



**Investigating an Outbreak of *Aspergillus fumigatus* Infection in a Racing Pigeon
(*Columba livia domestica*) Flock**

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Abstract: In this study, the clinical course, pathological findings, and potential risk factors of a systemic aspergillosis outbreak caused by *Aspergillus fumigatus* in racing pigeons (*Columba livia domestica*) were investigated. The disease was reported during the period of intense exercise in a 120 head racing pigeon flock. The exercise period coincided with the high environmental temperatures. Affected birds displayed symptoms, such as weakness, fatigue, depression, inability to stand, rapidly developing weakness, incoordination, convulsions, and death. Necropsy revealed varying sized, prominent nodular or plaque-like lesions on the lungs, air sacs, and visceral peritoneum. Single or multiple caseonecrotic fungal granulomas invading the existing and/or adjacent tissues were seen histopathologically. Malacic lesions associated with fungal elements were observed in the central nervous system. Histopathologic and cytological findings revealed the presence of characteristic *A. fumigatus* elements and lesions. Considering the fact that the environmental temperatures were above the seasonal norms during the emergence of the disease as well as subjugation of pigeons to intense exercise for race preparations, *A. fumigatus* colonization and its spread in racing pigeons was seen likely due to the combined effects of these risk factors.

Keywords: Aspergillosis, exercise, pathology, predisposing factors, racing pigeon

Bir Yarış Güvercini (*Columba livia domestica*) Sürüsünde *Aspergillus fumigatus* Salgınının Araştırılması

Öz: Bu çalışmada yarış güvercinlerinde (*Columba livia domestica*) *Aspergillus fumigatus*'un neden olduğu sistemik aspergillozis salgınının klinik seyri, patolojik bulguları ve muhtemel risk faktörleri araştırıldı. Yüzyirmi adet yarış güvercini bulunan sürüde yoğun egzersiz periyodu ile ilişkili hastalık salgını rapor edildi. Hastalığın ortaya çıkışı yoğun egzersiz programı ve hava sıcaklıklarının yüksek seviyelerde seyretmesi ile ilişkiliydi. Etkilenen kanatlılarda halsizlik, çabuk yorulma, depresif durum, ayağa kalkamama, hızlı gelişen zayıflama, inkoordinasyon, çarpınma gibi belirtiler ve ölüm görüldü. Nekropside akciğer, hava keseleri ve viseral peritoneumun yüzeyinde değişen büyüklüklerde, nodüller veya plak benzeri lezyonlar dikkati çekti. Histopatolojik olarak parenkimatöz organları etkileyen ve bu organların çevresindeki dokulara invazyon gösteren fungal heterofilik granülomlar belirlendi. Santral sinir sisteminde fungal yapılar ile ilişkili malasik lezyonlar görüldü. Histopatolojik ve sitolojik bulgular *A. fumigatus*'a ilişkin karakteristik bulguları ortaya koydu. Hastalığın çıkış döneminde hava sıcaklıklarının mevsim normallerinin üstünde seyretmesi ve bu tarihlerde yarışlara hazırlık için güvercinlere yoğun egzersiz yaptırıldığı göz önüne alındığında *A. fumigatus*'un güvercinlerde kolonize olarak yayılmasında bu faktörlerin olası rolü olduğu düşünüldü.

Anahtar kelimeler: Aspergillozis, egzersiz, patoloji, predispozan faktörler, yarış güvercini

Introduction

Despite being uncommon in mammals (Hazıroğlu et al., 2006; Arné et al., 2011), aspergillosis, primarily caused by *Aspergillus fumigatus* (Arné and Lee, 2020), is a major deadly mycotic disease of free-living, captive, and domestic birds (Akan et al., 2002; Atasever et al., 2004; Beytut et al., 2004; Beyaz et al., 2008; Özmen et al., 2013; Aslan et al., 2015; Gulcubuk et al., 2018). *Aspergillus* fungi are common in nature (Arné and Lee, 2020), and aspergillosis is commonly associated with contaminated feed or litter

(Martin et al., 2007). Fungi sporulate in favorable climatic conditions, increasing the number of conidia in the air and thus the risk of air-borne infection. Despite the presence of aerial mycoflora birds, immunosuppression plays a major role in the development of aspergillosis (Fullerger et al., 2006; Cafarchia et al., 2014).

