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Original Scientific Article

NO EVIDENCE OF AVIAN INFLUENZA VIRUSES IN BACKYARD POULTRY FROM RISK AREAS IN R. MACEDONIA (Virological and molecular surveillance 2009)

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Summary: Until 2009 no data are available for the circulation of avian influenza viruses (AIV) in backyard poultry in Republic of Macedonia. In 2009 virological and molecular surveillance was conducted in backyard poultry in the risk areas of the country according the National Annual Program for AI and Commission Decision 2007/268/EC. A total number of 2151 samples were analyzed. Two thousand and twenty cloacal swabs (sampled from backyard poultry - poultry sector 4 from the risk areas in Republic of Macedonia i.e., areas where migratory waterfowl gathers) and 131 organs/cloacal swabs (for daily routine diagnostics) were tested by virological and molecular methods. The virological diagnosis was performed by isolation on embryonated chicken eggs, followed by macro hemagglutination, hemagglutination on microtiter plate and inhibition of hemagglutination. The molecular diagnosis was performed by RRT-PCR for M-gene. All samples were negative for AIV. However, 4 samples were positive on avian paramyxovirus 1 (Newcastle disease virus). The wild birds, as a reservoir for AIV were not included in the surveillance which on the other hand does not ensure complete information for the AIV circulation in R. Macedonia and should be further investigated. This fact imposes the need for overall national action to ensure the concept of early detection and response in case of emergence of avian influenza.

Key words: avian influenza viruses; backyard poultry; cloacal swabs; Republic of Macedonia; surveillance

Introduction

In the last 10 years the field of avian influenza (AI) has undergone something of a revolution. According to Alexander [1], this happened not just because of the actual zoonoses and the potential threat of a pandemic emerging, which has highlighted public awareness, but also because many aspects of the epidemiology of AI infections in poultry and other birds appear to have changed dramatically.

The number of outbreaks of the highly pathogenic avian influenza (HPAI) disease has increased alarmingly in the last 10 years and, even more noticeably, the impact in terms of the number of birds involved and the costs of disease control have dramatically escalated [2]. In addition, the apparently unprecedented emergence and spread of the HPAI H5N1 virus in Asia and beyond has brought AI to the forefront of important animal diseases [1].

Influenza viruses are spherically or longitudinally shaped enveloped particles with an up to eight-fold segmented, single-stranded RNA genome of negative polarity. Influenza viruses hold generic status in the Orthomyxoviridae family and are classified into types A, B or C based on antigenic differences of their nucleo- and matrix proteins. AIV belong to type A [3].

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The main antigenic determinants of influenza A and B viruses are the hemagglutinin (H or HA) and the neuraminidase (N or NA) transmembrane glycoproteins, capable of eliciting subtype-specific and immune responses which are fully protective within, but only partly protective across, different subtypes. Because of the antigenicity of these glycoproteins, influenza A viruses currently cluster into 16 H (H1 - H16) and nine N (N1 - N9) subtypes. These clusters are substantiated when phylogenetically analysing the nucleotide and deducted amino acid sequences of the HA and NA genes, respectively [4].

To date, only viruses of the H5 and H7 subtypes have been shown to cause HPAI. It appears that HPAI viruses arise by mutation after low pathogenic avian influenza (LPAI) viruses have been introduced into poultry by several mechanisms [5]. AIV have been shown to infect birds and mammals. The main factor that influences susceptibility to infection is the receptor conformation on host cells (AIV bind preferentially to SA-α2,3-Gal and human IV to SA- α2,6-Gal terminated saccharides [5].

Influenza viruses have been shown to infect a great variety of birds [6, 7], including free-living birds, captive caged birds, domestic ducks, chickens, turkeys and other domestic poultry.

All available evidences suggest that in the conventional situation the primary introduction of LPAI viruses into a poultry population is a result of wild bird activity, usually waterfowl [5]. The greatest threat of spread of AIV is by mechanical transfer of infective faeces in which virus may be present at concentrations as high as 107 infectious particles/g and may survive for longer than 44 days [8].

