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MICROMORPHOLOGICAL CHANGES ON THE EMBRYONIC MEMBRANES OF TURKEY EGGS INFECTED WITH ASPERGILLUS FUMIGATUS AND THEIR IMPORTANCE FOR EMBRYONIC SURVIVAL

ABSTRACT: Aspergillosis is a frequent fungal disease of young and adult poultry in our commercial flocks. Infection can occur after hatching by inhalation of Aspergillus conidia which can be present in contaminated hatcheries, or later, by spores from moldy litter, dust, faeces or feed. Spores from the surface of egg shell can penetrate inside of an egg. The main characteristic of aspergillosis is granulomatous inflammation of respiratory system, although generalized form is possible as well. Multiple yellow nodules can be seen as major patomorphological changes and they are usually localized in lungs, air sacs, and can also be found in spleen, brain, subcutis and eyes. Egg embryos are quite susceptible to infection by Aspergillus fumigatus during incubation.

In this study, the history of a case on one local farm with mortality rate of 7.2% in the turkey flock is presented. At the same time, 28 day old 30 incubated hatching turkey eggs were sampled, that were unable to hatch. The aim of the present work was to determine the cause and to identify the agent of embryonic mortality. Total of the 30 eggs were opened, and 16 of them had thickened egg membranes in the area of air sac. Membrane thickening was visible and circumscript or diffuse presence of black–grey or grey–green fungal growth was observed. Only 3 samples air sacs were filled with developed stages of fungi. To evaluate histopathological lesions, changed egg membranes were processed by standard histological technique.

Dominant microscopic finding was thickening of egg membranes as a consequence of fungal growth and many of them penetrated deep towards embryo. Most of the hyphae were growing vertically through membranes. On the outside surface of the membranes, the elements of fungi (conidial heads with phialids and spores on them), could be clearly observed. These changes were responsible for embryonic death, which on the basis of the size of dead embryos occurred between 7th and 10th day of incubation. Aspergillus fumigatus was isolated and identified from the content of air sacs.

KEY WORDS: Aspergillus fumigatus, turkey hatching eggs, egg membranes, morphological changes
INTRODUCTION

The principal agent causing aspergillosis in poultry is *Aspergillus fumigatus*. The genus *Aspergillus* which consists of approximately 600 species belongs to the division *Deuteromycota* which is composed of anamorphs *Aspergillus fumigatus* and *Aspergillus flavus*. Both lack a sexual stage and this is why they belong to the next classification scheme: division Deuteromycota, class Deuteromycetas, order Moniliiales, family Moniliaceae, genus Aspergillus (S a i f et al., 2008). *Aspergillus fumigatus* is a ubiquitous, saprophytic mold that can be isolated from a variety of habitats worldwide. As a facultative pathogen, it can cause various disease manifestations, including life-threatening invasive aspergillosis in animals and humans (K u n k l e, 2003; O l i a s et al., 2010). Most frequently it occurs in turkey poults, chicks, ducklings and goslings (I v e t ić et al., 2003; B e y t u t et al., 2004; S p a l e v ić et al., 2010; K u r e l j u š ić et al., 2011). Aspergillosis is frequent fungal disease of young birds, although adult birds can be infected, too. There are two forms of the disease: acute aspergillosis usually characterized by severe outbreaks in young chickens and chronic aspergillosis which occurs in adult breeder. The fungal spores are ubiquitous in nature. Exposure of poultry to fungi or spores occurs after the introduction of contaminated litter (T s i o u r i s et al., 2008), but fungi from contaminated food can be directly inhaled by chickens while consuming food. According to M i lj o v ić et al. (2012) *A. fumigatus* was isolated from changed parts of the skin on the foot pads in broiler chickens. In Serbia, this agent was isolated in *Coracias cyanogaster*, *Coraciformes* and *Fringillidae-Passeriformes* imported as free-living birds in quarantine (M i lj o v ić et al., 2011). K a p e t a n o v et al. (2010) reported that early infection in hatcheries is possible if fungal contamination exists. Spores from the surface of egg shell can penetrate inside of an egg (S i n g et al., 2009). The main characteristic of aspergillosis is granulomatous inflammation of respiratory system, although generalized form is possible as well. Multiple yellow nodules can be seen as major pathomorphological changes and they are usually localized in lungs, air sacs, and changes can also be found in spleen, brain, subcutis and eyes (K u r e l j u š ić et al., 2012). The treatment of aspergillosis is very difficult, therefore preventive measures are essential. In this paper, we determined the causal agent of embryonic mortality.