Pulmonary infection, which includes diffuse miliary plaques or nodular fungal lesions of lungs and air sacs, is the most common type of aspergillosis. Depending on the severity of lesion, infected birds exhibit varying degrees of dyspnea, gasping, and accelerated breathing (Arné and Lee, 2020) The infection may spread to neighboring organs through local inva-

sion (Martin et al., 2007) or, in rare cases, through circulation to other organs and systems, most notably the eyes (Beckman et al., 1994) and brain (Hubben, 1958; Akan et al., 2002; Ozmen and Dorresteijn, 2004). Encephalitic lesions can be granulomatous or malacic in nature and are clinically manifested by ataxia, tremor, and other symptoms (Hubben, 1958; Akan et al., 2002; Arné and Lee, 2020). Other avian granulomatous infections with similar clinical signs may be confused with this disease. The presence of a fungal element in a tissue specimen, a touch-impression smear of the cut surface, or in culture and cytology aids in the final diagnosis (Arné et al., 2011; Arné and Lee, 2020)

The present study describes the clinical and pathological findings and potential risk factors for aspergillosis caused by *A. fumigatus* in racing pigeons, affecting the lungs, air sacs, visceral organs, and brain.

Materials and Methods

History

In October 2022, a disease outbreak resulting in deaths was reported in a flock of 120 racing pigeons (*Columba livia domestica*) aged 3 to 5 months. The breeder stated an average of 1-2 deaths daily at the onset of the disease that increased to an average of 8-10 deaths daily following the vaccination against Newcastle disease and infectious bronchitis while there were no reports of disease or death in the area. The occurrence of disease coincided with the exposure of pigeons to temperatures above seasonal norms (36 °C - the highest) and excessive physical exercise while preparing for competitions. Housing and rearing conditions like ventilation, relative humidity, and lighting were compliant with the standards. Fatigue, depression, inability to stand, rapidly developing weakness, incoordination, convulsions, and death were among the clinical symptoms seen in the affected birds. In the course of this outbreak, 75 pigeons died, 15 of which were presented to the Department of Pathology for necropsy. The study did not require ethical approval from the institutional committee since the samples used in the study were collected within the scope of diagnostic purposes.

Pathological examination

Gross pathological findings were documented during necropsy. Lactophenol cotton blue was used to stain touch-impression smears of the lesions' cut surfaces. Tissue samples were then fixed in 10% neutral buffered formalin for 48 h before being dehydrated in ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin. The paraffin blocks were cut into serial sections (4-5 µm) and stained with hematoxyline and eosin. To visualize the microbial agents, selected tissue sections were stained with periodic acid Schiff (PAS), Gridley fungus (GFS),

Brown-Breen (BB), and Ziehl-Neelsen (ZN) stains. Slides were examined using a light microscope (#BX51, Olympus) and a digital camera (#SC180, Olympus), and photomicrographs were taken.

Immunohistochemistry

To investigate the possibility of Newcastle virus infection, immunohistochemical staining was performed on sections of lesioned organs (brain, kidney, liver, heart, air sacs, and lung). After deparaffinization and rehydration, the tissues' peroxidase activity was inhibited, antigenic epitopes were exposed, and non-specific binding sites were blocked. The sections were then incubated for 1 h each with polyclonal rabbit anti-Newcastle virus and HRP-labeled anti-rabbit (#MRT621, Biocare, USA), before being stopped with 3,3'-diaminobenzidine tetrahydrochloride. As a positive control, tissues with confirmed Newcastle disease virus were used.

Result

Gross pathology

All of the birds were in poor physical condition, with moderate muscle mass loss. On the lungs, there were randomly distributed multifocal to coalescing consolidated areas with round, oval-shaped, raised dome-like, flat, or umbilical nodules in the center, accompanied by caseous necrosis (Figure 1a-b). Fibrinous adhesions from the pleura to the thorax were occasionally found in the lesion areas. In the tracheal lumen, mucoid exudate or, less commonly, caseous exudate adhering to the mucosa was observed. In sporadic cases, the mucosa at the level of pharyngeal papillae was quite swollen and hyperemic, with numerous gray-to-white nodular formations of varying sizes on the surface (Figure 1c). Hearts were generally hypertrophic, with thickened pericardial sacs and gray-to-white areas on the epicardium on occasion (Figure 1d). The air sacs had thickened and become opaque, with cream-colored plaque-like lesions protruding from the surface. The thoracic air sacs had the most prominent lesions. Plaque-like lesions of varying sizes were also observed at random on the surface of the kidneys and the intestine serosa. Kidney lesions were more common and larger near the air sacs (Figure 1e-f). Except for hyperemia in leptomeningeal vessels, no lesions were found in the central nervous system (CNS).

