Susceptibility of adult pigeons and hybrid falcons to experimental aspergillosis

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Avian susceptibility to aspergillosis

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Abstract

Aspergillosis caused by *Aspergillus fumigatus* seems to be more prevalent in some avian species than in others. We compared the development of aspergillosis in 8 month old Gyr-Saker hybrid falcons and 8 month old pigeons after a single intratracheal inoculation of different dosages of *A. fumigatus* conidia (10^7, 10^5 and 10^3). Clinical signs, including vomiting, discoloration of the urates, loss of appetite and dyspnoea, were observed in 4 out of 5 falcons and 4 out of 5 pigeons inoculated with 10^7 *A. fumigatus* conidia. Necropsy revealed the presence of granulomas in the air sacs and / or lungs in 4 out of 5 falcons and 4 out of 5 pigeons in the high dosage group. *A. fumigatus* was isolated from these granulomas in 3 falcons and 3 pigeons. The presence of fungal hyphae was detected with PAS staining in 3 out of 5 falcons and 3 out of 5 pigeons in the high dosage group. Avian respiratory macrophages were clearly present in and around the fungal granulomas. In the other dosage groups, no granulomas, positive *A. fumigatus* cultures or fungal hyphae were present, except for one falcon in the middle dosage group in which a sterile granuloma without fungal hyphae was noticed.

In conclusion, the study shows that adult falcons and pigeons are susceptible to aspergillosis after inoculation of a single dose of conidia intratracheally.

Introduction

Respiratory disease due to *Aspergillus* species is a major cause of morbidity and mortality in captive and free-ranging birds (Tell, 2005; Beernaert *et al*., 2008; Olias *et al*., 2010). In the genus *Aspergillus*, especially *A. fumigatus* and to a lesser extent *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans* are causative agents of aspergillosis (Jones and Orosz, 2000). *A. fumigatus* is a ubiquitous saprophytic fungus that sporulates abundantly and releases a huge number of conidia into the air. Inhaled conidia can reach the lungs and air sacs due to their small size (Fedde, 1998, Beernaert *et al*., 2010).

Although aspergillosis most likely occurs in all avian species, it is seen more often in captive waterfowl, wading birds, penguins, raptors, pheasants and passerines (Bauck, 1994, Kearns 2003). At present, it is still not clear why these birds appear to be more susceptible to aspergillosis. In literature, the factors necessary to induce clinical disease after exposure to *A. fumigatus* conidia are not known. Even though a multifactorial etiology seems to be common, the intrinsic susceptibility to aspergillosis may be host dependent.

The aim of the present study was to compare the intrinsic susceptibility of otherwise healthy adult hybrid falcons and pigeons to aspergillosis, after a single intratracheal inoculation with different numbers of *A. fumigatus* conidia.

Material and methods

***A. fumigatus* strain and inoculum preparation.** The *A. fumigatus* strain K125 (HE 864321) used in this study was isolated from a lung granuloma of a Gyr-Saker (*Falco rusticolus* x *Falco cherrug*) hybrid falcon, which died from severe aspergillosis. It was stored at - 80°C via Microbank™ (Pro-Lab Diagnostics, Novolab, Belgium) till

usage. Five-day-old cultures of this strain on Sabouraud dextrose agar (SAB) (CM0041, Oxoid Ltd., Basingstoke, Hampshire, England) were washed with 5 ml 0.01% Tween 20 in Hank's balanced salt solution (HBSS) to harvest *A. fumigatus* conidia. The conidia were washed three times in 0.01% Tween 20 in HBSS (3200 x g, ten minutes at 4°C) and the suspension was adjusted to a concentration of 10^3, 10^5 or 10^7 *A. fumigatus* conidia/ 0.5 ml in HBSS by haemacytometer count. Numbers of viable conidia were determined by plating serial 10-fold serial dilutions in 0.01% Tween 20 in HBSS on SAB plates. The number of colony forming units (CFU) / ml was calculated after incubation at 37°C for 20 hours. The final conidial suspensions had a viable count of 2.5 x 10^7, 1.89 x 10^5 and 2.25 x 10^3 CFU / ml.