The fact that AI represents one of the greatest concerns for public health that has emerged from the animal reservoir in recent times [5], leads to the considerable public and media attention having in mind its zoonotic potential. This situation has given rise to the fear that the virus might acquire the capacity of sustained human–to-human transmission and thus cause global influenza pandemic.

This fear has been the main driving force behind the international commitment to global HPAI control, to date amounting to an estimated US$1.02 billion disbursed funds and US$1.68 billion committed funds as of June 2007 [9], which in turn has significantly influenced national responses to outbreaks of H5N1. The latter have involved destruction of birds on a massive scale, regulation of poultry production and trade and, in some countries, large-scale vaccination campaigns. These responses often come at considerable cost to poultry producers.

HPAI, like other highly contagious animal diseases, affects animal production via three main pathways: direct losses, public impact and market reactions [10].

Firstly, HPAI causes direct losses to producers and other actors interlinked to the production and marketing of poultry through morbidity and mortality and the private costs associated with ex-ante risk mitigation (e.g. investment in animal housing) and/or ex-post coping measures during periods of downtime (e.g. bridging loans if the enterprise carries significant borrowings) and the need to reinvest in replacement birds. Secondly, animal diseases that are 'notifiable' can have severe impacts through government intervention, which carries a cost borne by the public at large and affects producers (and associated up- and downstream actors), irrespective of the disease status of their flocks. These costs include public investment in animal health infrastructure and epidemic preparedness. Thirdly, disease impacts arise through market reactions, which can be particularly severe on the demand-side for diseases that are associated with a public health risk. Analogous to disease control measures affecting producers even if their flocks have not contracted HPAI, market reactions can occur, irrespective of whether or not avian influenza has actually occurred in the country.

Geographically, R. Macedonia belongs to the Black sea - Mediterranean migratory flyway, surrounded by Albania, Greece, Bulgaria, Serbia and Kosovo. Three of these neighboring countries at some point have reported to OIE outbreaks of HPAI H5N1 (Albania 2006 - 1 case, Greece 2006 – 1 case, Bulgaria 2006 – 1 case and 2010 – 1 case). In the wider region, Romania has reported 109 cases of H5N1 in 2006, 1 in 2007 and 2 cases in 2010. Turkey has also reported 2 cases in 2005, 200 in 2006, 17 in 2007 and 7 cases in 2008.

As pointed by the claims of EU, surveillance of AIV for poultry and wild birds is mandatory for every Member state and comprises the implementation of surveillance programs according the EU legislative guidelines.

The National Annual programme for AI surveillance includes conducting surveillance programs in poultry and wild birds. Unfortunately, until now according to the National Annual program no surveillance has been conducted. This is a great disadvantage giving the fact that there is no available data for the previous years and it is anybody's guess if there is circulation of AIV or not.

Although knowing the fact that 3 of the surrounding countries have reported outbreaks of HPAI H5N1, the question of AIV circulation in R. Macedonia remained unanswered and thus emphasized the need of investigation due to the possibility of spreading the disease to Macedonia.

Therefore, the aim of this study was to assess the AIV situation in the poultry sector 4 in R. Macedonia, i.e., to affirm the presence and distribution and/or to confirm the absence of AIV in backyard poultry.

Materials and methods

The cloacal swabs were sampled from backyard poultry (poultry sector 4) from the risk areas in R. Macedonia i.e., areas where migratory waterfowl gathers. The poultry sector 4 defines backyard poultry production in villages with minimal biosecurity measures (birds are kept out most of the day with high possibility of contact with other chicken, ducks, domestic birds and wildlife). Those characteristics define the poultry sector 4 as the most prone sector for AI emergence, especially in locations near lakes and flooding areas where migratory waterfowl gathers.

The distribution of the sampled villages in the risk areas is given in Table 1, and their location is given in Figure 1. The distribution of samples per poultry species is given in Table 2.

As pointed in the Annual program for AI surveillance [11] and Commission Decision 2007/268/EC [12], the sampling was representative for the whole state.

A total number of 213 holdings in 59 villages (1-6 holdings per village) were sampled. According to the previously defined representative number for cloacal swabs per holding (n-11), 11 cloacal swabs were sampled wherever possible. In smaller holdings, the number of sampled cloacal swabs varied from 1 to 11 depending on the number of poultry reared in the holding. Besides these samples, the survey included 131 samples from domestic and wild birds submitted for daily routine diagnosis in the laboratories of the Faculty of veterinary medicine in Skopje (FVMS) (Table 3). Sample processing was performed according to the guidelines in Commission decision 2006/437/EC [13] and OIE [14].

