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CO-INFECTION WITH MAREK'S DISEASE VIRUS AND AVIAN LEUKOSIS VIRUS IN THE BANAT NAKED NECK HENS

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Summary

Marek's disease virus and avian leucosis virus are causes of contagious, immunosuppressive, and oncogenic diseases. Many reports have described single virus infection, but detailed description of co-infection with Marek's disease virus and avian leucosis virus has been provided in a few previous reports. The aim of this study was to determine the cause of health disorders in a flock of the Banat Naked Neck hens at the age of 11 weeks. Clinical examination of the flock showed signs of cachexia, apathy, dropped wings, nervous symptoms in the form of bilateral leg paresis, sporadic diarrhoea, and the mortality reaching 20%. Post-mortem examinations showed the following changes: chronic pseudomembranous typhlitis, intestinal ascaridiasis, splenomegaly, granulomatous pneumonia, multiple yellowish caseous foci in the liver, and white tumour masses in the heart. Histopathological examination of the spleen, proventriculus and heart tissue revealed polymorphic infiltrates, while granulomatous inflammation was observed in the lung and liver. Gomori methenamine silver stain method revealed septate and arborized hyphae resembling the *Aspergillus* spp. fungi in the liver and lung granulomas. Bacteriological examination of faeces revealed the presence of *Escherichia coli*, and parasitological examination of the small intestine determined the presence of oocyst of *Eimeria acervulina* and *Eimeria tenella* in the caecum. The genomes of Marek's disease virus and avian leucosis virus were determined by polymerase chain reaction in tissue samples. In this case, specific pathological lesions, as well as molecular finding confirmed co-infection with Marek's disease virus and avian leucosis virus.

Key words: avian leucosis virus, Banat Naked Neck hens, Marek's disease virus

Introduction

There is an increase in the number of laying hens kept in alternative production systems such as organic, free-range and low-input production systems. These rearing systems are less standardisable than intensive ones, as biosecurity is poor in backyard flocks, and poultry have frequent access to the outdoors where they come in contact with wild birds and other animals such as rodents that can transmit different diseases (Gauly et al., 2002; Whitehead and Roberts, 2014; Carrisosa et al., 2021; Dal Bosco et al., 2021). The Banat Naked Neck hen is autochthonous poultry breed that is very tolerant to poor rearing conditions. It belongs to the breeds that are of national interest, as part of the strategy of preservation of total biodiversity in the Republic of Serbia and within the program of preservation of autochthonous genotypes, which is supported by the government of the Republic of Serbia in the form of substantial annual subsidies (Milošević et al., 2013).

Oncogenic viral tumour diseases are one of the major threats to poultry industry as they are immunosuppressive and make poultry susceptible to various other illnesses. Under natural conditions, poultry can be infected by leucosis and Marek's disease virus (MDV). Avian leucosis virus (ALV) is a member of the genus *Alpharetrovirus*, family *Retroviridae*, associated with tumour formation, immunosuppression and decreased fertility that lead to enormous economic loss in the poultry industry, especially because there are no commercial vaccines to protect them from this infection. The virus has six subgroups, from A to E and J. It is known that subgroup A causes major disease and production problems in layers and that ALV-J (avian leucosis virus-J) has caused significant economic losses in meat-type chickens, but there are reports of field cases of leucosis

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caused by ALV-J in commercial egg-type chickens (Simon et al., 2005; Payne and Nair, 2012). Marek's disease is a contagious, lymphoproliferative disease of poultry caused by the virus that belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Mardivirus* (Witter and Schat, 2003; Nair, 2005). The virus is transmitted only horizontally (Mete et al., 2016). The disease can be acute or chronic, and clinical signs depend on the strain virulence, poultry age and previous health condition (Baigent et al., 2006.). Classical chronic form, caused by moderate and weak oncogenic strains of the virus, is characterized by changes in the nerves, i.e., paresis, then paralysis of the legs, torticollis, dropped wings, rarely changes in the iris and paralysis of the eyelids. Lymphomas occur in 5%-10% of infected birds (Rusov, 1997; Vučićević et al., 2014). This disease is the most commonly reported cause of mortality in backyard poultry, as many owners are not aware of the necessity of vaccination.