Experimental animals. Eighteen adult male Gyr-Saker (*F. rusticolus* x *F. cherrug*) hybrid falcons were obtained from 1 breeder. The birds' health was evaluated by a general examination, endoscopy, bacteriological, virological and parasitological examination. All airsacs on both sides from each falcon were visualised and were free of signs of aspergillosis. To detect anti-*Aspergillus* antibodies, the *Aspergillus* haemagglutination test (Hemkit® *Aspergillus* IHA, Ravo Diagnostika, Germany) was used. The *Aspergillus* haemagglutination test was negative for all falcons. Excreta were collected for five days from each falcon and mixed. Bacteriological analysis was performed using direct plating on brilliant green agar and enrichment on buffered peptone water/brilliant green tetrathionate broth. This test was negative for the presence of *Salmonella* spp. . PCR testing for the detection of Herpes virus infection on blood was negative. Parasitological analysis was performed using a saturated salt solution in water and microscopic examination. No endoparasites ova could be detected. All birds were considered healthy before the trial, especially free of signs of

aspergillosis. The falcons were perched according to standard falconry techniques with a 12-h photoperiod during the trial (Parry-Jones, 2008). One-day-old chicks were provided to each bird each day.

Twenty adult racing pigeons (*Columba livia domestica*) were divided into four groups. The birds' health was evaluated, by a general examination, endoscopy, bacteriological, virological and parasitological examination as described for the falcons. All pigeons were considered healthy before the trial. During the experiment, each bird was housed individually with a 12-h photoperiod. The birds received a commercial pigeon diet *ad libitum* and had free access to fresh drinking water.

All experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Merelbeke, Ghent University, Belgium (EC 2010/111; EC 2011/138).

Experimental design.

Falcons

Three groups of 5 falcons were inoculated intratracheally with 10^3, 10^5 or 10^7 *A. fumigatus* conidia in 0.5 ml HBSS, respectively, and one group of 3 falcons was sham-inoculated intratracheally with 0.5 ml HBSS. The inoculation was performed under general anaesthesia with Isoflurane (Isoflo®, Medini, Belgium) and a paediatric endotracheal tube (Ø 2.5 x 4.1-L. 165 mm)(Vygon, Ecouen, France) was used for the intratracheal inoculation. The animals were weighed daily and observed at least twice daily. At 28 days post inoculation (p.i.) all falcons were euthanized by an intravenous injection of 1 ml T61 (Intervet, Mechelen, Belgium) in the *vena basilica* under general anaesthesia.

Pigeons

Three groups of 5 pigeons were inoculated intratracheally with 0.5 ml of 10^3, 10^5 or 10^7 *A. fumigatus* conidia in HBSS, respectively, and one group of 5 pigeons was sham-inoculated intratracheally with 0.5 ml HBSS. The inoculation was performed under general anaesthesia with Isoflurane and an intravenous cannula (18 G x 1¾")(Vasovet, Tuttlingen, Germany) was used for the intratracheal inoculation. The animals were weighed daily and observed at least twice daily. At 28 days post inoculation (p.i.) all pigeons were euthanized by an intravenous injection of 1 ml T61 in the *vena basilica* under general anaesthesia.

Clinical follow up. The presence of ruffled feathers, dyspnoea, sneezing and stridor were scored daily. During the trial, animals with severe dyspnoea (open beak breathing) or extreme weight loss were considered irreversibly fatally ill and suffering and therefore were euthanized. This was noted as "mortality".

Environmental sampling. To measure the environmental load of *A. fumigatus* conidia, air samples from the experimental units were collected using the MAS-100 *Eco* impaction air-sampler (Merck, Whitehouse Station, NJ). A sampling volume of 100 l was chosen. Twice a week, samples were collected in triplicate on SAB agar plates and incubated at 37°C under aerobic conditions to quantify *A. fumigatus*.

Gross, histopathological and immunohistochemical examination. At necropsy, macroscopic lesions were noted. Samples of the lungs, air sacs, liver, spleen, kidney and granulomas were fixed in phosphate buffered formaldehyde solution, sectioned and stained with Haematoxylin and Eosin (HE) or Periodic acid Shiff reagent (PAS) for visualization of fungal elements.

