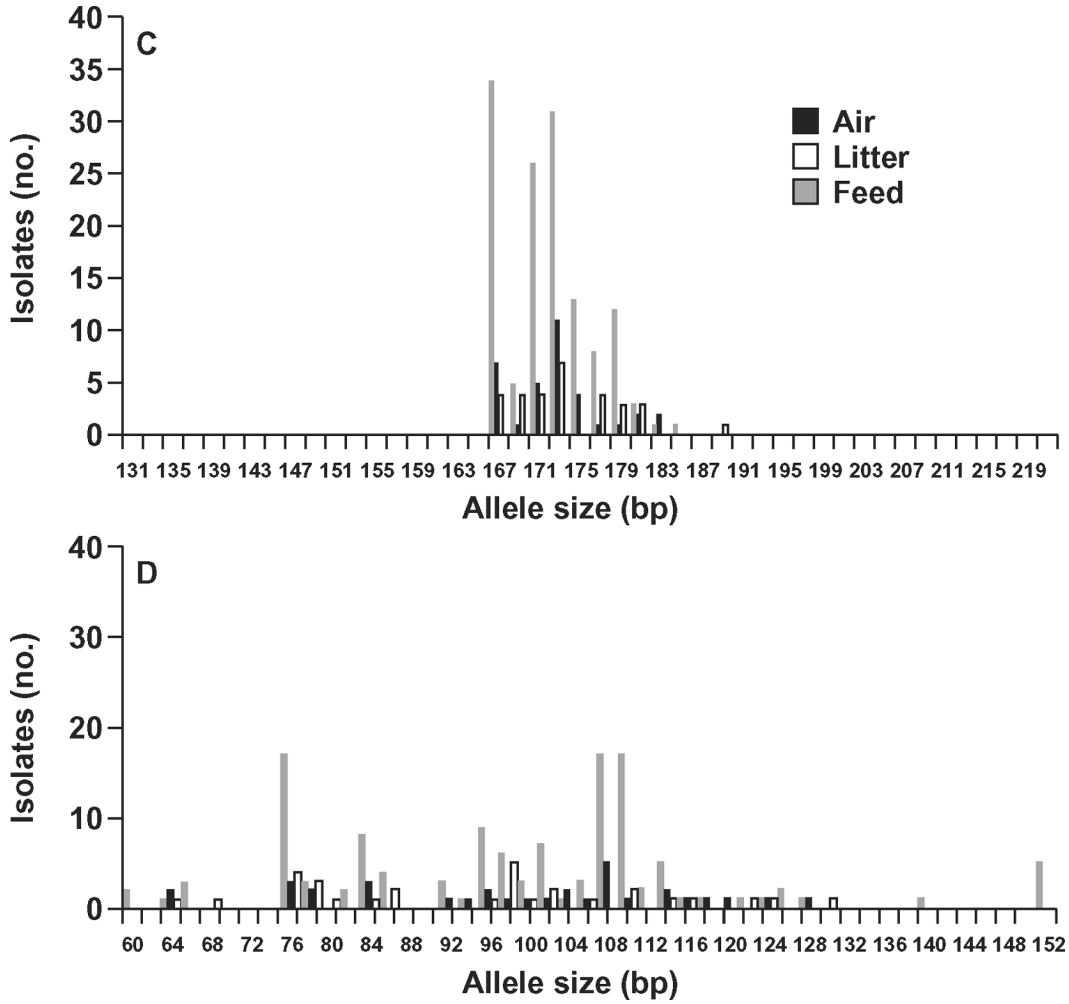

Figure 3. Allele size distributions of *Aspergillus fumigatus* isolates at microsatellite loci C and D upon analysis of 198 environmental isolates from air samples (solid bars), litter samples (striped bars), and feed samples (gray bars).

