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Pathological, Clinical and Mycological Findings in Experimental Aspergillosis Infections of Starlings

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With 6 figures

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Summary

A group of 24 wild starlings (*Sturnus vulgaris*), in undefined age categories but at least post-pubertal, constituted the material of this study. Six starlings were kept as a control group and 18 starlings served as the infection group. The starlings in the infection group were infected with inoculums of 1.35×10^6/0.2 ml *Aspergillus fumigatus* via intratracheal route whereas the control group was administered only placebo in the same way. Six, four, two, four and two birds died on 2, 3, 4, 5 and 6 days post inoculation respectively. At the necropsy of the dead birds, caseous foci were determined in the lungs, on the air sacks, myocardium, thoracic wall and abdominal serosa. In the histopathological examination of the white-yellowish caseous foci ranging from pinhead to chick pean in size, necrotic granulomatous foci consisting of macrophages, heterophil leukocytes and gigant cells encapsulated with a fibrous tissue were observed. Hyphae and spores of *A. fumigatus* were determined in these foci using the Gridley staining method.

Introduction

Aspergillosis is a fungal disease mainly located in the respiratory system and characterized with lesions in the internal organs, eyes and, in certain cases, the brain in poultry. *Aspergillus fumigatus* has been determined as the most pathogenic among the causative agents of aspergillosis with widespread resistant spores present in the natural environment (Raines et al., 1956; Teme, 1984; von Sandersleben and Atmungsorgane, 1985; Reece et al., 1986; Akan et al., 1996, 2002; Richard, 1997; Gylstorrff and Grimm, 1998).

Poultry aspergillosis, mainly observed in the young of turkeys and chicken, is also reported in other poultry species, including ducks, geese, quails, ostriches, parrots, canaries, pigeons, flamingos and penguins (Olson, 1969; Teme, 1984; von Sandersleben and Atmungsorgane, 1985; Barton et al., 1992; Perelman and Kuttin, 1992; Singh et al., 1994; Fitzgerald and Moisan, 1995; Richard, 1997; Gylstorrff and Grimm, 1998).

In the acute form of the disease, which develops within 24–48 h in young animals, the rate of mortality may vary from 70% to 90%, whereas in the sub-acute form of the disease which develops within 8–10 days in animals up to the age of 2 weeks various clinical phases are reported (Teme, 1984; von Sandersleben and Atmungsorgane, 1985; Reece et al., 1986; Roy et al., 1991; Richard, 1997; Gylstorrff and Grimm, 1998).

Basically, pathological findings observed in all poultry species are the same (Teme, 1984; von Sandersleben and Atmungsorgane, 1985; Barton et al., 1992; Fitzgerald and Moisan, 1995; Richard, 1997; Gylstorrff and Grimm, 1998). Macroscopic findings differ with regard to the location of the disease. Lungs are the internal organs reported to be most affected. Lesions may range from miliary to larger granulomatous foci (Olson, 1969; Geisel, 1982; Reece et al., 1986; Perelman and Kuttin, 1992; Singh et al., 1994; Richard, 1997) which are grey-yellow-white in colour, dry in consistency and protrusive with regard to the surface of the internal organ in which they are located (von Sandersleben and Atmungsorgane, 1985; Reece et al., 1986; Perelman and Kuttin, 1992; Richard, 1997; Gylstorrff and Grimm, 1998; Akan et al., 2002). Thickening and dullness of the walls of the air sacs have been reported to develop because of the infection (Teme, 1984; von Sandersleben and Atmungsorgane, 1985; Reece et al., 1986; Roy et al., 1991; Richard, 1997; Gylstorrff and Grimm, 1998; Akan et al., 2002). Microscopic examination has revealed the presence of granulomatous foci and caseous necrosis with a surrounding region of proliferation including giant cells, macrophages, hetereophils and lymphocytes and an outer capsule of connective tissue. Fungal hyphae with or without septa and fungal spores located within regions of necrosis may easily be observed by means of special staining methods (Raines et al., 1956; Hasegawa et al., 1971; Reece et al., 1986; Perelman and Kuttin, 1992; Singh et al., 1994; Fitzgerald and Moisan, 1995; Richard, 1997; Gylstorrff and Grimm, 1998).

The first case of poultry aspergillosis in Turkey was reported by Başkaya and his coworkers in turkeys in the National Forest and Farm of *Atatürk*, in 1953. Cases determined in pullets (Merdivenci, 1971; Minbay and Akay, 1981; Erer et al., 1986), chicks and young pigeons (Merdivenci and Zehavi, 1976), quails (Akan et al., 1996), broiler chicken (Akan et al., 2002), geese (Türkütanıt, 1999), and ostriches (Karaman et al., 2002) have followed this first report. In studies carried out on the cases mentioned above, histopathological findings have been determined, agent isolation has been performed and transfer of infection to other animal species has been attempted.

