



Original Contribution

THE ROLE OF AVIAN METAPNEUMOVIRUS IN RESPIRATORY COMPLEX DISEASE CIRCULATING IN BROILERS IN NORTHERN IRAN

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ABSTRACT

Avian metapneumovirus (aMPV) is the etiologic agent of a respiratory disease of poultry. In a 6 months period from October 2012 to March 2013, broiler flocks with respiratory signs are examined in northern Iran. The purpose of the given research was to define presence of antibodies of the birds' avian metapneumovirus among broilers and detection of virus. Birds were not vaccinated against this virus. Total 525 blood samples and trachea/nasal turbinates swabs were obtained from 35 commercial broiler flocks and pooled (5 swabs/pool), then RT-PCR method was done on these 105 pooled samples. Presence of antibodies against the bird's metapneumovirus in each serum sample has been tested by method of immune-enzyme analysis. 10 (28.5%) flocks had positive antibodies. Of the 35 flocks, 8 were positive by RT-PCR with Nd/Nx primers (23%). This is the first report of APV infection in northern Iran. Vaccination programs should be adjusted to include the APV vaccine to aid in the control of this respiratory disease in the Iranian poultry industry.

Key words: Avian metapneumovirus (aMPV), Broilers, Respiratory complex, Iran.

INTRODUCTION

Infections of the respiratory tract have significant economical impact on poultry production worldwide. Various pathogens have been known as causing respiratory diseases, acting either in a primary or secondary role. Avian metapneumovirus (aMPV) is a respiratory virus that infects a range of avian hosts, including chickens and turkeys. aMPV is a member of the subfamily *Pneumovirinae* under the family *Paramyxoviridae* (1). Based on genetic and antigenic properties, aMPV can be classified into 4 subgroups: A, B, C, and D. While subtype A and B viruses are mainly prevalent in Europe (2, 3), Asian countries (4), subtype C is most prevalent in the United States (5). An additional subtype D has been first reported in France (6). aMPV causes turkey rhinotracheitis (TRT), an acute respiratory tract infection in turkeys of all ages. The virus is also associated with swollen head syndrome (SHS) in

broilers and broiler breeders (7) and egg production losses in layers (8). In recent years, outbreaks of respiratory disease have been increased in poultry farms in Iran. Clinical signs of disease are including tracheal rales, nasal and ocular discharge, and swelling of the face and head which followed by high mortality. Studies showed that viral and bacterial factors are involved in causing of this condition (9-11), thus, clinicians have used the term of "respiratory complex" for this condition. The aim of this study was to investigate of aMPV in broilers farms with respiratory distress.

MATERIAL AND METHODS

SAMPLE COLLECTION

Thirty-five commercial broiler flocks suffering from respiratory disease, in the north of Iran, were examined over a 6 months period from October 2012 to March 2013. Fifteen serum samples and trachea/nasal turbinates swabs were obtained from birds per flock and pooled (5 serums/tube and 5 swabs/pool). Swabs and serums were stored in cool boxes at 4 °C and transported to the laboratory within two days.

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All the flocks were between 5-7 wks of age and were not vaccinated against aMPV.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Specific aMPV-antibodies in serum samples were detected by a commercially available enzyme-linked immunosorbent assay (ELISA) system (Avian Rhinotracheitis Antibody Test Kit[®], CK 120, BioChek, Netherlands) according to the manufacturer's recommendation. Briefly, samples were diluted in a 1/500 ratio and the OD was measured on an ELISA microplate reader (SFRI IRE96, France) at 405 nm. Results were determined by calculating the sample to positive (S/P) ratio. Samples with S/P ratios of 0.5 or greater (titres higher than 1656) are considered as aMPV antibody positive.

DETECTION OF THE aMPV GENOME BY RT-PCR

RNA EXTRACTION

Extraction of RNA was performed on the pooled material for swabs from each flock with RNX[™]-Plus reagent (CinnaGen, Iran) according to the manufacturer's procedure. Swabs were placed in PBS and were scraped on the side of the tube to facilitate removal of contents from the swab head. 1 ml of RNX solution was added to 100 mg of each pooled swabs contents, then 200µl of chloroform were added to the mixture. After centrifuging the samples at 12000 rpm for 15 min., the aqueous phase was transferred to another tube. RNA was precipitated with addition of equal volume of isopropanol. After washing the sediment with 75% ethanol, it was eluted in 50 µl of distilled water and stored at -70 °C until used.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

The reverse transcription (RT) reaction was performed with RevertAid[™] First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to the manufacturer's recommendation. Then the PCR was performed using primers, Nd (5'-AGC AGG ATG GAG AGC CTC TTT G-3') and Nx (5'-CAT GGC CCA TTA ACA TGT T-3'). PCR was performed to amplify a 115 bp fragment of the N protein gene of the aMPV, using the AccuPower PCR PreMix kit (BioNeer, South Korea) containing Taq DNA polymerase 1 µL, each dNTP(dATP, dCTP, dGTP, dTTP) 250 µM, Tris-HCl (Ph:9.0) 10 mM, KCl 40 mM and MgCl₂ 1.5 mM. Amplification was performed using Eppendorf thermal cycler for 35 cycles of pre denaturation at 94 °C for 15 min, denaturation at 94 °C for 20 sec., annealing at 51 °C for 45 sec., extension at 72 °C for 45 seconds and finally at 72 °C was performed with a 10-minute final extension phases. The products were analyzed in a 1% agarose gel containing ethidium bromide, using an ultraviolet transilluminator.