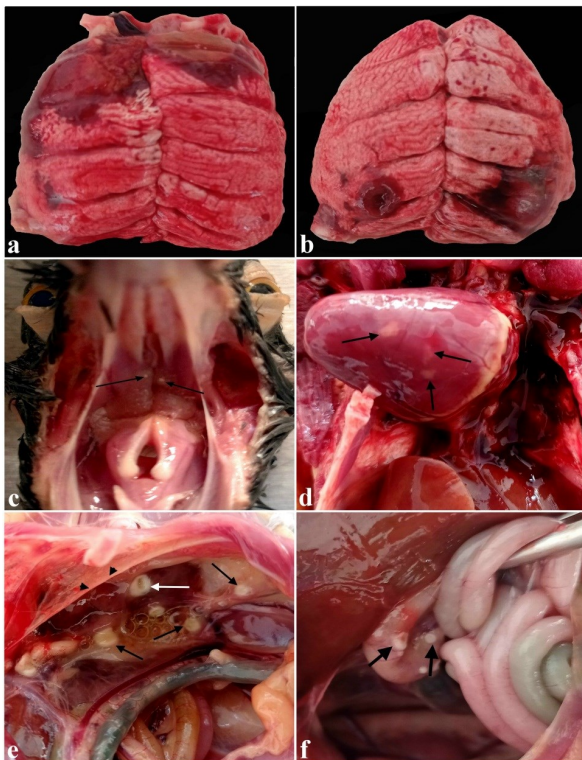


Figure 1. Macroscopic lesions of aspergillosis in racing pigeons. a) extensive consolidated areas with necrosis on the cranial regions of the lung; b) varying sized, multifocal consolidated areas with round to oval, raised dome-like, or umbilical nodules on the lung; c) The mucosa at the level of pharyngeal papillae is prominent with gray to white nodular lesions (arrows) on its surface; d) gray to white amorphous areas on the surface of epicardium (arrows); e) the air sacs are thickened and opaque (arrowheads) and plaque-like lesions on air sacs (black arrows) and surface of the kidneys (white arrow); f) flat plaque-like lesions on the serosa of the intestines (arrows).

Histopathological findings

On gross examination, the nodular or plaque-like lesions seen in various organs and tissues were heterophilic granulomas with intralesional fungal hyphae. Granulomas were observed replacing, distorting, or compressing existing tissue. The granulomas had caseous necrotic exudate in the center, which was surrounded by a palisade of radially organized multinucleated giant cells and an intense infiltration of macrophages mixed with heterophils without an outermost fibrous capsule. Giant cells were occasionally missing from the granulomas. At the periphery of granulomas, clusters of lymphoid cells were seen sporadically. In the lesion areas, there was total or segmental fibrinoid necrotic vasculitis with thrombosis, hemorrhages, and fungal mycelium invading

the vessel wall, as well as perivascular inflammatory cell infiltration. With mixed inflammatory cells and edema, the visceral peritoneum was slightly thickened.