The aim of this study was to evaluate clinical, macroscopic, microscopic and mycological findings observed in starlings. This research is the first experimental study carried out on a migratory bird, the starling (*Sturnus vulgaris*), in which *A. fumigatus* was isolated from a naturally infected bird.

Material and Methods

A group of 24 starlings (*S. vulgaris*) in undefined age categories but at least post-pubertal, constituted the material of this study. The animals were captured in the wild and were grouped into two as infected (18) and control (six) after clinical and mycological examinations. The birds were kept in cages and given clean water and a feed containing a barley and wheat mixture. Upon the necropsy, histopathological examination and agent isolation procedures on a dead starling found in the countryside, the case was diagnosed as *A. fumigatus* infection. The inoculum administered for the experimental infection of starlings was prepared from the isolated *A. fumigatus* strain. *Aspergillus fumigatus* spore suspension at 530 nm wave length and an optical density range between 0.09 and 0.11 was prepared by using a Shimadzu-UV 1208 model spectrophotometer (Kyoto, Japan). The suspension was diluted with physiological saline to a concentration of $1.35 \times 106/0.2$ ml spore/ml and administered to 18 birds in the infection group intratracheally, whereas only placebo (0.9% NaCl) was given to six animals in the control group.

Necropsies were performed on the dead birds, and samples taken from internal organs displaying lesions were fixed in 10% buffered formalin. Following trimming and blocking in paraffin, 5–6 μ thick cross sections were prepared and stained with haematoxylin-eosin. Part of the cross sections were stained in accordance with the Gridley method and examined under light microscope for mycological diagnosis as described by Luna (1968). Using this method, fungi were dyed as dark brown where tissue was dyed yellow in cross sections.

Tracheal swap samples of all control animals (alive) and entire internal organs of the all infected animals (dead) with or without lesions at necropsy were cultured in Sabourand-Dextrose Agar (SDA; Oxoid, Hampshire, UK) for a mycological examination. Tissue samples were inoculated on SDA and incubated at 25°C at aerobic conditions for isolation of the agent. Czapex-Dox agar (Oxoid) was also used for differential diagnosis (Kennedy and Sigler, 1995). In order to avoid bacterial contamination, 20 IU/ml penicillin G potassium (1.000.000 IU; İ.E. Ulagay, Turkey) and 40 μg/ml streptomycine sulphate (1 g; İ.E. Ulagay) were added to the media. Lactophenol cotton blue stain was used for the microscopic examination of isolates (Kennedy and Sigler, 1995; Jordan and Pattison, 1996) and 20% potassium hydroxide was used for direct microscopic examination of internal organ samples and tracheal swaps. Macroscopic and microscopic examinations of colonies were carried out using the method described by Arda (1981).

Results

Clinical findings

Two days after inoculation, general clinical (ruffled feathers, green watery diarrhoea and anorexia) and respiratory tract signs (wheezy sound and dyspnoea) referring to aspergillosis were observed. Upon inoculation, all birds died (six, four, two, four and two birds died on the day 2, 3, 4, 5 and 6 respectively). Neither death nor clinical signs of aspergillosis were observed in control group.

Macroscopical findings

Necropsies of starlings which died after inoculation revealed the presence of white-yellowish caseous nodules in the lungs, air sacs, myocardium, thoracic wall and abdominal serosa ranging from pin point to chickpea in size (Fig. 1). The cut surface of these nodules were seen to be dry and displayed caseous structure in the centre.

Microscopical findings

Cross sections were prepared from similar nodular structures located in various internal organs. Histological examination revealed the presence of early lesions in the lungs characterized with infiltration of partly lymphocytes, a small number of macrophages, and giant cells as well as heterophil leucocytes without necrosis. Fungal hyphae and spores were also observed in these foci (Fig. 2). In larger granulomatous areas, necrotic regions infiltrated with macrophages, giant cells, lymphocytes, and heterophil leucocytes were surrounded by fibrous tissue from the outside. These structures were found to be located mainly near the para-bronchioles. Short and thin fungal hyphae with or without septa and fungal spores were determined within these granulomatous foci (Fig. 3). Further-

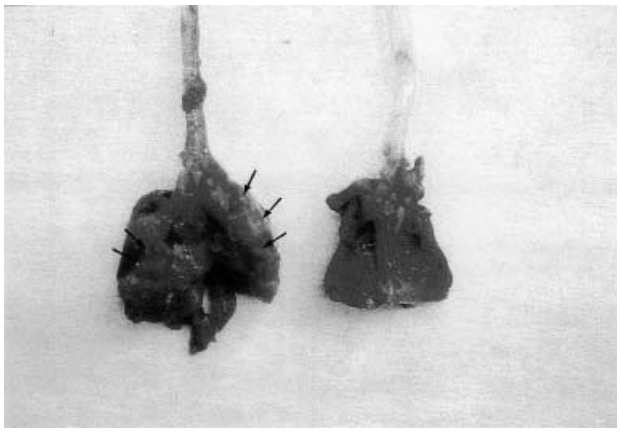

Fig. 1. Yellowish caseous foci in lung tissue on the left (arrows); the appearance of intact lung tissue on the right.