Co-infection with ALV and MDV that cause contagious, immunosuppressive, and oncogenic diseases, has an important epidemiologic influence in poultry production. Some studies have described pathomorphological findings in co-infection with oncogenic viruses (Payne and Venugopal, 2000; Zhou et al., 2018; Liu et al., 2019).

In the conventional farming system, where poultry are kept in the outdoor systems, characterized by a high risk of acquiring infections, the prevalence of parasitic infection is high (Marcos-Atxutegi et al., 2009). In a situation where the organism has already been altered by oncogenic viral diseases, associated parasitic infestations can be fatal to the host. The most significant parasitic infections that influence poultry production are ascaridiasis and coccidiosis (Nnadi and George, 2010; Abebe et al., 2016). *Ascaridia galli* is the most frequent nematode disease of different avian species. In poultry that is extensively reared, the incidence of infection is >80%. It is accompanied by various clinical signs that include loss of appetite, anorexia, weight loss, ruffled feathers, dropped wings, retarded muscle and osteological development, altered hormone levels, depression and increased mortality, and in laying hens even lower egg production (Dahl et al., 2002; Thapa et al., 2017). Direct losses are caused by obstruction of the intestine and damage to the intestinal tract in hens, and indirect losses are due to reduced function of the immune system, which makes the birds more susceptible to secondary infections (Permin et al., 2006).

Coccidiosis is a disease caused by protozoan parasites from the genus *Eimeria*. It can cause increase of mortality, poor performance, lost productivity and great economic losses in poultry industry. This parasitic infection is followed by high morbidity, which manifests itself in the acute form, bloody enteritis with high mortality, or occurs in a subclinical form. The most important *Eimeria* spp. in poultry are *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. mitis* and *E. praecox*. Differences among these species include invasion of certain part of the gut, pathogenicity, and the type of lesion. Clinical signs are ruffled feathers, listlessness, mucoid to bloody diarrhoea, reduced weight gain or weight loss, and in severe cases mortality (Lucas et al., 2019).

Aspergillosis is a non-contagious disease that primarily affects the lower respiratory system of birds and is caused by a fungal species of the genus *Aspergillus*. The disease occurs under the following conditions: immune compromised bird, overwhelming number of spores, stress, mismanagement problem in commercial and back yard poultry (Saif, 2008; Girma et al., 2016).

The aim of this study was to determine the cause of death in the Banat Naked Neck hens.

Materials and Methods

In September 2021, the backyard flock of the Banat Naked Neck hens, with 50 birds at the age of 11 weeks in extensive breeding was clinically examined. Food consumption was reduced. Wheat, barley, corn and feed concentrate were used in the diet, and water was provided *ad libitum*. The flock was not vaccinated against infectious diseases and was not in contact with other types of poultry.

In this study, during the farm visit we noticed leg paralysis, the birds were unable to move both legs, had weakened or dropped wings, and food consumption was reduced leading to severe weight loss, prominent sternum, and fat loss followed by death. Disorders of the digestive system in the form of bloody and black faeces were reported sporadically. The morbidity rate in the flock was 90%-100% and mortality was up to 20%. During clinical examination of the flock, samples of faeces were taken for bacteriological and parasitological analysis, which were further performed in the accredited laboratory of the Serbian Institute of Veterinary Science. *Ascaridia galli* and coccidiosis lesions were physically verified upon dissection of the whole intestine and caeca, as described by Sharma et al. (2015). Samples of caeca were taken for microscopic confirmation of coccidiosis, while the presence of adult *A. galli* was checked by visual examination. Collected samples were processed for microscopic examination to diagnose the presence of coccidian oocyst. Homogenized mixture of the faecal sample and distilled water was processed using sugar solution and distilled water for floatation and sedimentation methods, then placed on a glass slide and observed under the compound microscope. *Eimeria* oocysts were observed at 400× for confirmation of coccidiosis (Dryden et al., 2005; Fatima et al., 2015).

After necropsy of five birds, samples of the spleen, liver, lung, proventriculus and heart tissue were taken for histopathological examination and fixed in 10% buffered formalin, routinely processed and embedded in paraffin blocks. Paraffin sections about 5 μm thick were stained with haematoxylin-eosin (HE) method and Gomori methenamine silver (GMS) method.