To visualise respiratory macrophages in lungs, airsacs and granulomas, a concanavalin A staining was performed (Greenfield et al., 1988). Briefly, antigen retrieval in the deparaffinized sections, breaking the protein cross-links formed by formalin fixation and uncovering hidden antigenic sites, of the lungs, airsacs and granulomas was performed using a pressure cooker. The sections were heated for 15 min at 850 W and 15 min at 300W in the microwave oven. Subsequently, they were cooled down for 20 min and thereafter treated with 3% hydrogen peroxide in methanol for 5 minutes at room temperature to block endogenous peroxidase activity. After rinsing with phosphate buffered saline (PBS), the sections were incubated with peroxidase-labelled concanavalin A (L6397, Sigma-Aldrich, St-Louis, USA) at 20 µg/ml for 60 min in a humid chamber at room temperature. After rinsing with PBS, the reaction product was developed with a hydrogen peroxide and diaminobenzidine solution (prepared following manufacturer's instructions) for 5 min. Finally, the sections were counterstained with haematoxylin and mounted for examination.

Mycological examination and Microsatellite Length Polymorphism. To isolate *A. fumigatus* from the birds, samples of the trachea, lungs, air sacs, heart, pericardium, liver, kidney, brain, pectoral muscle, and abdominal fluid were inoculated on SAB plates and incubated for 72 h at 37°C at aerobic conditions. After identification of the isolated fungi, Microsatellite Length Polymorphism (MLP) was conducted on each colony to confirm that mycoses during this study originated from the inoculated strain and performed as previously described (Van Waeyenberghe *et al*., 2011).

***Galleria mellonella* virulence assay**. To assess the virulence of isolate K125 and K24, 10 sixth instar larvae of *G. mellonella* were injected with 1 x 10^6 *A. fumigatus*

conidia of K125 and K24 in 10 µl PBS, respectively, into the haemocoel through the last left proleg using a Myjector U-100 Insulin syringe. After infection, the larvae were incubated in plastic containers, and the number of dead larvae were scored daily. Larvae were considered dead when they displayed no movement in response to touch. All tests were performed in triplicate.

Results

Clinical signs

Falcons

Clinical signs were only noticed in the high and middle dosage group. In the high dosage group, vomiting was observed on day 2 p.i. in 4 out of 5 birds. One bird also vomited 10 days p.i.. A greenish coloration of the urates was seen in 3 out of 5 birds. A loss of appetite was noticed in 4 out of 5 birds during the first days p.i.. One bird also showed a reduced appetite from day 10 to day 14, one bird from day 13 till day 28 and one bird from day 20 till day 28.

In the middle dose group, one bird vomited on day 17 and 18 p.i. and showed greenish coloration of its urates from day 19 till day 23 p.i.. From day 18 till day 22 p.i., the bird also exhibited a loss of appetite.

A summary of the clinical signs is presented in table 1. An overview of the weight loss in the different groups is presented in figure 1. No mortality was observed in any group.

Pigeons

In the high dosage group, dyspnoea and a reduced appetite were observed after 2 days p.i. in 4 out of 5 birds. On day 4 p.i., 1 of these 4 birds died in its cage. The respiratory symptoms of the other 3 birds remained present the following 14 days. On

day 13 p.i., 1 of the remaining 4 birds was found dead with blood in the oral cavity. from 15 days p.i. onward, the dyspnoea of the other birds improved and the appetite returned. In the three other groups, none of these symptoms were observed. A summary of the clinical signs is presented in table 1. An overview of the weight loss in the different groups is presented in figure 2.

Pathological, mycological, histopathological and immunohistochemical findings. In the high dosage group of the inoculated falcons, necropsy revealed the presence of granulomas in the air sacs in 4 out of 5 birds. In 2 of these birds, granulomatous lesions were also observed in the lung. Lesions were found at the left as well as the right side of the respiratory system in 2 out of 4 birds. *A. fumigatus* was isolated from the lesions of the airsacs in 3 birds. In the middle dosage group, 1 bird had a granulomatous lesion in the air sacs, though *A. fumigatus* could not be isolated. No lesions were observed in the low dosage group and the negative control group.

In the high dosage group of pigeons, necropsy revealed the presence of granulomatous lesions in the lungs in 4 out of 5 birds. In two of these birds, granulomatous lesions were also noticed in the airsacs and kidney. The lesions were all bilateral in nature. *A. fumigatus* was isolated from the lesions in 3 birds. No macroscopic lesions were observed in the other groups.