MATERIALS AND METHODS

Total of 30 samples of 28 day old incubated non-hatching eggs were obtained from one local turkey hatchery. The egg shell was pure and non-deformed. Samples were taken when turkey chicks were brought from the roller space from several boxes. The samples were collected during a period when the farm, which was dealing with fattening of turkeys which were originally from the hatchery, experienced a problem of increasing mortality rate of 7.2%.
The results of the tests were reported previously by Kurčulj ušić et al. (2012). After laboratory processing of the egg shell surface, the 30 non-hatching eggs were opened and 16 of them had thickened egg membranes in the area of air sac. To evaluate histopathological lesions, changed egg membranes were processed by standard histological technique. Samples were fixed in 10% buffered formalin, routinely processed and embedded in paraffin blocks. Paraffin sections about 5 μm were stained with hematoxylin-eosin (HE), periodic acid Schiff (PAS) and Grocott methods. The content of air sacs and surface of egg membranes were inoculated into Sabouraud dextrose agar (SDA) with 20 IU/ml penicillin G and 40 mg/ml streptomycin sulphate, and incubated at temperature of 25°C under aerobic conditions for isolation. Primary isolation was successful after 5 days. Subcultures were made on SDA without antibiotics (5 to 5 days). Cultures were stained with Lactophenol-cotton-blue (Hi Media) drops. The identification was done according to the description in available literature (Quinn, 2002). No bacterial growth was observed in aerobic culture on blood agar plates for 2 days at 37°C.

RESULTS AND DISCUSSION

After opening the turkey incubated non-hatching eggs, changes on their membranes were observed. Out of a total of 30 eggs, 16 had thickened egg membranes in the area of air sac, which were, from the outside towards the air sacs, circumscriptively or diffuse covered with black, bluish-green and white color fungal colonies (Figure 1: a, b, c).

Fig. 1. – Macroscopic changes on the egg membranes

In the mycological examination on Sabouraud dextrose agar with antibiotic, fungal colony showed growth after 24 hours at 25 °C, but after four days it was white and about 2 cm in diameter. By the seventh day, the colony diameter increased to 3.5 cm and there were color changes in the central part of the colonies, from bluish-green to grey-green, and by the 10th day the color of the cultures was greenish (Figure 2a). Staining of the fungus with a drop of Lactophenol-cotton-blue showed spore heads and arrangements of phialides and spores (Figure 2: a, b) Aspergillus fumigatus.
Stained culture *A. fumigatus* with Lactophenol-cotton-blue, magnification 400x (b).

Below the inner layer of egg membrane a yellow-red yolk mass was detected which was, more or less, attached to the inner layer of the membrane in a form of pseudomembrane. Some parts of the egg membrane were black stained from penetrated elements of molds. Also, the thickness ranged up to 1 cm, and consisted of mycelium and yolk content. In our opinion, this thickened membrane formed as a result of the growth of *A. fumigatus*. As a consequence of thickened membrane the oxygen supply to the embryo was decreased, which necessarily led to its death. In our case, and based on the size of the turkey embryos (Figure 3), we assumed that they died between the seventh and tenth day of age. However, the question is whether the death of embryos was a result of suffocation or due to toxicosis.

Dominant microscopic finding was the thickening of egg membranes as a consequence of fungal growth which penetrated deeply towards the embryo (Figure 4). This was in accordance with the results presented by Ivetić et al. (1999).
A. *fumigatus* penetrating into sheath and directly towards the embryo tissue, PAS staining, magnification 200x.

Most of the hyphae grew vertically through membranes. On the outside surface of the membranes, elements of fungi were observed, and conidial heads with phialides and spores were bigger in diameter than the hyphae (Figure 5). This finding is consistent with the findings of Femenia et al. (2007) for the case of artificial infection of 1-day old turkey by intra-air-sac inoculation of spore suspension of 3-day old *A. fumigatus* culture, initially isolated from a human with invasive aspergillosis (CBS 144.89) containing $10^7$ spores/animal, when similar finding was obtained.