During the pathomorphological examination, pooled samples of altered organs (spleen, liver, lung, proventriculus and heart tissue) were taken for molecular tests. Detection of Marek's disease virus DNA was done using real time polymerase chain reaction (PCR) (Hennig et al., 2003), and the genome of avian leucosis virus using gel-based PCR method (Mohammadi et al., 2008).

Results

In all five necropsied chickens, multiple yellowish-white caseous foci, 3 to 5 mm in diameter, were observed in the liver and white solid tumour masses in the heart. Consolidation of lung parenchyma with granulomatous pneumonic lesions was prominent in two birds (Figure 1).

During necropsy, all five corpses were found to have cachexia, and internal organ inspection revealed serous atrophy of epicardial adipose tissue, hypertrophic proventriculitis, presence of one specimen of *Ascaridia galli* in the gizzard of one bird, mild haemorrhagic enteritis sporadically, and marked splenomegaly (Figure 1) (2-3 times larger than the normal size). In three birds, heavy infection of the intestine with *Ascaridia galli* was observed, where 15-35 adult *Ascaridia galli* worms were noticed. Pathomorphological examination of the caecum revealed chronic pseudomembranous typhlitis, while microscopic examination of native smears of the caecal superficial mucosal layer indicated the presence of developmental forms of *Eimeria* species.

Polymorphic infiltrate was observed by histopathological examination of the spleen, heart, liver and proventriculus (Figures 2, 3 and 4). Consequent atrophy of the parenchyma was present in the liver, heterophilic and histiocytic infiltrates were observed in the spleen in addition to lymphoblastic proliferation, and lymphoblastic infiltration with capillary congestion and small haemorrhages were observed in the proventriculus. Also, in the altered lung, well-organized granulomatous pneumonia with GMS staining positive hyphae was observed (Figure 5).

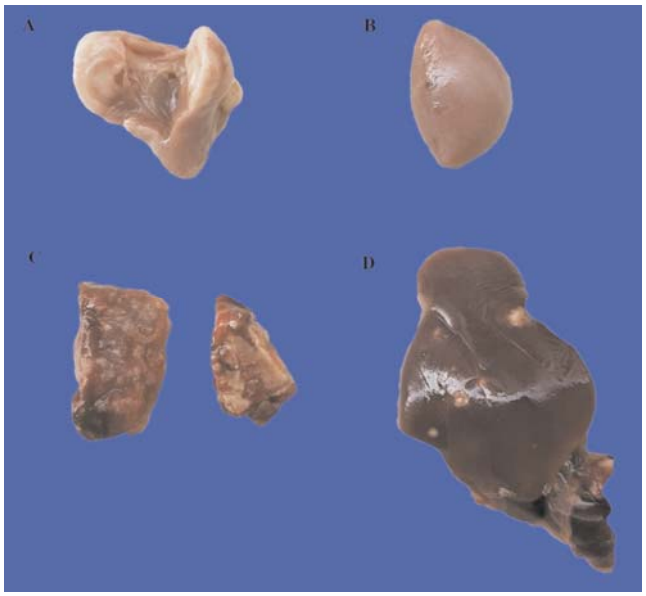

Figure 1. Macroscopic finding of the heart, spleen, lung and liver of Banat Naked Neck hens: (A) solid tumour of the heart; (B) splenomegaly; (C) granulomatous pneumonia; (D) granulomatous hepatitis