RESULTS AND DISCUSSION

ELISA was used to detect seroprevalence to avian metapneumovirus in 525 sera collected from 35 broiler flocks aged 5-7 weeks. Results showed that 28.5% of the tested samples contained APV antibodies, while 71.5% were negative (**Table 1**). The RT-PCR technique amplified the 115 bp fragment from clinical samples. Of the 35 flocks, 8 were positive by RT-PCR with Nd/Nx primers (23%). positive control resulted in the expected fragment, whereas no amplification was found in the reaction corresponding to the negative control (**Figure 1**).

Table 1. Avian metapneumovirus ELISA results in broiler flocks (Northern Iran)

Number	Negative	Positive
35	25 (71.5%)	10 (28.5%)

The clinical diseases that may result from avian metapneumovirus (aMPV) infections of turkeys or chickens have been termed turkey rhinotracheitis (TRT), avian pneumovirus infection of turkeys (APV), swollen head syndrome (SHS), and avian rhinotracheitis (ART), based on clinical signs and lesions. However, these clinical signs and lesions are not specific for aMPV infections and can be

confused with disease resulting from mono- or co-infections with other respiratory pathogens.

Nevertheless, it is now universally accepted that the conditions referred to as TRT, SHS, or ART can occur as a result of infection with aMPV. The more severe form of associated disease probably results from dual or secondary infection with other organisms, and for SHS, the

characteristic swollen head appears as a result of co-infection with secondary adventitious bacteria, usually *Escherichia coli* (1). For the first time in northern regions of Iran, detection of aMPV and investigation of its specific antibodies in broiler farms concerning respiratory distress has been revealed. Because all studied flocks were between 5-7 weeks of age at sampling time

and none was vaccinated, results suggest field exposure of these flocks to aMPV and exclude the possibility that the detected antibodies were due to maternal antibodies or vaccination. These findings confirm that aMPV is endemic in Iranian broiler flocks. Seroprevalence of APV has been found in other countries in the region (12-14).



Figure 1. RT-PCR of Iranian strains of aMPV. lane 1, negative control; lanes 2, 3 and 4 positive field strains; lane 5, negative samples; lane 6, positive control (vaccine strain); M, molecular size maker (100 bp)

The results showed that 23% broiler flocks were serologically positive for aMPV, which supports previous researchers' findings in Iran (15, 16). Although no bacterial examination was done on positive samples, but it seems that aMPV is responsible for these symptoms. On the other hand, role of environmental factors and bacterial infections are very important in severity of diseases condition.

In 2007, researchers have attempted to examine the aMPV in commercial laying flocks with no history of vaccination. All positive samples were examined for of infectious bronchitis virus (IBV), Laryngotracheitis (LT), Newcastle disease (ND) and *Mycoplasma gallisepticum* (MG) studied and only aMPV was detected (17). The aMPV infection is associated with high morbidity, and mortality may rise to 50 percent with the involvement of secondary agents such as *Mycoplasma* species, *Bordetella avium* and *Ornithobacterium rhinotracheale* (18).

According to the previous studies, detection of anti-APV antibodies among broilers was significantly lower than among layer and broiler breeder flocks. This may be due to the short life span of broiler flocks and the time of blood sample collection, which was at the acute stage of the respiratory disease (14, 16).

The signs and lesions observed in the field and the detection of aMPV by RT-PCR, suggest that aMPV might have been responsible for the disease outbreaks. However, as the clinical signs observed could have been produced by other pathogens, differential diagnosis with other respiratory agents was performed (data not shown). NDV and avian influenza virus (AIV) were not detected in any of the flocks. According to results, we suggest that the aMPV detected in this study might have been responsible for the respiratory disease in these flocks. Of course, as mentioned, some other factors such as IBV and MG can contribute to create of clinical symptoms.

Homayounfar et al. (15) in a study to detection of aMPV in poultry farms in northwest Iran reported that the nucleotide sequence of the gene G indicates that the positive samples belonged to the subtype B of virus. Phylogenetic tree evaluation of Iranian aMPV field strains and vaccinal strain showed that they are located in separated branches. Gharaibeh and Algharaibeh (14) and Banet Noach et al. (19), showed APV subtype B is circulating in Jordanian and Israeli poultry, respectively. It seems that serotype B is dominant in Middle East.

In chickens, the role of APV as a primary pathogen is less well established, although the

virus is commonly associated with SHS. APV infection is more severe when accompanied by secondary bacterial infection, as the virus tends to invade more internal organs and the virus titers are higher. The rate of spread and virulence vary greatly, but simultaneous infections with *Pasteurella* spp., *Bordetella avium*, *E. coli*, Newcastle disease virus or *Ornithobacterium rhinotracheale* exacerbates disease (20, 21). Loss of cilia on the epithelial surface of the upper respiratory tract can be associated with other infectious agents and may enhance infection with avian metapneumo virus, allowing deeper penetration of the virus into the respiratory tract (22). Experimentally, infected birds often show recognizable signs of rhinotracheitis, but these are milder than those seen in the field (23). Chickens show, at most, only mild respiratory disease in laboratory infections and nasal mucus may often only be discernible after light squeezing behind the nostrils. Presumably, the difference in pathogenicity between laboratory and field infections is related to the conditions under which the birds are kept and the presence or absence of exacerbative organisms. In laboratory studies concurrent infection of turkey poults with aMPV and respiratory bacteria, such as *E. coli*, *Bordetella avium* and *Riemerella anatipestifer* (22, 24) and low virulent (lentogenic) Newcastle disease virus (25), significantly exacerbate and prolong clinical signs and morbidity.

CONCLUSIONS

Further studies such as virus isolation and molecular techniques in order to characterization of current subtypes in the field are useful. These data will suggest review of the vaccination against APV in broiler flocks in Iran.

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