A very interesting microscopic finding was pseudogranuloma-granuloid formation of round or verrucoid shape, located on the surface egg membrane (Figure 6).
Similar granular formations were described by Biondić et al. (1981), which consisted of numerous hyphae with or without protein mass. According to some authors (Tóth et al., 1979) these granuloid formations indicate the toxic effects of the molds. Immunocompromised condition is presumably induced by products of *A. fumigatus* existing in the body or environment, because this fungus can produce toxins such as gliotoxin, which has immunosuppressive properties. The weakened bird, susceptible to subsequent diseases, ultimately died due to the acute airsacculitis and pneumonia.

The presence of *A. fumigatus* in the air of chicken hatchery is common, and it is often isolated in embryonated chicken eggs (Spalević et al., 2008). The authors stated in their study that the control and disinfection of eggs should be conducted on the farm, as well as the control of litter, nest boxes, more frequent egg collection and careful evaluation of the quality of the eggs, with removal of all damaged and dirty eggs. Turkey hatching eggs are usually washed and sanitized before incubation because of the high percentage of soiled and stained eggs which is why this procedure is required. Eggs are sanitized by formaldehyde fumigation to eliminate pathological microorganisms that may reduce turkey performance and spread disease. Fumigation of the hatching eggs, hatchery facilities, equipment, and vehicle is an effective way of control of pathological microorganisms in the hatchery.

**CONCLUSION**

Hyphae of *Aspergillus fumigatus* were found in the inner layer of the egg membrane and sometimes they penetrated deeply towards the embryo. Egg membranes that suffered the previously described changes were responsible for embryonic death, which, on the basis of the size of the dead embryos occurred between the seventh and tenth day of incubation.

In this case, *Aspergillus fumigatus* was the cause of embryonal mortalities, which was confirmed by mycological and histopathological examination.
REFERENCES


МИКРОМОРФОЛОШКЕ ПРОМЕНЕ НА ОПНАМА ЕМБРИОНИРАНИХ ЂУРЕЋИХ ЈАЈА ИНФИЦИРАНИМ СА ASPERGILLUS FUMIGATUS И ЉИХОВ ЗНАЧАЈ ЗА ЕМБРИОНАЛНО ПРЕЖИВЉАВАЊЕ

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Резиме

Аспергилоза је често гљивично обољење младих, али могу да оболе и одрасле јединке. Инфекција може да настане већ након излегања, удишањем спора аспергилуса присутних у контаминираним инкубатору, или касније, спорама из фекеса, простирке, прашине или хране. Иначе споре могу да пенетрирају и љуску јајета и дођу у његову унутрашњост. Болест се карактерише запаљењем, пре све га респираторног система, мада понекад протиче и у генерализованој форми. Патолошке промене у виду мултипних жућкастих чворића локализоване су најчешће у плућима, ваздушним кесама, а могу бити захваћени слезина, мозак, поткожно ткиво и очи. Аспергилоза се тешко лечи због чега је неопходно да се предузме све превентивне мере којима се смањују или елиминишу услови неопходни за настанак болести.

Нашим истраживањем је обухваћено 30 ембрионираних ћурећих јаја, из којих су у току инкубационог периода нису излегли ћурићи, а са циљем да се утврди узрок ембрионалног замиралета. Јаја су потицала од родитељског јата ћурака инкубираних у инкубаторској станици из околине Београда. Свих 30 јаја је отворено, а код 16 макроскопским прегледом је установљено забележивање ембрионалних опна у подручју ваздушне коморе која је са спољашње стране дифузно или циркумскритно прекривена црно-сивим или сиво-зеленим колонијама односно расти плесни. Код три случаја већи део ваздушне коморе испуњавали су развојни елементи плесни.

За микроскопско испитивање узете су промењене јајчане опне и обрађене стандардном хистолошком техником, а добијени ткивни резови обојени су HE, PAS и Грочот методом.

Микроскопском сликом доминирало је задебљање јајчаних опна као последица инфилтрације сплетом хифа од којих многе дубље пенетрирају према ембриону. Већина њих на излазу из опни заузима вертикалан положај. На спољашњој
површини опни јасно су се видели слободни елементи плесни (конидијалне главе са фијалидама и спорама на њима). Из садржаја ваздушних комора изолован је *Aspergillus fumigatus*.

Овако промењене опне јајета сигурно су одговорне за ембрионално угина-ће које се у овом случају, судећи по величини ембриона, догодило од седмог до десетог дана старости.

КЉУЧНЕ РЕЧИ: *Aspergillus fumigatus*, ембрионирана ћурећа јаја, јајчане опне, морфолошке промене

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