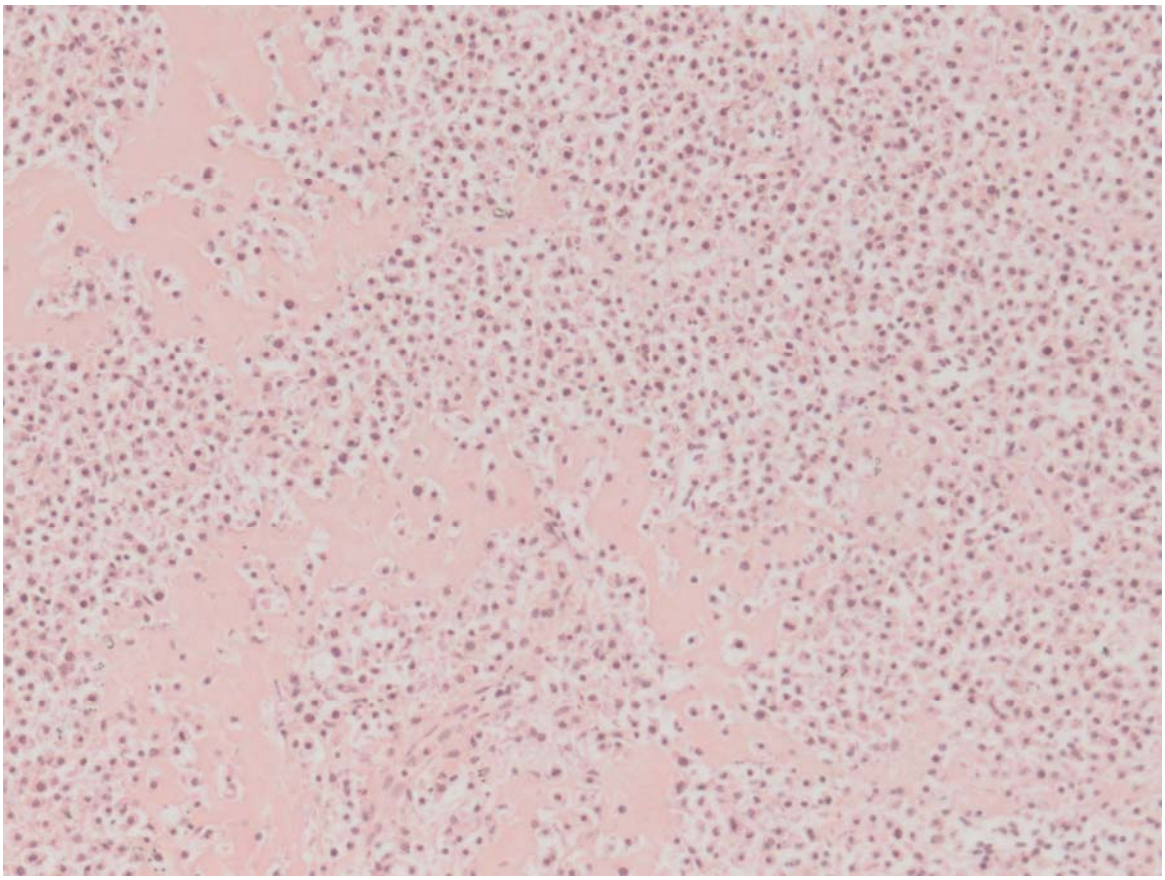

Figure 2. Microscopic finding of the spleen of Banat Naked Neck hens: polymorphic infiltrate. (HE, magnification ×40)

