UDK 616.921.5:598.2/.9(497.4)
Original scientific paper
Received: 13 October 2005
Accepted: 27 September 2006

AVIAN INFLUENZA SURVEILLANCE IN BACKYARD FLOCKS AND MIGRATORY BIRDS IN SLOVENIA

NADZOR INFLUENCE U DVORIŠNE PERADI I PTICA SELICA U SLOVENIJI

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Summary

Blood samples and clocal swabs were taken from free-living migratory birds and backyard flock birds. Enzyme linked immunosorbent assays (ELISA) and haemagglutination inhibition test (HI) was used to screen free-living migratory birds and backyard flocks for antibodies against avian influenza viruses (AIV). Pools of cloacal swabs were used for the attempt of isolation of AIV. Samples were inoculated of 9 to 11 day-old chicken embryos. Amnioallantoic fluid were collected and analysed for haemagglutination activity. Pools of cloacal swabs were used for the detection of AIV by RT-PCR. Sera samples of four out of twenty three geese were positive on presence of specific antibodies against AIV. All attempts of virus isolation were negative. No AIV nucleic acid was detected by RT-PCR.

Key words: Migratory birds; Backyard flocks; Avian influenza; Seroprevalence; RT-PCR

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INTRODUCTION

Since the mid-1970 influenza viruses have been isolated from different avian species representing most of the major families of birds through the world including birds from the order Passeriformes [1]. Hubalek reviewed some microorganisms associated with migratory birds and pointed out that the migratory birds are of concern for transport and dissemination of certain pathogenic organisms and might be involved in dispersal of avian influenza virus [2].

In this study prevalence of avian influenza viruses in free-living migratory birds and backyard flock birds were investigated. Blood samples of birds were tested on presence of specific antibodies to avian influenza viruses. Cloacal swabs of birds were tested on the presence of avian influenza viruses.

There was no previous research on presence of avian influenza viruses in migratory birds and backyard flock birds in Slovenia.

MATERIALS AND METHODS

Samples

A total of 187 blood samples and 232 cloacal swabs were taken from 24 different species of free living migratory birds: Blue Tit *(Parus careuleus)*, European Robin *(Erithacus rubecula)*, Blackcap *(Sylvia atricapila)*, Garden Warbler *(Sylvia borin),* Leeser Whitethroat *(Sylvia curruca),* Common Whitethroat *(Sylvia communis),* Siskin *(Carduelis spinus),* Pied Flycatcher *(Ficedula hypoleuca),* House Sparrow *(Passer domesticus),* Bluethroat *(Luscinia svecica),* Common Redstart *(Phoenicurus phoenicurus),* Icterine Warbler *(Hippolais icterina),* Great Tit *(Parus major),* Blackbird *(Turdus merula),* Great Reed Warbler *(Acrocephalus arundinaceus),* Wryneck *(Jynx torquila),* Sedge Warbler *(Acrocephalus schoenobaenus),* Reed Warbler *(Acrocephalus scirpaceus),* Grasshopper Warbler *(Locustella naevia),* Chiff Chaff *(Phylloscopus collybita),* Willow Warbler (*Phylloscopus trochilus)*, Marsh Warbler *(Acrocephalus palustris),* Red – Backed Shrike *(Larus colluria)* and River Kingfisher *(Alcedo althis).* Migratory birds were caught into mist nest and ringed with standard ornithological procedures in the course of their autumn migration. Migratory birds were bled from jugular vein. Collected blood was stored in Microtainer® (BD, USA) tube and centrifuged at 2500 rpm. Special small cloacal swabs (Copan, Italia) were used for the sampling procedure avoiding cloacal damage.

188 blood samples and cloacal swabs were taken from different species of backyard flock birds: chickens, ducks, geese, turkeys, pheasants and quails. Back yard flock birds were bled from wing vein – vena ulnaris cutanea. Cloacal swabs were taken according to the protocol [3].

Minimal essential medium (MEM), (Sigma, Sigma-Aldrich Chemie GmbH, Germany) with penicillin 10000 IU/ml, streptomycin 1000 µl/ml and amphotericin B 5 µg/ml (Antibiotic-Antimycotic, Gibco, Invitrogen, United Kingdom) added was used as transport medium. Swabs were stored at –70 °C prior the attempt of isolation of virus and detection of viral nucleic acid by RT-PCR. Sera of all birds were stored at –20 °C until the serological examinations were performed.

Serological investigations

Total of 90 sera samples of migratory birds and 120 sera samples of backyard flock birds were tested for the presence of antibodies against avian influenza virus by avian influenza virus ELISA kit (Flockscreen GUILDHAY Lim.). ELISA positive or suspicious samples were retested by haemagglutination inhibition (HI) assay according to the instructions [3]. Reference AIV subtypes strains were used in HI assay (VLA, Weybridge, UK).

Isolation of virus in chicken embryos

Cloacal swabs were used for the attempt of isolation of AIV. Pools of samples of swabs were made prior the isolation by combining three swabs into one pool. Preparation of swabs and inoculation of 9 to 11 day-old embryos were performed as described [3] with exception that supernatant was not filtered. Inoculated eggs were incubated at 37 °C for 5 days. Amnioallantoic fluid were collected and analysed for haemagglutination activity [3].

