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ASSESSMENT OF SEROLOGICAL TESTS OF THE INFLUENZA A INFECTION IN WILD MIGRATORY AND ZOO BIRDS DURING THE EPIZOOTIC IN BULGARIA, 2015

Georgi STOIMENOV[1], Gabriela GOUJGOULOVA[2], Georgi GEORGIEV[3], Chavdar FILIPOV[1], Roman PEPOVICH[1], Kalin HRISTOV[1], Branimir NIKOLOV[1]

[1]University of Forestry, 10 Kliment Okhridsky Blvd., 1797, Sofia, Bulgaria
[2]NDSRVMI, 15 Pencho Slaveikov Blvd., 1606, Sofia, Bulgaria
[3]Risk Assessment Center on Food Chain, 136 Tsar Boris III Blvd., Sofia, Bulgaria

Corresponding author e-mail: georgi.stoimenov.vm@gmail.com

Abstract

The aim of our study was to assess the possibility of serological tests for a detection of antibodies against Influenza A virus in wild migratory, zoo birds and alive birds, presented on markets. The samples were collected in Bulgaria during the epizootic in 2015. Totally 209 specimens have been tested, of which 179 only by ELISA and 30 both by ELISA and HI assay. Some differences during the testing of two yolk sacs of eggs, from the found death Dalmatian pelicans, have been demonstrated, where ELISA and AGID were negative, but HI was positive; the following VNR found them to be partially positive. A possible explanation for the observed contradiction could be given by the specific haemagglutinin, located on the surface of viral particle. The obtained positive serum samples of wild birds from Sofia Zoo and those from a market for alive birds have shown that, the supervision of Avian influenza should not be focused only on the migratory birds, because the disease can be introduced by an import of exotic birds and their offer through auctions and markets.

Key words*: Avian influenza A, AGID, Bulgaria, ELISA, HI.*

INTRODUCTION

Influenza A infections are among the most dangerous and significant illnesses in many species of animals and people. Their zoonotic potential has always inspired a great interest among scientists and not once has struck fear among the populations of the world. Avian Influenza is particularly crucial since waterfowl migratory birds are the main reservoir and vector of infection. They can emit the virus 30 days after infection, which is a prerequisite for its dissemination over long distances during the migration of birds and subsequent introduction into populations of domestic flocks. Poultry farming is one of the traditional breeding industries for Bulgaria. There are a lot of industrial poultry sites almost everywhere in the country. At the same time, there are many "backyard" farms where the level of biosecurity is low. In addition, Bulgaria's market share of fattened duck liver in Europe is over 20%, and it is known that a large number of low pathogenic avian influenza A viruses circulate among the ducks. The combination of these factors and the passage of large populations of wild birds throughout Bulgaria in two main migration routes, create conditions for outbreaks of avian influenza A. The strict compliance of biosecurity measures in industrial poultry farms and increased alertness of veterinary services and farmers in backyard farms are the necessary requirements for better control of the disease.

Although there are different studies on serum antibody responses in experimentally and naturally infected ducks (Suarez and Schultz-Cherry, 2000), the immune response in birds due to infection with Influenza A has not been well studied. This response is predominantly based on the virus-producing neutralizing antibodies directed against hemagglutinin (HA) and neuraminidase (NA) glycoproteins (Marinova-Petkova, 2012).

MATERIALS AND METHODS

Blood samples were derived from a farm for hunting birds (Yambol region), hunting birds

(shot around Ogosta dam), zoo birds and eggs from wild birds found dead in the Srebarna Biosphere Reserve.

Table 1. Characterization of samples, included in provided serological tests

Species	*Type of sample*	*Number of samples*	*Method*			
Pheasants	Serum	169	ELISA			
Wild birds zoo Sofia	Serum	10	ELISA	HI		
Wild birds	Serum	6	ELISA	HI		
Wild birds	Serum	10	ELISA			
Dalmatian pelicans	Yolk sac	2	ELISA	HI	AGID	VNR
Mallards	Serum	10	ELISA	HI		
Swans	Serum	2	ELISA	HI		

Antigens used in serological reactions

APMV1 (NDV), H5N1, H5N2, H5N3, H5N9, H1N1, H2N3, H3N8, H4N8, H6N2, H7N1, H7N3, H7N7, H8N4, H9N2, H10N9, H11N9, H12N5, H13N6, H14N5, H15N9-Instituto Zooprofilattico delle Venezie.