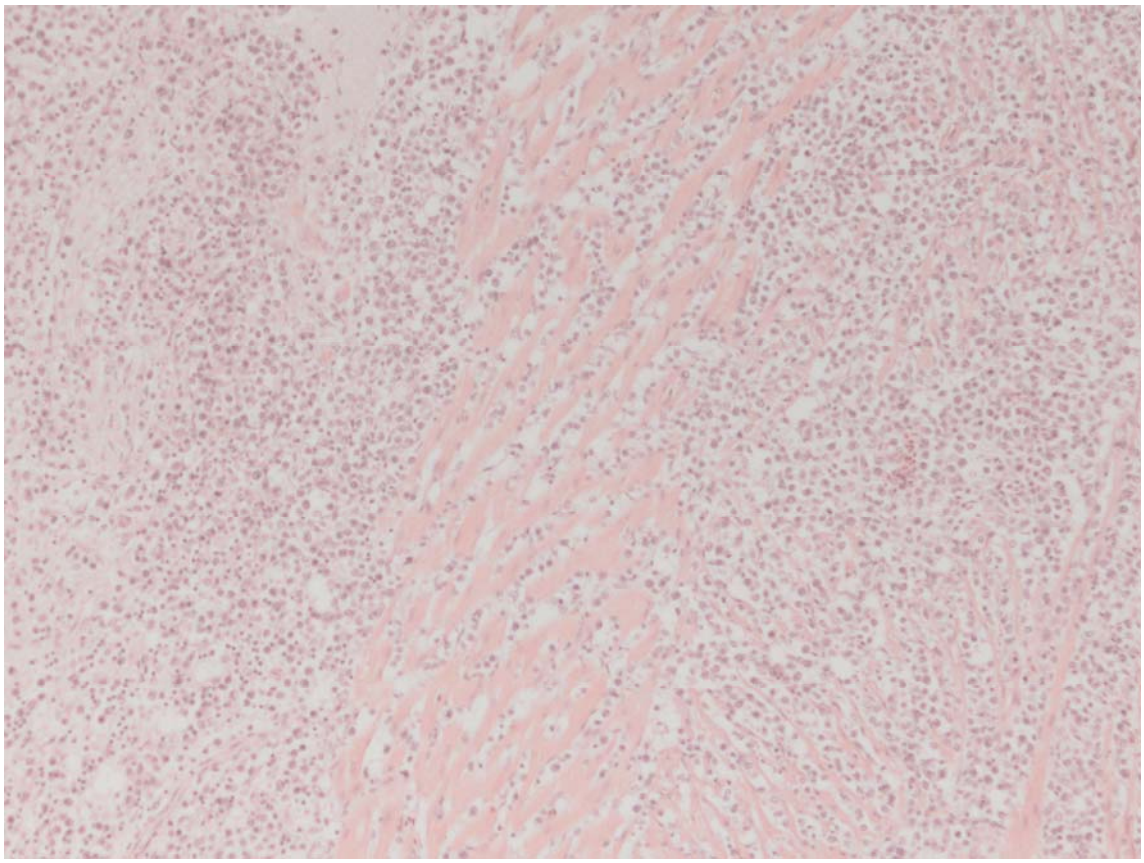

Figure 3. Microscopic finding of the heart of Banat Naked Neck hens: polymorphic infiltrate. (HE, magnification ×20)