Multifocal heterophilic granulomas were found in the parabronchi of the lungs. Heterophilic fibrinous exudate or less commonly diffuse granulomatous infiltrates filled the secondary bronchi, infundibulum, and atria surrounding the granulomas (Figure 2a). In severe cases, coalescence of these lesions affected a larger area opening to the adjacent parabronchi, where numerous dichotomously branched septated hyphae, conidia, and a few conidiophores (characteristics of *Aspergillus* spp.) were seen (Figure 2b). Widespread thrombotic necrosis areas affecting the adjacent granulomas along with intense fungal elements invasion were seen in some cases. The pleura and air sacs had thickened with fibrin-rich or caseonecrotic exudate with intralesional fungal elements (Figure 2c-d). The tracheal submucosa thickened due to edema and inflammatory cell infiltration. Caseous exudate occasionally covered epithelial damage in the mucosa. Small granulomas were seen scattered throughout the oropharynx's submucosa. Mild-to-moderate macrophage infiltrates mixed with heterophils and edema thickened the pericardium, occasionally invading the myocardium (Figure 2e). The pericardial lesion was more visible at the heart's base. Similar lesions were found in the adventitia of the vessels that enter and exit the heart. In one case, small granulomas were also found in the subendocardium. Typical granulomas were also observed on the surface of kidney (Figure 2f). The leptomeninges and choroid plexuses in the CNS (n=2) were thickened with edema and inflammatory cell infiltrations. Malacic lesions with inflammatory cells infiltrates were found adjacent to these lesions (Figure 3a-b). In the liver (n=3), there was inflammatory cell infiltration consisting of macrophages and fewer heterophils scattered randomly in the parenchyme or perivenular areas, with occasional lymphoid cell hyperplasia and necrotic foci. In the liver, parenchymal degeneration observed, which was more prominent in subcapsular and periacinar regions and was accompanied by fatty degeneration in some areas.

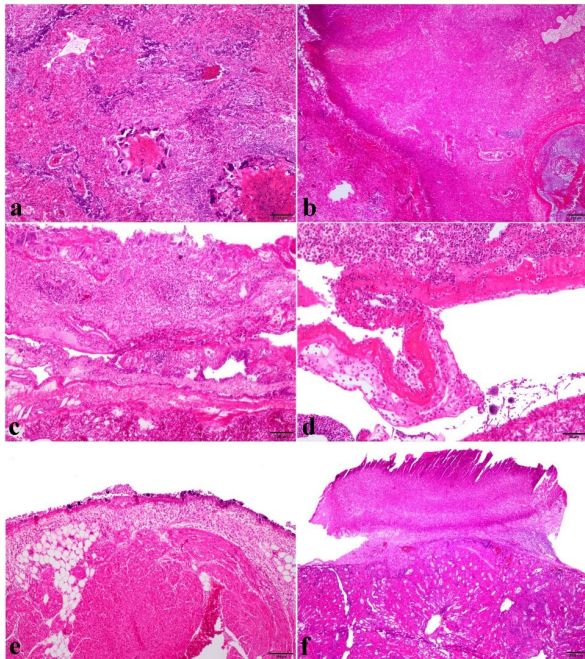


Figure 2. Histopathological lesions of aspergillosis in racing pigeons, H&E stainings. a) varying sized granulomas on the lung, characterized by central caseonecrotic exudate surrounded by a palisade of radially organized multinucleated giant cells and epithelioid macrophages, Bar: 100 µm; b) a large granuloma with intralesional intense fungal elements and thrombosed vessel on the lung parenchyma, Bar: 200 µm; c) thickened pleura with fibrin-rich exudate comprising of macrophages, heterophiles, and multinucleated giant cells, Bar: 100 µm; d) caseified necrotic exudate that includes hyphae, conidia, and conidiophores covered the air sacs, Bar: 50 µm; Inset: close view of fungal elements in caseified necrotic exudate; e) thickened pericardium by moderate infiltrates of macrophages admixed with heterophils and edema invading the myocardium, Bar: 100 µm; f) laminar appearance caseous exudate typically protruding from the surface of kidney, Bar: 200 µm.

In the center of granulomatous foci and encephalomalacic areas, GFS and PAS staining revealed branched, septated fungal hyphae and spores invading the surrounding tissue (Figure 3c-d). The BB and ZN stains were negative. H&E stained tissue sections and lactophenol cotton blue stained touch-impression smears revealed the characteristic of *A. fumigatus* as septate hyphae and typical unbranched conidiophores, each with a single conidial head or vesicle. There were typical columnar and uniseriate conidial heads, as well as short conidiophore stipes with conical-shaped terminal vesicles. On the upper two thirds of the vesicle, conidial vesicles were seen supporting a single row of phialides (Figure 4).