Biological systems

As biological systems in the virus neutralization reaction, we used 9-11-day old SPF embryonated chicken eggs.

Methods used for a detection of antibodies against avian influenza A virus obtained from eggs of dead wild birds

Processing of the yolk was performed according to the standard operating procedure of the Reference Laboratory in Ames, Iowa, USA:

1. Break the eggs and put the contents in a Petri dish.
2. Using a syringe, take 1 ml of yolk.
3. Mix egg yolk with 1 ml of sterile PBS in a tube.
4. Vortex the yolk-PBS mixture at maximum speed for 10-15 s.
5. Leave the mixture for 1h at room temperature, then repeat step 4.
6. Centrifuge at 1500 x g for 30 min.
7. We separated the supernatant and used it for AGID, ELISA, HI and VNR.

We have been working with several commercial ELISA kits: INGEZIM INFLUENZA A - Avian Influenza Virus Antibody ELISA Kit and Avian Influenza Virus Antibody test kit, MultiS-Screen, IDEXX. We followed the manufacturer's protocols. The standard OIE procedure was followed for HI (OIE, 2015).

Virus neutralization procedure

We made ten-fold dilutions of yolk and mixed it with an equal amount of field isolate [as we used A / Dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1)]. This was followed by each dilution inoculated into the allantoic cavity of three 10-day-old embryonated chicken eggs (ECE).

Results interpretation

In case of presence of antibodies, they should neutralize the virus and no mortality or agglutination of erythrocytes should be noted. When there is no complete neutralization of the virus, it can be read by the degree of agglutination.

RESULTS AND DISCUSSIONS

Using this serology test, of all 209 samples, 14 were found to be positive. Antibodies in two of them (mallard ducks shot around the dam Ogosta, region Montana) have been defined by us as subtype H7 by hemagglutination inhibition test (HI) (Table 2).

Table 2. Summarized positive results from ELISA testing and HI subtyping

Bird species	Methods		
	Ingenaza ELISA	IDEXX ELISA	HI for subtypes H5 and H7
Mallard 1 (Montana)	Positive	Positive	Positive for H7 Titter 1:16
Mallard 2 (Montana)	Positive	Positive	Negative for H5 and H7
Mallard 3 (Montana)	Positive	Positive	Positive for H7 Titter 1:16
2 Wild birds (Sofia zoo)	Positive	Positive	Negative
9 Mallards (Live birds market)	Positive	Positive	Negative

On March 25, 2015, together with the organ samples of the found death pelicans in the Srebarna Reserve, we received eggs found around them. We processed yolk according to the SOP (reference laboratory at Ames, Iowa, USA). The results are summarized in Table 3.

Table 3. Results of detected antibodies in yolk sac, tested in three methods

Methods	Yolk sac 1	Yolk sac 2
Ingenaza ELISA	Negative	Negative
IDEXX ELISA	Negative	Negative
Immunodiffusion test	Negative	Negative
HI	*1:512	*1:32
	**1:16	**1:8

* Used A / Dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1) antigen-field isolate

** Standard antigen H5N3, A / duck / Italy / 775/2004, IZV, Italy

Virus neutralization

The neutralization test was applied for detection of antibodies in the two yolk sacs of eggs from the found death Dalmatian pelicans. With the prepared mixture of antigen and antibody we infected 3 embryos into their allantoic cavity. After that we observed the embryos for mortality. All chicken embryos were found dead after 48 hours and their allantoic fluids were tested for hemagglutination activity via a hemagglutination assay (HA). We detected hemagglutination activity in all dilutions. The HA positive allantoic fluids were examined for hemagglutination inhibition (HI) using 4 hemagglutination units per well and hyperimmune standard serum (H5N1, H5N3) produced from Instituto Zooprofilattico delle Venezie (Comin et al., 2013; Molesti et al., 2014). The standard OIE procedure was followed for both the HA and HI assays (OIE, 2015). The titter in HI was 1:64 for the concentrated yolk and 1:256 for all other dilutions.