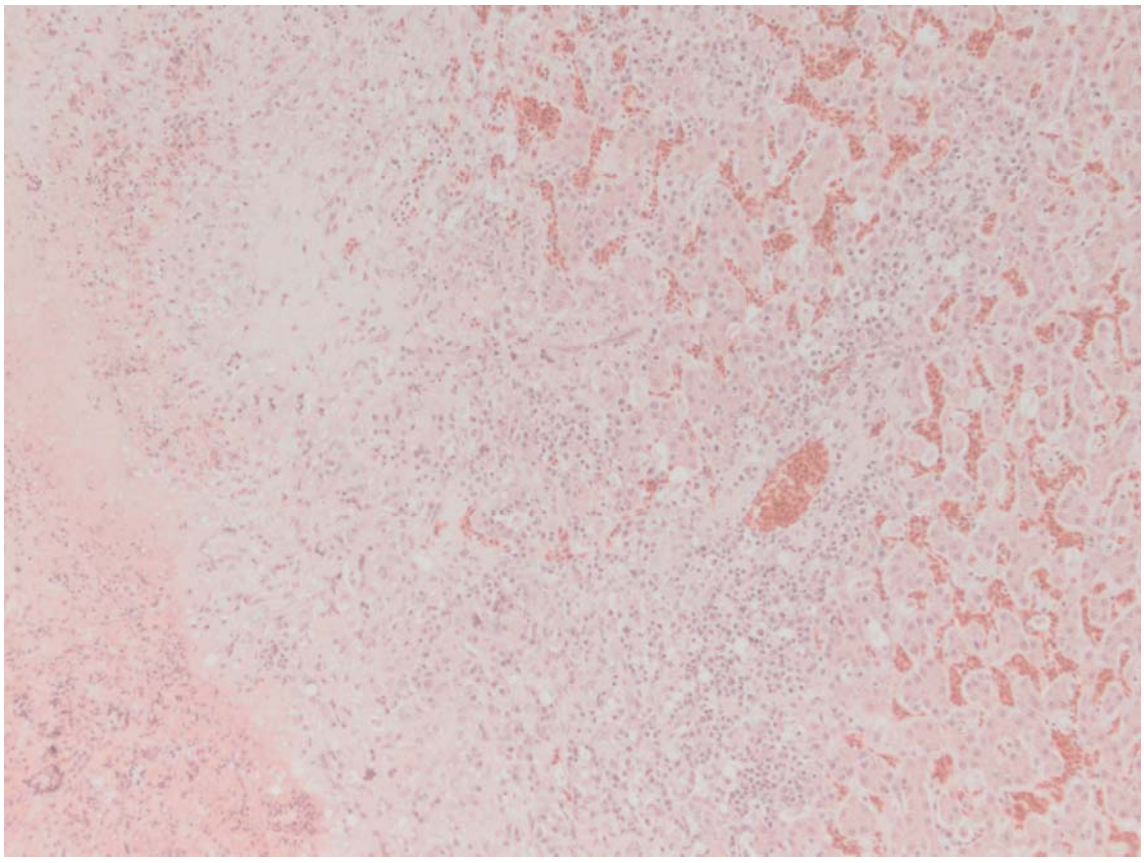

Figure 4. Microscopic finding of the liver of Banat Naked Neck hens: polymorphic infiltrate. (HE, magnification ×20)

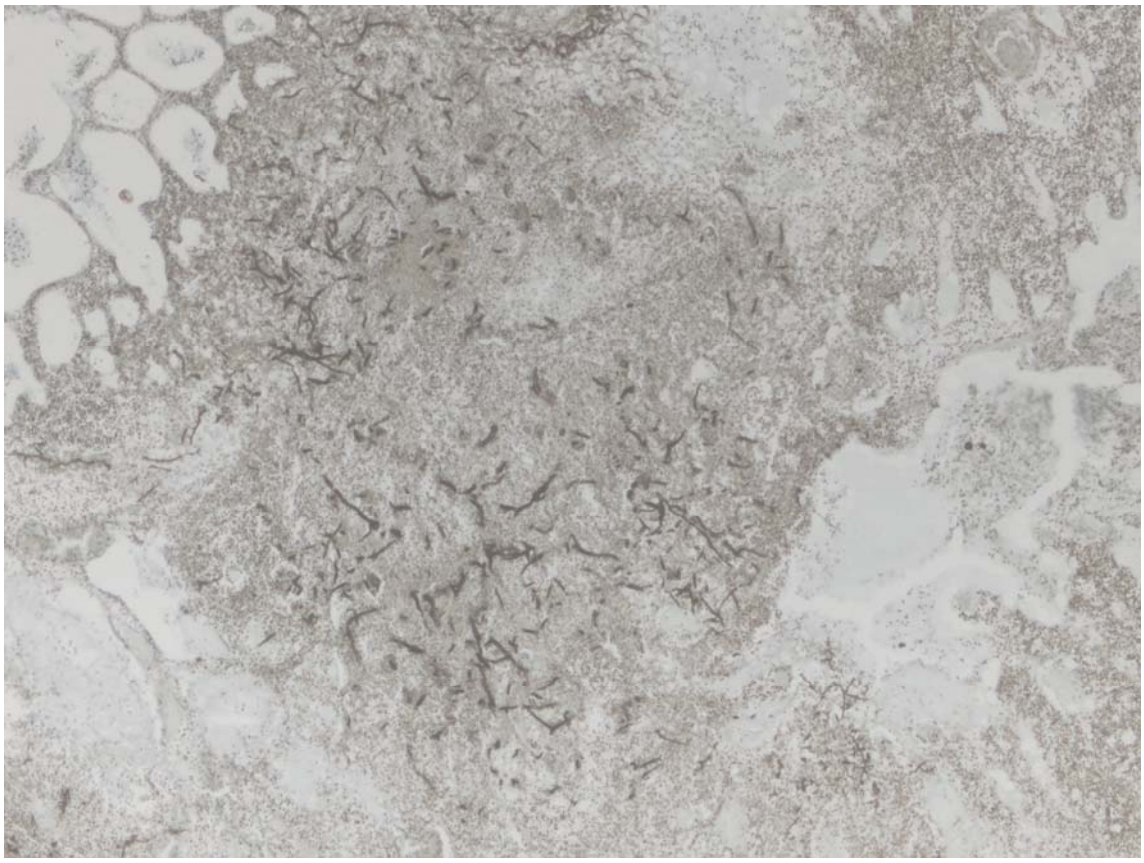

Figure 5. Microscopic finding of the lung of Banat Naked Neck hens: arborized and septate hyphae. (GMS, magnification ×10)

Bacteriological analysis of faeces revealed the presence of *E. coli*, and parasitological analysis of faeces confirmed the presence of *Eimeria acervulina* and *Eimeria tenella* oocysts.