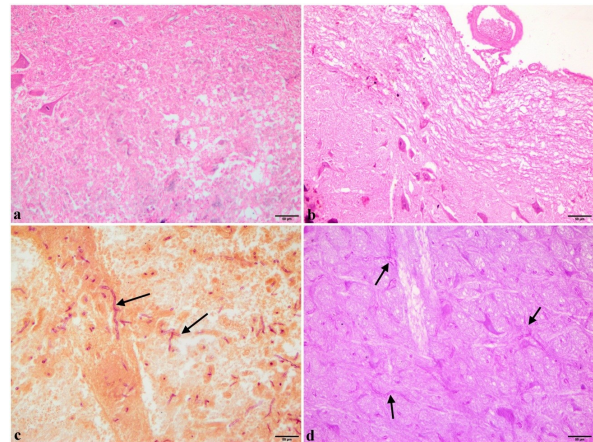


Figure 3. Mycotic encephalomalacia in racing pigeons. a-b) cerebrum showing extensive malacia, H&E, Bars: 50 µm. GFS (c) and PAS (d) stainings showing numerous dichotomously branched, septate fungal hyphae, typical of *Aspergillus* spp. in the areas of malacia, Bars: 50 µm.

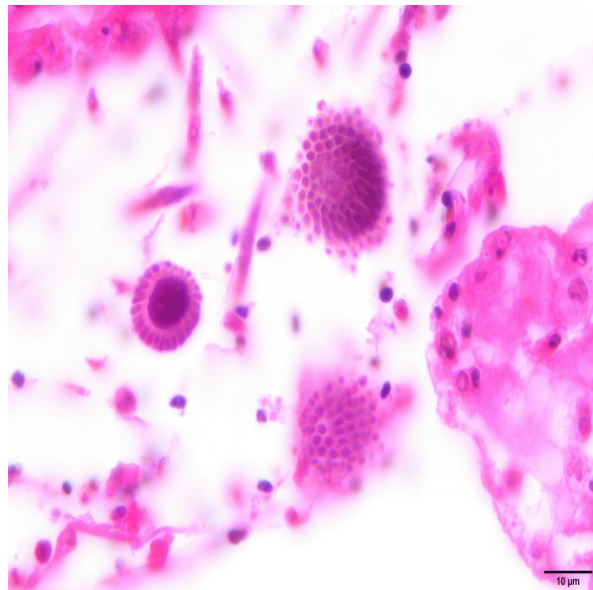


Figure 4. Tissue section showing fungal elements (hyphae, conidia, and conidiophores) specific to *A. fumigatus*. H&E staining, Bar: 10 µm.

Immunohistochemical findings

There was no anti-Newcastle disease virus antibody immunoreactivity.

Discussion and Conclusion

Despite being uncommon in mammals (Tunca and Hazıroğlu, 2004; Hazıroğlu et al., 2006; Tunca et al., 2006), fungal infections especially aspergillosis continues to result in considerable morbidity and mortality.

ty especially in birds (Akan et al., 2002; Atasever et al., 2004; Beytut et al., 2004; Beyaz et al., 2008; Özmen et al., 2013; Aslan et al., 2015; Gulcubuk et al., 2018). In this epidemic, clinical, macroscopic, microscopic, and cytological examinations revealed *A. fumigatus* systemic infection. The clinical signs of the disease differ depending on the organs and tissues involved, as well as the disease's progression (localized or systemic) (Arné and Lee, 2020). In this outbreak, clinical respiratory signs associated with disease were subtle that might be linked with the lesser extent of the pulmonary lesions. Previous research has shown that mild pulmonary symptoms can occur in spontaneous outbreaks (Jensen et al., 1997) and experimentally infections of *A. fumigatus* (Thierry et al., 2013). Signs of the involvement of nervous system like inability to stand up, incoordination and convulsions were seen in this study as described previous reports (Richard et al., 1982; Arné and Lee, 2020). Earlier studies have shown that these findings are caused by granulomatous (Ozmen and Dorrestein, 2004) or malacic meningoencephalitis (Akan et al., 2002). In our study, nervous signs in some pigeons might have resulted from malacic lesions caused by fungal elements. Despite the neurological symptoms, some studies have found that histopathological lesions in the CNS may be absent in aspergillosis outbreaks. These findings suggest that neurological symptoms may occur as a result of fungi-produced toxins (Richard et al., 1981; Özmen et al., 2013)