The extraction of RNA was performed with RNEasy Mini kit according to the producer's manual [15].

Detection of M-gene was conducted on Bio-Rad's IQ5 RRT-PCR. The method was performed as pointed in the protocol of the Waybridge reference laboratory [16].

The following reagents were used:

1. Primers and probes:
   - Sep 1 AGA TGA GTC TTC TAA CCG AGG TCG (Operon)
   - Sep2 TGC AAA AAC ATC TTC AAG TCT CTG (Operon)
   - SePRO FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA (Operon)
2. Real time PCR master mix: Qiagen Onestep RT-PCR kit

The virological methods (virus isolation on embryonated chicken eggs - ECE and identification by hemagglutination inhibition - HI test) were performed according to the Commission decision 2006/437/EC [13] and OIE [14]. These methods are not specific for AIV and are used for detection of other avian viruses that can be propagated on ECE and identified by HI test. The HI test was performed with specific antisera for avian paramyxovirus 1, AIV H5, AIV H7 and avian adenovirus.

Table 1: Distribution and number of sampled poultry in risk areas

Risk area	Sampled villages	Nº of samples
Artificial lake Mavrovo	6	188
"Sini virovi" Ohrid	4	164
Ohrid lake	20	690
Prespa lake	7	270
Fishery Bel Kamen	3	124
Fishery Bukri	2	68
Dojran Lake	4	139
Flooding area Monospitovsko blato	5	209
Flooding area Katlanovsko blato	5	103
Artificial lake Veles	3	65
TOTAL	**59**	**2020**

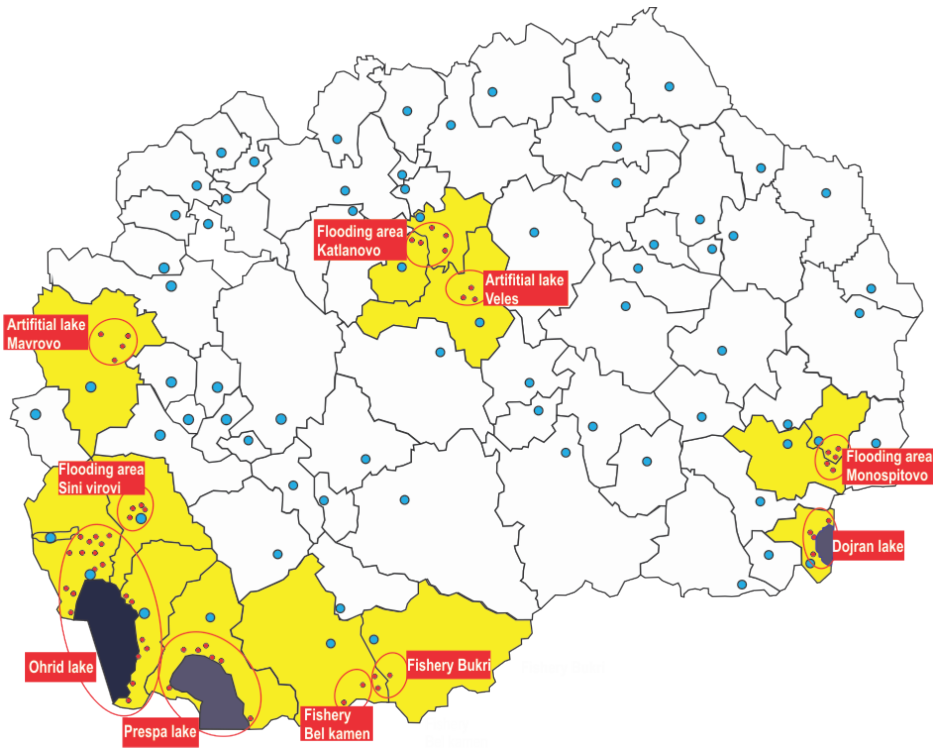

Figure 1: Location of the sampled villages in the risk areas of R. Macedonia

Table 2: Sampled poultry species

Poultry species	No of samples
Chickens	1847
Ducks	124
Geese	21
Turkeys	23
Guinea fowl	2
Pheasants	3