The genome of Marek's disease virus serotype 1 and genome of avian leucosis virus were confirmed by PCR analysis.

Discussion

Concurrent infections with oncogenic viruses and parasites are economically very important to the poultry industry, especially in the conventional farming. There is no infrastructure for biosecurity and poultry is exposed to environmental stresses, which lead to huge economic losses for farmers (Abbas et al., 2015).

Many reports have described histological changes associated with a single virus infection, but detailed description of histological findings from naturally occurring cases of co-infection has been provided in a few reports (Wen et al., 2018; Lui et al., 2019). In the present study, we describe clinical signs and pathological findings associated with MDV and ALV co-infection in a naturally infected flock of the Banat Naked Neck hens. In this case, co-infection with two oncogenic viral diseases induced immune suppression and increased susceptibility to secondary infections including coccidiosis, ascaridiasis and aspergillosis.

Marek's disease (MD) is a major cause of mortality in backyard chickens worldwide (Mete et al., 2013) because backyard poultry are primarily kept for a hobby with poor biosecurity measures and vaccination strategies (Elkhoraibi et al., 2014). Cell-associated alphaherpesvirus that causes Marek's disease is highly contagious, environmentally resistant, and continues to be a significant problem in the alternative production systems due to the lack of vaccination strategies for backyard poultry flocks. The findings by Spalević et al. (2016) proved the genome of Marek's disease virus serotype 1 as a cause of impaired health in the flock of Holland white crested chickens. Clinical signs in this case manifested as cachexia, feed intake decrease, and sporadic signs of diarrhoea. Pathomorphological changes in certain internal organs are in correlation with our findings. White nodules in the heart, also hypertrophic proventriculitis, are consistent with previous reports. Histopathological examination revealed that the proventriculus wall was thickened, while congestion of capillaries with small haemorrhages and signs of glandular cystic dilatation were observed in the mucosa. Multifocal lymphoid infiltrations and heterophilic and histiocytic infiltrates in different organs are similar to the histopathological changes described by other authors (Pejović et al., 2007; Spalević et al., 2016; Mete et al., 2016).

Our findings are also in correlation with the findings reported by Wen et al. (2018), who describe clinical signs and pathological findings associated with MDV and ALV-J co-infection in a naturally infected flock of egg-laying hens. Using immunohistochemical method and sequences of the

viral oncogenes isolated from the diseased egg-laying hens, they found that concurrent infection with MDV and ALV-J contributed to tumorigenicity observed in the flock.

In the present study, sporadic occurrence of bloody and black faeces could have been a result of haemorrhages and erosions in caecal mucosa due to the infection with *Eimeria,* but mild haemorrhagic enteritis in the small intestine could have occurred due to the *A. galli* larvae emerging from the mucosa. *E. acervulina* and *E. tenella* have been established as the major causes of bloody diarrhoea in poultry coccidiosis (Makai et al., 2007; Ngongeh et al., 2019). Coccidiosis is a challenge for backyard flocks as they are floor-raised and have continual exposure to *Eimeria* spp. oocysts from contaminated environmental areas (Godwin and Morgan, 2015).

Atrophy of epicardial adipose tissue may have occurred as a consequence of fat mobilization, which is in correlation with the findings reported by Sharma et al. (2018), who demonstrated that hens with high *A. galli* burden had consistently lower lipid reserves compared to uninfected hens. *A. galli* alters nutrient utilization and absorption from feed, which leads to reduction in the final growth. Due to the increased use of energy reserves from the body to elevate the immune response against parasites such as *A. galli* infection may affect stored energy reserves such as liver lipids in laying hens (Das et al., 2010; Sharma et al., 2019). The presence of *A. galli* infection also leads to cannibalism, which endangers the welfare and behaviour of chickens (Gauly et al., 2007). Direct losses are caused by obstruction of the intestine and damage to the mucous membrane of the intestinal tract in hens, and indirect losses are a consequence of reduced function of the immune system, hence increased susceptibility to secondary infections. Subclinical *A. galli* infections may have an immunosuppressive effect, allowing *E. coli* to establish itself (Permin et al., 2006). According to the research by Dahl et al. (2002), even subclinical *A. galli* infection seems to enhance the establishment of *P. multocida*.