Mold-contaminated litter and feed is the main source of Aspergillosis (Martin et al., 2007). Under favorable climatic conditions, fungi sporulate in these materials, increasing the mycofloral load in the air. Birds exposed to large amounts of conidia are more likely to become infected. Nonetheless, the factors that cause immunosuppression in birds are equally important in the progression of infection. These factors include inadequate ventilation, high temperature and humidity, mucosal irritants, physical activity, and exogenous administration of corticosteroids (Fuller et al., 2006; Cafarchia et al., 2014). Physical stress, such as intense exercise, activates the sympathomedullary pathway and the hypothalamus-pituitary-adrenal axis, allowing stress hormones to be released (Caplin et al., 2021). Excess catecholamine secretion during increased physical activity may contribute to immunosuppression (Kruk et al., 2020). During the summer and autumn, racing pigeons in this outbreak were subjected to intense exercise and a special nutritional regimen for long-distance flight. This rigorous exercise regimen may be one of the factors that predisposes pigeons to aspergillosis by causing immunosuppression. Furthermore, environmental temperatures were above seasonal norms during the current outbreak, indicating that heat stress might have played a role in the disease's emergence. Moreover,

the increased mortality following vaccination in the current outbreak was consistent with the findings of a previous study reported that vaccine administration is a risk factor for aspergillosis (Barton et al., 1992).

Because the infectious agent usually enters through the air, the pulmonary system is the primary infection site. Conidia inhaled are typically eliminated by an activated immune system. The conidia of *A. fumigatus* are very small organisms that can cross the physical barrier and infiltrate deep into the respiratory system to initiate the infection before the host immune response is effective (Martin et al., 2007; Arné et al., 2011; Arné and Lee, 2020). Consolidation in the lungs with caseous necrotic nodules and fibrinous adhesions, as well as plaque-like lesions in the air sacs and visceral peritoneum, was also observed in our study, as previously reported (Akan et al. 2002; Arné and Lee, 2020). The present study's widespread and severe pulmonary lesions suggest that hyphae adhesion and colonization most likely began in the lung and thoracic air sacs and then spread to other tissues. Because of the presence of smaller lesions in organs adjacent to the air sacs, as well as hyphal lesions in brain, endocardium, and parenchyma of other organs, we believe that systemic infection occurred via both hematogenous route and by direct invasion. The presence of multiple parenchymal lesions in the same organ suggests that hematogenous spread is a continuous process.

Ocular lesions have been reported in previous aspergillosis outbreaks, with two different localizations involving either the corneal and conjunctiva or the vitreous humor (Richard et al., 1984; Beckman et al., 1994; Dalton and Ainsworth, 2011). While corneal and conjunctival involvement develops as a result of direct contact with superficial fungal elements (Beckman et al., 1994; Dalton and Ainsworth, 2011), choroid and ciliary retinal involvement develops as a result of fungi being spread hematogenously from pulmonary lesions (Richard et al., 1984). Histopathological evidence of corneal and intraocular lesions was not found in this study. Other factors may be associated with the absence of external eye lesions. In fact, a previous study suggested that mycotic keratoconjunctivitis develops as a result of corneal damage caused by toxic gases, such as ammonia (Beckman et al., 1994).

Multifocal to coalescing distributed fungal granulomas surrounded by giant cells in lungs, air sacs, and visceral peritoneum in addition to diffuse granulomatous infiltration in lungs in severe cases was consistent with previous studies (Richard and Thurston, 1983; Akan et al., 2002; Cafarchia et al., 2014). We easily detected the fungal elements using PAS and GFS stainings, as previous reported (Ozmen and Dorrestein, 2004). According to one experimental study (Richard and Thurston, 1983), granulomas

begin to be surrounded by a fibrous capsule during the subacute stage, and the fibrous capsule becomes more prominent later on. In our study, granulomas that were not surrounded by a prominent fibrous capsule suggests that infection occurred early in the disease's progression. Infected birds with non-viable *A. fumigatus* conidia do not exhibit the typical pneumonia lesions surrounded by giant cells (Kunkle and Rimler, 1996)

The present study demonstrates that *A. fumigatus* caused this outbreak with a mortality rate of more than 60%. In racing pigeons, high environmental temperature and intense physical activity played a role in the systemic course of the disease involving the CNS by probable immunosuppression.

Declaration of Competing Interest

The authors declare that no commercial funding was obtained that may be construed as potential conflict of interest.

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