Table 3: Samples for daily routine diagnosis

Species	Sample	No of samples
Chickens (Holding with high rate mortality)	Carcass	12
Gulls, herons, mallard ducks (Unspecified number per each species)	Cloacal and tracheal swabs	37
Mallard ducks	Organs	11
Accipiter	Organs	2
White stork	Organs	1
Ostrich	Organs	2
Common quail	Carcass	2
Geese	Organs	1
Pigeons	Carcass	12
Chickens	Carcass	51
TOTAL		131

Results

The 2020 analyzed cloacal swabs gave negative result for the presence of AIV by both virological and molecular diagnosis.

The 131 analyzed samples for daily routine diagnosis also gave negative result for the presence of AIV by VI and PCR. Four samples (three from pigeons and one from poultry) were positive on avian paramyxovirus 1 (Newcastle disease virus).

Discussion

Until 2009, no molecular or virological surveillance was conducted to determine the presence and circulation of AIV i.e., no data were available for assessing the AI situation in R. Macedonia.

The research of Dodovski et al. [17] from September 2008 till March 2009, showed seropositive flocks by ELISA but negative on hemagglutination inhibition test for H5/H7, which may be due to circulation of non H5/H7 viruses. So, this fact imposed the question: If seropositive flocks are present - is there a circulation of the virus? Following this fact, Cvetkovikj et al. [18] in 2009 performed virological and molecular surveillance in the commercial poultry sector resulting with negative results for circulation of AIV.

According the 2009 surveillance there is no evidence of AIV subtypes circulating among backyard poultry population, which of course does not represent a constant result and imposes the need of further permanent surveillance.

So, what are the possible reasons for the negative results? There are several possible issues that may lead to this situation and are addressed below.

R. Macedonia is defined by the World Bank [19] as a country with low or moderate risk for AI outbreak, which contributes the fact that the surveillance yielded with negative result.

According to World Animal Health Information Database (WAHID) Interface [20] in 2009, in the Black Sea-Mediterranean flyway AI was reported only in 4 countries with 6 outbreaks - 3 for Low Pathogenic Notifiable Avian Influenza (LPNAI) and 3 for Highly Pathogenic Notifiable Avian Influenza (HPNAI). On the other hand, in 2008, 9 countries with total number of 141 outbreaks for HPNAI were reported and no LPNAI was reported. This fact shows the trend of decreasing prevalence of Notifiable Avian Influenza (NAI) in the countries belonging to the Black Sea - Mediterranean flyway.

The poultry sector 4 in R. Macedonia is defined as a sector with low biosecurity level and high risk for AI occurrence. In R. Macedonia the backyard poultry is reared mostly for egg production and kept in closed and covered fences, thus minimizing the possibility of contact with the wild birds or their faeces.

Besides that, R. Macedonia does not possess large water areas attractive for the migratory waterfowl – representing the potential danger of input and spread of AIV in poultry.

This surveillance was based on sampling cloacal swabs from backyard poultry. So, did the cloacal swabs influence the negative result?

For most bird species it is prudent to collect both tracheal/oropharingeal and cloacal swabs for testing, or only tracheal/oropharingeal when gallinaceous birds are subject of the surveillance. However, usage of cloacal swabs in surveillance of AI in backyard poultry is not uncommon.

Buscaglia et al. [21] in the surveillance in Argentina from 1998 to 2005, demonstrated no positive result in both serological and virological surveillance in backyard poultry using sera and 18.000 tracheal and cloacal swabs (only 10% tracheal swabs per year and 90% cloacal). After 2006, (isolation of H5N1 virus from mallard duck near Zagreb) Croatia has a regular monitoring for avian influenza in wild birds and poultry (especially in the backyard flocks using cloacal swabs). All samples were HPAI virus negative but LPAI viruses (H2N3, H3N8, H5N3 and H10N7) were isolated from wild birds [22]. Racnik et al. [23] also demonstrated negative result in backyard poultry using cloacal swabs as samples in the first Slovenian surveillance for avian influenza in migratory birds and backyard flocks.