Pathological features induced by aspergillosis indicate permanent organ damage (Nururrozi et al., 2020). Our findings are consistent with the previous research, which reports that granulomas caused by *Aspergillus* usually occur in the lungs and air sacs (Ivetić et al., 2003; Saif 2008; Kureljušić et al., 2011; Kureljušić et al., 2012). In this case, co-infection with ALV, MDV and ascaridiasis were found, where all agents induced immune suppression and increased susceptibility to secondary infections, so it is not surprising that the flock was infected by aspergillosis as well.

Conclusion

Comprehensive understanding of contagious diseases of backyard poultry flocks is important to provide poultry health information to flock owners, veterinarians, and animal health officials, as they may spread unchecked. Concurrent diseases are common in free-range flocks. Helminth infections and poultry coccidiosis are usually conjoint and they occur as a result of impaired management, due to the lack of the basic biosecurity knowledge. We recommend that the infections be diagnosed regularly with focus on the determination of subclinical occurrence, and controlled promptly. Cases of Marek's disease are not uncommon in our country due to the lack of vaccination strategies for backyard poultry flocks, but this was for the first time that we detected MDV and ALV-J as a concurrent infection. Birds with aspergillosis must be culled because of irreversible pathological damage. Farmers should be aware of the importance of management, biosecurity measures, transmission of pathogens among flocks, regular deworming and vaccination. In this way, we would reduce the impact of infections on the poultry production, and it will improve the overall health and welfare of backyard flocks and farm profitability.

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KOINFEKCIJA VIRUSOM MAREKOVE BOLESTI I VIRUSOM PTIČJE LEUKOZE KOD KOKOŠI PASMINE BANATSKI GOLOŠIJAN

Sažetak

Virus Marekove bolesti i virus ptičje leukoze uzrokuju zarazne, imunosupresivne i onkogene bolesti. Mnoga izvješća opisuju infekciju pojedinim virusom, ali tek mali broj izvješća podrobnije opisuje koinfekciju virusom Marekove bolesti i virusom ptičje leukoze. Cilj ovog istraživanja bio je utvrditi uzrok zdravstvenih poremećaja u jatu kokoši pasmine Banatski gološijan u dobi od 11 tjedana. Klinički pregled jata pokazao je znakove kaheksije, apatije, spuštena krila, živčane simptome u obliku pareze nogu, sporadični proljev te 20%-tnu smrtnost. Postmortem pregledi pokazali su sljedeće promjene: kronični pseudomembranski tiflitis, crijevnu askaridijazu, splenomegaliju, granulomatoznu pneumoniju, višestruka žućkasta sirasta žarišta u jetri i bijele tumorske tvorbe u srcu. Patohistološki pregled tkivnih uzoraka slezene, proventrikla i srca otkrio je polimorfne infiltrate, dok je u plućima i jetri zabilježena granulomatozna upala. Metenamin srebrno bojenje po Gomoriju otkrilo je septirane i arborizirane hife nalik gljivama *Aspergillus* spp. u jetrenim i plućnim granulomima. Bakteriološke pretrage fecesa pokazale su prisutnost *Escherichia coli*, dok je parazitološka pretraga tankog crijeva utvrdila prisutnost oocita *Eimeria acervulina* te *Eimeria tenella* u cekumu. PCR-om su genomi virusa Marekove bolesti i virusa ptičje leukoze utvrđeni u tkivnim uzorcima. U ovom istraživanju su specifične patološke promjene i molekularni nalazi potvrdili koinfekciju virusom Marekove bolesti i virusom ptičje leukoze.

Ključne riječi: virus ptičje leukoze, kokoš pasmine Banatski gološijan, virus Marekove bolest