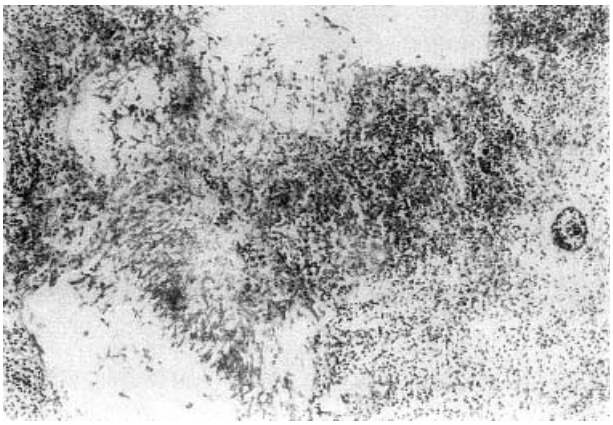

Fig. 2. Necrosis, inflammatory cells and fungal hyphae in tissue. Lung, H × E, 200.

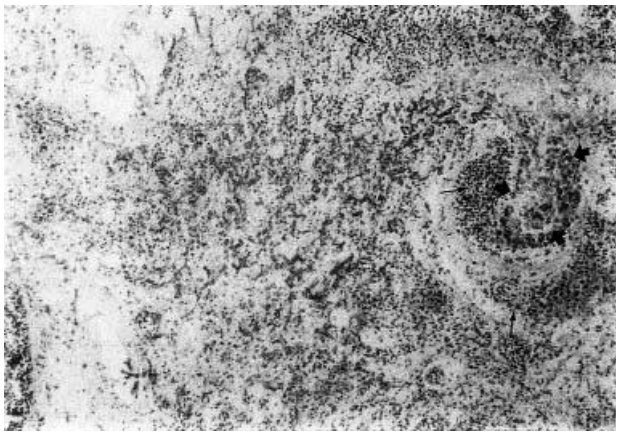

Fig. 3. Invasion of tissue with fungal hyphae, thrombosis (bold arrows) and haemorrhage (thin arrows) in blood vessels. Lung, H × E, 200.

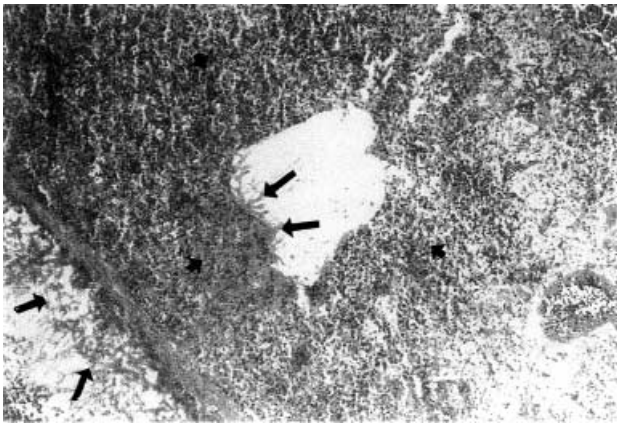

Fig. 4. Invasion of tissue with fungal hyphae originating from para-bronchioles (thin arrows), inflammatory cell infiltration (bold arrows). Lung, H × E, 200.

Fig. 5. Fungal hyphae and conidiophores in tissue. Lung, Gridley, 200.

more, similar fungal forms were also observed in intact tissues. In certain cross sections, conidiophores were found to exist in granulomas and the lumina of para-bronchioles. Thrombosis in the lumina of blood vessels and haemorrhages in the parenchyma were also detected (Fig. 4). Furthermore, a great number of fungal hyphae and spores were detected in the lumina of para-bronchioles. These were observed to invade tissue in certain regions (Fig. 5). Findings similar to those of the lungs were observed in the air sacs, myocardium, thoracic wall and abdominal serosa.