The histopathological findings of the observed lesions in falcons and pigeons showed a severe heterophilic and granulomatous inflammation, with large accumulations of necrotic heterophils, surrounded by a continuous rim of epithelioid, multinucleate giant cells and macrophages. PAS staining revealed the presence of fungal elements in 3 of the 6 air sac granulomas in the falcons and in 3 out of 4 granulomas in pigeons. The spleens of the falcons and pigeons were evaluated for the presence of circovirus

226 inclusions. No circovirus inclusions were noticed in the spleen. A summary of the
227 pathological, mycological and histopathological lesions is presented in table 2. No
228 lesions or fungal elements were detected in the other organs. In granulomas of the
229 airsacs and lungs, concanavalin A staining revealed a large number of macrophages
230 and giant cells surrounding the necrotic foci (figure 3).
231
232 **Environmental sampling**. In 8 measurements, on average 140 +/- 86 *A. fumigatus*
233 conidia / m^3 air were detected in the experimental unit of the falcons and on average
234 71 +/- 31 *A. fumigatus* conidia / m^3 air were detected in the experimental unit of the
235 pigeons.
236
237 **Microsatellite length polymorphism**. The genotypes of the *A. fumigatus*, isolated
238 from the lesions of the falcons, were identical to the genotype of the inoculated strain
239 in 2 out of 3 falcons. In one falcon, besides the genotype of the inoculated strain, a
240 second genotype was obtained from three out of four lesions.
241 The genotypes of the *A. fumigatus*, isolated from the lesions of the pigeons, were all
242 identical to the genotype of the inoculated strain.
243
244 ***Galleria mellonella* virulence assay**. No differences in survival rate of the larvae
245 were observed between the two *A. fumigatus* isolates. After 72 h, all larvae inoculated
246 with the different *A. fumigatus* conidia were found dead.
247
248 **Discussion**
249 In the present study, the occurrence of aspergillosis after single exposure to different
250 dosages of *A. fumigatus* conidia was examined in two avian species. A single dose

exposure of 10^7 *A. fumigatus* conidia was capable to cause disease in 8 month old Gyr-Saker hybrid falcons and pigeons. Although several authors claim that birds of prey, especially gyrfalcon (*Falco rusticollis*) and its hybrids, golden eagle (*Aquila chrysaetos*), osprey (*Pandion haliaetus*), goshawk (*Accipiter gentilis*), roughlegged hawk (*Buteo lagopus*) and red-tailed hawk (*Buteo jamaicensis*), are highly susceptible to aspergillosis (Redig, 1993; Joseph, 2000; Tell, 2005; Silvanose, 2012 personal communication), the expected difference in species susceptibility between 8 month old hybrid falcons and 8 month old pigeons was not observed. On the other hand, age-related susceptibility to aspergillosis is reported for falcons (Joseph, 2000), pigeons (Beernaert *et al*., 2008), turkeys (Femenia *et al*., 2007) and white storks (Olias *et al*., 2010). Therefore, infection trials with young hybrid falcons and pigeons should be performed to determine the influence of age in the development of aspergillosis within the used model.

In our study, adult pigeons developed aspergillosis after intratracheal inoculation of 10^7 *A. fumigatus* conidia. In a study of Beernaert *et al*. (2008), adult pigeons did not develop aspergillosis after intratracheal inoculation of even 10^8 conidia of a different *A. fumigatus* strain. This may suggest that virulence of the *A. fumigatus* strain involved is more important than species susceptibility in the development of aspergillosis. These differences in virulence of *A. fumigatus* strains were already demonstrated in turkeys (Peden and Rhoades, 1992) and in mouse models of invasive pulmonary aspergillosis (Mondon *et al*., 1996; Aufauvre-Brown *et al*., 1998). In the non-vertebrate host model of *G. mellonella*, no differences in virulence between the two strains were observed. Besides, Olias *et al*. (2011) demonstrated that under field conditions strain pathogenicity does not play a major role.

Although animal movements may contribute to generate a conidial aerosol (Dyar *et al*., 1984; Arné *et al*., 2011), the *Aspergillus* conidial concentrations did not exceed the general indoor concentrations of 175 conidia/m^3 (Ault and Schott, 1993). In poultry houses, concentrations up to 2.1 x 10^3 conidia/m^3 were recorded in spring (Ault and Schott, 1993). However, Vanhee *et al*. (2009; 2010) reported much lower concentrations indoor, in poultry houses and pigeonries as observed in our study. The falcons in the present study inhaled approximately 50 *A. fumigatus* conidia from ambient air each day. This concentration did not harm healthy adult hybrid falcons as the birds of the control group did not show any signs of disease or pathological lesions. MLP demonstrated that the inoculated strain was responsible for the disease in the experimental birds. The detection of additional strains is not uncommon as birds may be infected by several strains (Olias *et al*., 2011).