Aspergillus fumigatus isolates collected from the lesions were proved to represent a unique genotype (C173D112 for the first turkey and C169D102 for the second one). Genotype C169D102 was detected in air samples at wk 13 and 14 (3 and 2 wk before slaughtering) and in litter samples of wk 14. Genotype C173D112 was never detected in air, litter, and feed samples during the study.

DISCUSSION

Nature and Evolution of Fungal Contamination

In animal facilities, environmental conditions are usually favorable for the development of molds and yeasts. Concomitantly, birds are one of the few species that can naturally acquire fungal infections (especially aspergillosis and candidosis) in the absence of immunosuppression. In the past, several investigations aimed at describing the relative abundances of fungal species and their concentrations in facilities where birds were housed (Lovett et al., 1971; Bacon and Burdick, 1977; Pinello et al., 1977; Sauter et al., 1981; Dyar et al., 1984; Mulhausen et al., 1987; Kaboret et al., 1996; Dykstra et al., 1997). In these studies, different sampling methods were used, and the comparison of their results with those obtained in the present investigation is difficult. In most of the previous studies, the estimation of air contamination was based on the use of settle (sedimentation) plates. This sampling technique is particularly efficient at detecting large fungal spores, whereas the proportion of spores corresponding to important "small-spored" genera (such as *Aspergillus* and *Penicillium*) is underestimated. For this reason, pumping samplers (like the Air Strategie Bioimpactor) are now frequently used instead of settle plates. Previous investigations described the global fungal contamination and did not focus on potentially pathogenic fungi. Furthermore, none of the previous studies in confinement houses included information about the population structure of *A. fumigatus*.

The definition of acceptable threshold levels is required to correlate outbreaks of avian mycosis with the use of contaminated litter material or feed. This would also allow defects in facility ventilation to be identified and specific cleaning procedures to be monitored. In hospitals, many studies have attempted to evaluate the importance of fungal aerobiology in relation to the acquisition of invasive

fungal infections by immunocompromized patients. Most authors recommend *Aspergillus* air counts of <5 cfu/m^3 in protective isolation suites (Morris et al., 2000; Alberti et al., 2001). The present investigation demonstrated a constant fungal contamination in the facility throughout the 16-wk study period. However, mold counts were generally low (<30 cfu/m^3 and <1.5 cfu/g), except for *A. flavus* in the first weeks of the surveillance program (up to 150 cfu/m^3). These values are within the ranges reported in similar investigations concerning the global fungal contamination of indoor air of dwellings where humans lived or worked: 3 to 6000, with an average of 654 cfu/m^3 (Kozak et al., 1980); 165 to 850 cfu/m^3 (Nevalainen et al., 1991); 100 to 2,300 cfu/m^3 in "moldy" dwellings (Nevalainen et al., 1991); and 500 to 1,000 cfu/m^3, the current standard (Miller et al., 1988). The amount of viable and culturable microscopic fungi in indoor air is very variable. The liberation of the spores into the air depends on the physiological properties of individual species of microscopic fungi colonizing the litter or the feed. *Aspergillus* species form small xerophilic spores (2 to 3 μm in diameter for *A. fumigatus*) in high amounts, making very fragile chains. The formation and emission of these spores from their sources depend on different parameters, including environmental temperature, moisture, and air disturbance within the facility. The most striking result of the present study was the predominance of *A. flavus* spores in the first weeks of brooding. The high temperature maintained inside the confinement house for the chicks may account for this result. The temperature was very high (34°C) when the 1-d-old chicks were introduced in the facility. It regularly decreased to 20°C (at wk 7). The species *A. flavus* has a worldwide distribution but is more frequently isolated in tropical or subtropical areas (Domsch et al., 1993) where it may constitute the first fungal contaminant of the air. Its growth is optimal in low soil moisture and when temperatures are from 36 to 38°C.

Another interesting finding is the isolation of *C. albicans* in many environmental samples (68.7%), including air samples from wk 4 to the end of the study. The yeast *C. albicans* belongs to the normal digestive flora of birds. In the case of confinement structures densely stocked with birds, the environmental contamination by dropping is massive. In the present study, densities of *C. albicans* remained very high in litter samples (63.2 cfu/g), even after new litter was added at wk 10.