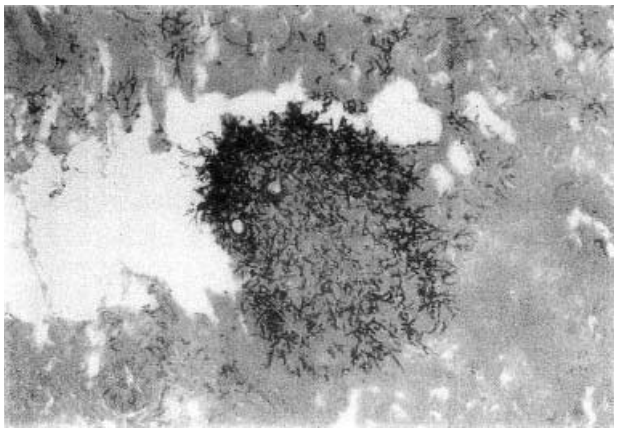

Fig. 6. Invasion of tissue with fungal hyphae and spores originating from the lumina of para-bronchioles. Lung, Gridley, 200.

The Gridley staining method was performed for differential diagnosis of fungal hyphae and spores located in typical granulomas detected in the lungs and other internal organs (air sacs, myocardium, thoracic wall and abdominal serosa) and those found in a free form in intact tissue. The Gridley staining method revealed the fungal structures to belong to *A. fumigatus* (Fig. 6).

Mycological Findings

After the culture of the tissue samples collected during the necropsy of all dead birds (lungs, air sacs, myocardium, thoracic wall and abdominal serosa), the formation of blue-green colonies was observed and *A. fumigatus* was diagnosed according to the prescribed mycological examination. However, no mycological findings in tracheal swaps were observed in the control group.

Discussion

Aspergillosis identified as one of the most significant diseases of poultry is a fungal infection frequently observed in the world and in Turkey. Aspergillosis is reported to cause great economic loss in mainly turkeys, chicken pullets and chicks, pigeons, and quails (Başkaya et al., 1953; Merdivenci, 1971; Merdivenci and Zehavi, 1976; Minbay and Akay, 1981; Teme, 1984; von Sandersleben and Atmungsorgane, 1985; Barton et al., 1992; Perelman and Kuttin, 1992; Akan et al., 1996; Richard, 1997; Gylstorrff and Grimm, 1998; Akan et al., 2002).

Pathological lesions in naturally and experimentally acquired aspergillosis cases exhibit characteristic forms. The characteristic forms of caseous nodules have been reported by many researchers mainly in the lungs and air sacs as well as other organs including the myocardium, kidneys, liver and spleen (Olson, 1969; Geisel, 1982; von Sandersleben and

Atmungsorgane, 1985; Erer et al., 1986; Perelman and Kuttin, 1992; Singh et al., 1994; Richard, 1997; Gylstorrff and Grimm, 1998). Lesions have also been observed in the glandular stomach and B. fabricius (Minbay and Akay, 1981). Similar to the reports of other researchers, in the present study, typical caseous nodules which ranged from pin point to chickpea in size, were observed in the lungs, air sacs, myocardium, thoracic wall and abdominal serosa. Contrary to some reports in the present study no lesions were detected in the brain (Raines et al., 1956; Hasegawa et al., 1971; Akan et al., 2002), eyes (Richard, 1997), glandular stomach and B. fabricius (Minbay and Akay, 1981) and skin (Richard, 1997).

The macroscopic appearance of caseous nodules may be confused with tuberculosis, Marek disease and tumours. Exact differential diagnosis can be made according to detection of fungal hyphae and spores upon histopathological examination (Geisel, 1982; von Sandersleben and Atmungsorgane, 1985; Erer et al., 1986; Richard, 1997; Gylstorrff and Grimm, 1998). Researchers (Minbay and Akay, 1981; von Sandersleben and Atmungsorgane, 1985; Reece et al., 1986; Richard, 1997; Gylstorrff and Grimm, 1998; Akan et al., 2002) have presented explanations for the microscopic appearance of lesions and the typical structure of hyphae and spores upon the examination of lungs and internal organs displaying lesions in various poultry species. Similar to the results of the present study other researchers have also observed the presence of *Aspergillus* hyphae and spores in caseous necrotic masses, as well as cell infiltrations consisting of giant cells, heterophils and macrophages together with necrotic granulomatous foci surrounded with an outer layer of connective tissue. Fungal hyphae and spores were easily demonstrated using the Gridley dye method. Some bronchioles were found to contain a great number of conidiophores and fungal hyphae and spores. The above mentioned morphological features contribute to the diagnosis of *A. fumigatus* (Geisel, 1982; von Sandersleben and Atmungsorgane, 1985; Erer et al., 1986; Richard, 1997; Gylstorrff and Grimm, 1998; Akan et al., 2002).

Macroscopic, microscopic and mycological findings determined on starlings in this study were found to be similar to lesions detected in various poultry species diagnosed with aspergillosis. This research is the first experimental study carried out on starlings in which an *A. fumigatus* strain was isolated.

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