RNA extraction and detection of virus by PCR amplification

Pools of cloacal swabs were used for the detection of AIV by RT-PCR. For the preparation of RNA, swab's medium were directly homogenised with Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The RNA was eluted in sterile nuclease-free water and stored at –70 °C.

For the amplification of 244 bp of M protein gene, the primer pair M52C and M253R were used [4]. A single-tube RT-PCR system (ACCESS RT-PCR system, Promega Corp., Madison, WI, USA) was used for the genomic RNA amplification.

Table 1. Results of serological and virological examinations in migratory birds

Examination method	Positive birds /Total birds tested
Serology	0/90
Virology	0/72
RT-PCR	0/147

Table 2. Results of serological and virological examinations in backyard flocks.

Examination method	*Positive birds*/Total birds tested					
	Chickens	*Ducks*	*Geese*	*Turkeys*	*Pheasants*	*Quails*
Serology	0/22	0/28	4/23*	0/7	0/29	0/11
Virology	0/34	0/27	0/16	0/5	0/14	0/14
RT-PCR	0/7	0/31	0/27	0/7	0/20	0/17

* four of twenty three geese sera samples were positive for the presence of antibodies against type avian influenza virus in ELISA; antibodies against subtype H6 were determined in HI test

The RT-PCR was performed by uninterrupted thermal cycling with the following programme: 45 min. at 48 °C for RT, 94 °C for 2 min. for AMV RT inactivation and RNA/cDNA/primer denaturation, 40 cycles of 30 sec. at 94 °C, 1 min. at 55 °C and 2 min. at 68 °C for the PCR and a final extension step at 68 °C for 7 min. The reaction's products were analysed by electrophoresis on a 1,8% agarose gel stained with ethidium bromide.

RESULTS

Serology

All investigated sera of free-living migratory birds were negative on presence of specific antibodies against avian influenza virus (Table 1 and 2).

Four of twenty-three geese sera samples were positive in ELISA on presence of specific antibodies against avian influenza virus; antibodies against avian influenza virus subtype H6 were determined in HI test. All other investigated backyard flock birds were negative.

Virus isolation

Amnioallantoic fluid obtained from inoculated eggs after 5 days of inoculation reacted negative for haemaglutination activity in all performed attempts of isolation in free living migratory birds and backyard flock birds were negative.

PCR

No AIV RNA was amplified from swab's medium of free living migratory birds and backyard flock birds by RT-PCR using a pair of primers that amplify a 244 bb fragment of M protein coding gene.

DISCUSSION

In these days when AIV has emerged as an important pathogen in the poultry industry and is of major global health concern [5] active and passive surveillance of AIV

in free-living birds and backyard flocks is very important. Based on the fact that the number, variety and widespread distribution of influenza viruses has been far greater in waterfowl than in other birds [6-9] monitoring of AIV of free living birds is focused mainly on waterfowl and other birds from order Anseriformes. However the fact that the next highest virus isolation rates of AIV were from migratory birds from order Passeriformes should not be ignored [10]. Intensive monitoring of these birds during spring and autumn migration is of great importance. In Slovenia, sampling and testing free-living passerine migratory birds on AIV is a part of our active surveillance scheme. Our results showed that all out of 147 free living migratory birds tested for the presence of viral nucleic acid by RT PCR were negative, as well as virus isolation attempts (72) were also negative.

In active surveillance of backyard flocks all virus isolation and RNA detection attempts were negative. Serological examination showed that four out of twenty-three of geese were positive in ELISA test. Positive samples were rechecked in HI test. All samples were positive on H6 subtype of AIV. All four birds were without clinical signs.

On the basis of this data we can speculate that avian influenza viruses are present in our country and systematic further research is needed to determine the role of free living migratory birds, backyard flocks and other birds in the ecology of AIV in Slovenia.

Acknowledgements

We thank to our veterinary technicians Marko Bogataj and Darja Krelj from Institute of poultry health, Veterinary Faculty in Ljubljana. We thank also to Cvetka Marhold and Marko Zadravec, students of veterinary medicine, Veterinary faculty of Ljubljana.

The study is carried out in the behalf of VARS and CRP (Ciljni raziskovalni program) project. The project was founded by the Ministry of Education, Science and Technology and the Ministry of Agriculture, Forestry and Food. We thank to the Museum of natural history, Ljubljana for expertise assistance.

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Sažetak

Uzeti su uzorci krvi i obrisci nečisnica slobodnoživućih ptica selica i dvorišne peradi. Imunoenzimni test i inhibicija hemaglutinacije korišteni su kako bi se u slobodnoživućih ptica selica i dvorišne peradi utvrdila nazočnost protutijela za viruse influence ptica. Skupni uzorci briseva nečisnica korišteni su za izdvajanje virusa influence ptica. Uzorci su inokulirani u 9 do 11 dana stare kokošje embrije. Sakupljena je amnioalantoisna tekućina i pretražena na sposobnost hemaglutinacije. Skupni uzorci brisova nečisnica korišteni su za dokaz virusa influence ptica pomoću RT-PCR. Četiri od 23 seruma gusaka bila su pozitivna na nazočnost specifičnih protutijela za virus influence ptica. Svi pokušaji izdvajanja virusa dali su negativan rezultat. Postupkom RT-PCR nije dokazana nazočnost nukleinske kiseline virusa influence ptica.

Ključne riječi: Ptice selice; Dvorišna perad; Influenca ptica; Seroprevalencija, RT- PCR