After 10 and 17 days, two falcons (one of the high dosage and one of the middle dosage group) vomited and showed a loss of appetite but recovered completely. Interestingly, these 2 birds had sterile granulomas in the airsacs. One pigeon also had a sterile granuloma in the lung after clinical recovery. Clearance of the fungal infection in these birds might explain the sterile granulomas. In turkeys and chickens respectively, clearance of fungal infections from the lung and air sacs was also demonstrated after 7 to 10 days p.i. and up to 3 weeks p.i., respectively (Taylor and Burroughs 1973; Femenia *et al*. 2007). This finding proves that aspergillosis in clinically healthy birds can present as a self-limiting disease, also in supposedly susceptible species. However, apart from the health condition of the host, this seems to be also dependent on the infection dose.

Host defence mechanisms against *A. fumigatus* include innate as well as adaptive immunity. Respiratory macrophages, belonging to the innate immune system, prevent

300 germination and establishment of early infection (Van Waeyenberghe *et al*., *In Press*).
301 Nevertheless, inhalation of an overwhelming amount of conidia results in germination
302 of conidia inside avian respiratory macrophages and colonization of the respiratory
303 tract (Van Waeyenberghe *et al*., *In Press*). In case of infection, respiratory
304 macrophages are highly present in and around the fungal granulomas as demonstrated
305 in this study. Demarcation of the fungal burden with these macrophages could support
306 clearance of the disease as observed in several birds.
307 In conclusion, clinically healthy falcons seem equally susceptible to develop
308 aspergillosis than pigeons after single dose exposure to *A. fumigatus* conidia and the
309 development of aspergillosis is dose-dependent under experimental conditions.
310 According to the available literature, this study demonstrates for the first time that a
311 single dose exposure to *A. fumigatus* conidia is sufficient to cause a clinical disease in
312 falcons. This should be considered in future, as under clinical conditions aspergillosis
313 is not always a multifactorial disease and can also be induced by an overwhelming
314 amount of conidia.
315

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387 inside avian respiratory macrophages is associated with cytotoxicity. *Veterinary*
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392 Table 1. Clinical signs in falcons and pigeons inoculated with 10^7 (high dosage), 10^5

393 (middle dosage) or 10^3 (low dosage) *A. fumigatus* conidia in the trachea.

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	Falcons				Pigeons			
	High dosage group (n = 5)	Middle dosage group (n = 5)	Low dosage group (n = 5)	Negative control group (n = 3)	High dosage group (n = 5)	Middle dosage group (n = 5)	Low dosage group (n = 5)	Negative control group (n = 5)
Loss of appetite	4	1	0	0	4	0	0	0
Vomiting	4	1	0	0	0	0	0	0
Discoloration urates	3	1	0	0	0	0	0	0
Dyspnoea	0	0	0	0	4	0	0	0
Mortality	0	0	0	0	2	0	0	0

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Table 2. Pathological, mycological and histopathological findings in falcons and pigeons inoculated with 10^7 (high dosage), 10^5 (middle dosage) or 10^3 (low dosage) *A. fumigatus* conidia in the trachea.

	Falcons				Pigeons			
	High dosage group (n = 5)	Middle dosage group (n = 5)	Low dosage group (n = 5)	Negative control group (n = 3)	High dosage group (n = 5)	Middle dosage group (n = 5)	Low dosage group (n = 5)	Negative control group (n = 5)
Granuloma in the lung	2	1	0	0	4	0	0	0
Granuloma in the airsac	4	0	0	0	2	0	0	0
Granuloma in other organs	0	0	0	0	2	0	0	0
Positive culture	3	0	0	0	3	0	0	0
Presence of fungal hyphae	3	0	0	0	3	0	0	0

425 Figure 1: Total weight loss as a percentage of the initial weight of the falcons,
426 inoculated with a high dosage (10^7), middle dosage (10^5) or a low dosage (10^3) of *A.*
427 *fumigatus* spores and a negative control (NC) group.
428
429 Figure 2: Total weight loss as a percentage of the initial weight of the pigeons,
430 inoculated with a high dosage (10^7), middle dosage (10^5) or a low dosage (10^3) of *A.*
431 *fumigatus* spores and a negative control (NC) group.
432
433 Figure 3: Fungal granuloma in the airsac of a falcon, stained with peroxidase-labelled
434 concanavalin A (20 µg/ml). Darkly stained macrophages (arrow) are distributed at the
435 edge of the granuloma.
436