However, to estimate the ecology of AIV in non commercial poultry, most surveillance strategies are based on sampling both tracheal and cloacal or only tracheal swabs.

Knowing the fact that gallinaceous birds typically shed AI viruses in respiratory secretions, a tracheal or oropharyngeal swab is the primary source of virus detection from chickens and turkeys. As pointed in the Diagnostic manual for avian influenza (2006/437/EC), for investigation of a holding suspected of being infected with the AI virus, the standard set of samples for virological testing is a combination of cloacal and tracheal/oropharyngeal swabs.

This point could easily take us to the answer that maybe the negative result was influenced by sampling only cloacal and not both cloacal and tracheal/oropharyngeal swabs.

Avian influenza has had an enormous economic and social impact on affected countries and the risk of this animal disease situation evolving into a global human influenza pandemic is of great public concern. Therefore, it is of interest of all countries, developed or developing, to invest in the containment of AI because there is continuing risk of AI spreading that no poultry-keeping country can afford to ignore. [24]

The establishment of efficient preparedness and control of AI has to be substantiated by activities at national, regional and global level [24].

1. At national level, each state has to strengthen Veterinary Public Health Services to better manage national surveillance systems, to improve early detection and response, define strategies and monitor their implementation. Likewise, disease prevention and control programmes have to be implemented including stamping out, biosecurity and vaccination as appropriate as well as developing and implementing better diagnostic tools.
2. At regional level, activities should be aimed to strengthen regional co-operation for harmonization of the surveillance and control strategies as well as promoting the regional diagnostic and surveillance networks etc.
3. Globally, the activities are aimed at donor support to provide the scientific and technical advice required by countries.

In this context, R. Macedonia needs to implement surveillance programmes mostly aiming on strengthening the national preparedness plan that can only be efficient with collaboration of veterinary services, national reference laboratory, ornithologists, etc.

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NI DOKAZOV O OBSTOJU VIRUSA PTIČJE GRIPE PRI DVORIŠČNI PERUTNINI NA OGROŽENIH OBMOČJIH V R. MAKEDONIJI (Virološki in molekularni nadzor 2009)

I. Cvetkovikj, A. Dodovski, I. Naletoski, S. Mrenoski, D. Mitrov, K. Krstevski, I. Dzadzovski, A. Cvetkovikj

Povzetek: Do leta 2009 nimamo podatkov o razširjenosti virusa ptičje gripe (AIV) pri dvoriščni perutnini v Republiki Makedoniji. V skladu z nacionalnim letnim programom nadzora AI in odločbe Komisije 2007/268/ES je bil v letu 2009 izveden virološki in molekularni nadzor dvoriščne perutnine na ogroženih območjih države. Skupno smo analizirali 2151 vzorcev. Dva tisoč in dvajset kloakalnih brisov (vzorci dvoriščne perutnine perutninskega sektorja 4 iz ogroženih območij v Republiki Makedoniji, med njimi iz območij, kjer so zbirališča vodnih ptic selivk) in 131 organskih/kloakalnih brisov (za vsakodnevno rutinsko diagnostiko) je bilo testiranih z virološkimi in molekularnimi metodami. Virološka diagnoza je bila izvedena z izolacijo oplojenih kokošjih jajc, kateri je sledila makrohemaglutinacija, nato hemaglutinacija na mikrotiterski plošči in zaviranje hemaglutinacije. Molekularna diagnostika je bila izvedena z RRT-PCR za M-gen. Vsi vzorci so bili AIV negativni, medtem ko so bili 4 vzorci pozitivni na ptičji paramiksovirus 1 (virus Newcastle). Divje ptice kot primarni gostitelj AIV niso bile vključene v nadzor, kar ne zagotavlja popolne informacije o razširjenosti AIV v R. Makedoniji in jo je treba še dodatno raziskati. To pomeni, da se je treba lotiti izdelave koncepta nacionalnih ukrepov za zgodnje odkrivanje in odziv ob pojavu ptičje gripe.

Ključne besede: virus ptičje gripe; dvoriščna perutnina; kloakalni bris; R. Makedonija; nadzor