During the analysis of the results from the applied tests (HI, ELISA), for the presence of antibodies against Influenza A virus in wild birds, we were impressed to detect antibody response in the mallard ducks, shot around Ogosta dam, Montana. From the same ducks we received cloacal swabs and tracheal swabs and the applied subsequently rRT-PCR method for the M-gene showed negative results. In serum, tested by ELISA, the results were positive. By HI, two of the three positive samples, detected antibodies against the H7 subtype, and the third sample was negative for the H5 and H7 subtype. Possible explanation of our results may be the duration of virus-carrying and virus-excretion in ducks. Intratracheal and oral infection of ducks (*Cairina moschata*) with H3N6 subtype revealed that the virus was secreted with the high titer in faeces until the 6th day after inoculation (Webster et al., 1978). On the 7th day, only 50% of the ducks emit the virus, and on the 8th day only 1 out of 4 birds. Zarkov et al. (2011) infected intravenously with H6N2 subtype (virus isolated from mallard duck) and found that the virus excretion on day 7 after inoculation was only 50%. On the 21st day, only 29%, while on day 28 it is no longer possible to detect the virus in the cloacal swabs and oropharyngeal swabs from the infected birds (Marinova-Petkova, 2012).

Basing on this information, we can build a hypothesis that serum samples were most likely to have been taken after the period of virus-excretion and the body had enough time to produce antibodies.

The immune response in ducks due to infection with Influenza A has not been well studied, although there are various studies on serum antibody response in experimental and naturally infected ducks (Suarez and Schultz-Cherry, 2000). In an experimental infection with LPAI H7N2 virus, in white Peking ducks, Kida et al. (1980) established virus-excretion until the 7th day after infection, but the antibody response was poor. With HI low antibody titers are established (Marinova-Petkova, 2012).

The results of the study of egg yolk of found dead pelicans in the biosphere reserve Srebarna are interesting. We also found some differences during the testing with the different methods of the yolk. ELISA and AGID were negative, whereas HI showed positive results. A possible reason for the positive reactions in HI test and the negative ones of AGID and ELISA is that the haemagglutinin is located on the surface of the viral particle. They first managed to carry out antigenic irritation. In contrast, the type-specific proteins against which the body builds antibodies, are located inside the virus and are more readily available to immunocompetent cells. The contact with them occurs only after the destruction of the virions (Zarkov, 2007). Some authors' publications have found a great variety in the comparative results of HI, ELISA and AGID in terms of their sensitivity and specificity. In some cases, the applied ELISA testing is more sensitive, in other ones the HI method is more sensitive, in third case the AGID showed higher sensitivity. This fact could be explained by different ways of preparation of diagnostic tests, because there is no standard operating procedure for this. It is well seen in the results of Jin et al. (2004). They compare two types of ELISA: one developed by them and the other was a commercial product. As differences in results are 6%.

Virus neutralization reaction similar to HI proves antibodies against hemagglutinin

protein of the virus. In our case, all chicken embryos were found dead after 48 hours and their allantoic fluids were tested for hemagglutination activity via a hemagglutination assay (HA). We detected hemagglutination activity in all dilutions. The subsequent HI virus titer was 1:64 for concentrated yolk and 1:256 for subsequent dilutions. We did not have complete virus neutralization, but partially the virus was neutralized, due to the fact that there were not enough antibodies in the yolk. Many studies on the birds have shown a strong correlation between antibodies in egg yolk and/or hatchlings and circulating levels of maternal antibodies (Staszewski et al., 2007; Martyka et al., 2011).

We assume that the titer of antibodies found in yolk are low during the acute stage of infection, death has occurred relatively quickly, and the pelican organism did not have enough time to build up more antibodies.

The right approach to prevent obtaining contradictory results is to test the samples with several tests. For example, blood serums were tested with both ELISA and HI test, and the yolk samples were run through four different tests.

CONCLUSION

Studies have shown that yolk samples from wild birds can be successfully used for serological studies. The antibody titer found in the yolk sac in highly pathogenic avian influenza infection is low as the disease is in an acute form and there is not enough time to build up antibodies due to the rapid death of the birds. The positive serum samples from the zoo Sofia and those from the live bird market show that surveillance of Avian Influenza A should not only focus on migratory birds, as the disease can be introduced by imports of exotic birds (zoos birds) and by offering them in markets.

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