Potential Health Problems Related to Fungal Contamination

Out of the different fungal species that were regularly isolated during the surveillance program, 3 are considered major opportunistic pathogens for turkeys: *A. fumigatus*, *C. albicans*, and, to a lesser extent, *A. flavus*. However, no case of clinical aspergillosis or candidosis was detected in the facility, and only 2 carcasses were condemned at slaughter inspection. Low levels of environmental contamination by *A. fumigatus* may account for this situation. However, the correlation between contamination and the occurrence of clinical cases of avian aspergillosis has been suspected but not clearly demonstrated. In a case report following an outbreak of aspergillosis in a flock of turkeys, high *Aspergillus* counts (>106 cfu/g) were reported from wood shavings that were used as litter (Dyar et al., 1984). These counts decreased after the litter was treated with $CuSO_4$, and the outbreak was controlled. In care units, where immunocompromised humans are hospitalized, there is no consensus on the spore density at which the risk of aspergillosis is increased. Recently, 2 studies in hematology and oncology wards found no correlation between the density of airborne *Aspergillus* and the occurrence of aspergillosis (Hospenthal et al., 1998; Leenders et al., 1999).

Although *A. fumigatus* is the most common etiologic agent of *Aspergillus* infection, it is not the only pathogenic species in the genus. *Aspergillus flavus* can also cause avian infections (Knudtson and Meinecke, 1972; Ghazikhanian, 1989; Barton et al., 1992; Richard, 1997). In the present study, no case of *A. flavus* infection was observed, even during the few weeks when the environmental contamination with this species was massive. However, the potential health problems in turkeys should not be underestimated, because *A. flavus* is a toxinogenic species. The presence of mycotoxins inside as well as on the surface of *A. flavus* spores has been demonstrated. Furthermore, it has been found that the potency of the respiratory route is higher than the alimentary route. The dose of mycotoxin required to cause particular effects is 1 order of magnitude less when administered by the respiratory tract than by ingestion (Hendry and Cole, 1993).

During the present investigation, the thermophilic species *Ochroconis gallopava* (*Dactylaria gallopava*) was not isolated. This species tends to grow in warm environments and is typically found in soil and decaying vegetation. *Ochroconis gallopava* has been isolated from fowl broiler-house litter in the United States (Randall et al., 1981) and is incriminated as an agent of epidemic encephalitis in birds. However, no case of avian *Ochroconis* infection has ever been described in Europe.

A. fumigatus genotyping

The analysis of PMM from 198 *A. fumigatus* isolates confirmed the very high polymorphism of fungal populations in the environment. Most of the 73 distinct genotypes were detected only once. Not surprisingly, the extent of genetic diversity seemed to be the same among isolates from the air, the feed, or the litter material. Differences could not be detected among sampling sites, either. Similar results were reported when the genetic diversity of *A. fumigatus* isolates from a hospital environment were investigated with the same molecular technique (Bart-Delabesse et al., 1998, 1999). When clinical and environmental isolates were compared, no particular *A. fumigatus* genotype could be associated with virulence in humans (Bart-Delabesse et al., 1998, 1999) and birds (Lair-Fulleringer et al., 2003). The absence of particular virulent *A. fumigatus* isolates was confirmed by Peden and Rhoades (1992), who inoculated

isolates from diverse origins (environmental, mammalian, avian) in air sacs of turkeys.

Several studies have already reported the application of the PMM technique for the genotyping of *C. albicans* (Field et al., 1996; Bretagne et al., 1997; Botterel et al., 2001). The performance of this typing system has been evaluated on collection and clinical strains (from human cases of candidosis). Unfortunately, we did not have the opportunity to use the PMM technique for the yeast isolates that were collected during the present study. The population structure of *C. albicans* within a group of birds has never been described.

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