

Isolation of H9N2 Subtype of Avian Influenza Viruses during an Outbreak in Chickens in Iran

Mehdi Vasfi Marandi* and Mohammad Hassan Bozorgmehri Fard

Poultry Diseases Section, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

ABSTRACT

Avian influenza is an important disease of poultry with the potential to cause major epidemics resulting in significant economic losses. The presence of avian influenza viruses (AIV) in chickens in Iran has not been previously reported. An avian influenza outbreak in broiler, layer and breeder farms occurred during a very hot summer in July 1998. Three AIV isolates designated as 101, 102 and 103 were isolated from lung, tracheal cloacal samples of layer, breeder and broiler chickens, in embryonated chicken eggs. The presence of AIV in allantoic fluid cells was confirmed by indirect immunofluorescence assay using a monoclonal antibody against type A nucleoprotein. The viruses were further classified as H9N2 subtype in hemagglutination inhibition and neuraminidase inhibition tests using 15 hemagglutinin and 9 neuraminidase subtype specific antisera. The pathogenicity of AIV isolates was carried out in 4-6-wk-old chickens. No birds died within 10 days after inoculation of infectious allantoic fluids. Therefore, the representative Iranian layer, breeder and broiler AIV strains were classified as non-highly pathogenic avian influenza virus pathotype. Isolation of the same subtype and pathotype of AIV from different flocks suggested that the H9N2 AIV subtype is a common pathogen involved in poultry industry respiratory disease outbreak. *Iran. Biomed. J.* 6 (1): 13-17, 2002

Keywords: Avian influenza, Avian influenza virus, H9N2 subtype, Pathotype

INTRODUCTION

Influenza viruses (IV) belong to the *Orthomyxoviridae* family and to the genus *Influenza virus*. These viruses are classified into three types A, B and C, on the basis of their internal nucleoprotein and matrix protein antigens. These two antigens are regarded as common antigens to all strain of IV of the same type. IV is further categorized into subtypes, according to their surface hemagglutinin (H) and neuraminidase (N) glycoproteins [1]. Avian influenza viruses (AIV) belong to type A, and 15 H subtypes (H1 to H15) and 9 N subtypes (N1 to N9) have been reported [2, 3]. Based on the pathogenicity of AIV to domestic poultry, these viruses are sub-classified into two pathotypes of highly pathogenic avian influenza (HPAI) and non-highly pathogenic avian influenza (nHPAI) viruses including mildly pathogenic, low pathogenic and non pathogenic AIV [4]. Influenza A viruses of subtype H9N2 and H5N1 have recently been isolated respectively from children with influenza-like symptoms and from those died from

severe influenza disease. These two subtypes are now considered as zoonosis subtypes of IV [5, 6, 7].

Avian influenza is important poultry's disease with the potential to cause major epidemics resulting in significant economic losses worldwide. To date, the HPAI viruses that produce acute clinical disease "fowl plague", have been associated only with H5 and H7 subtypes. These viruses are listed as a group disease by the Office of International des Epizooties [4, 8]. In chickens, AI due to nHPAI viruses is most frequently subclinical disease but can be associated with mild clinical manifestations including respiratory diseases, decrease in egg production, diarrhea and renal syndromes in field outbreaks [9, 10]. Although viral pathogenicity is a major determinant of the severity of AI disease, factors such as age, species, environmental and management conditions and concurrent infections with other pathogens also may increase the severity of nHPAI [3, 9, 11]. Wild birds have been incriminated in a number of outbreaks of nHPAI in domestic poultry. However, no reservoir has been identified for HPAI viruses [12].

*Corresponding Author; Fax: (98-21) 693 3222; Email: mvmarand@chamran.ut.ac.ir

Influenza A viruses of subtype H9N2 are now considered to be widespread in poultry [13]. The existence of avian influenza specific antibodies in sera obtained from turkey flocks in Iran has been demonstrated by Samadieh *et al.* [14]. However, the presence of AIV in chickens has not been previously reported. A respiratory disease outbreak was occurred during a very hot summer (32-42° C), in a layer farm (Tehran) in July 1998. The 32-wk-old layer flock was vaccinated against main respiratory diseases including Newcastle disease (ND), infectious bronchitis (IB) and infectious laryngotracheitis (ILT). This infected flock had a decrease in feed consumption, and egg production with mortality. The similar respiratory disease outbreaks were observed in 6-wk-old broiler and 38-wk-old breeder flocks located in Tehran and Qazvin provinces, respectively. This study describes the isolation and identification of H9N2 subtype of AIV from layer, breeder and broiler flocks in Iran.

MATERIALS AND METHODS

Serum and tissue sample collection. For virus isolation 105 tissue samples including tracheal, lung, liver, spleen, kidney and brain as well as cloacal swabs were collected at necropsy or from chickens submitted to the Poultry Clinic of the Faculty of Veterinary Medicine, University of Tehran. Blood samples (300) from three layer, breeder and broiler flocks were collected after acute phase of disease with one week interval until 6 weeks. In each blood sampling times, about 15-20 blood were prepared and their related sera were kept in a separate vial and stored at -20°C for antibody titration by hemagglutination inhibition (HI) test.

The HI test for antibody titration. The HI test was done using 96 'U'-well microtiter plates, doubling dilution in PBS, 1 % v/v red blood cells (RBC) and 4 HA units of AIV antigen or 8 NDV antigen in 25 µl amounts as described previously by Manvel *et al.* [15].

Virus isolation in embryonated chicken eggs (ECE). Tracheal, lung, liver, kidney, spleen, brain and cloacal swabs sampled were pooled in batches of five into antibiotic solution and homogenized as described by Swayne *et al.* [16]. For each suspension, five 9 to 11-day-old ECE (Behparvar Co., Iran) were inoculated with 0.2 ml of the suspension into the allantoic cavity and candled

daily. All ECE with dead or dying embryos were chilled and harvested. Allantoic fluids were tested for hem-agglutination activity and surviving ECE were chilled on day 7 and tested for HA activity.

Indirect Immunofluorescence assay (IIF). The detection of type A influenza nucleoprotein in IIF assay was carried out according to the procedure described by Shalaby *et al.* [10] on embryo cells fixed on the slides [10]. The primary antibody was a monoclonal antibody (mAb) against type A influenza virus nucleoprotein (supplied by Dr. T. Mochtari, Faculty of Health, Tehran University of Medical Sciences, Iran). The second antibody was a goat anti-mouse FITC IgG antibody (Sigma). A mAb against avian reovirus was used as negative control.

The HI and NI tests for subtyping. The HI and NI tests for H and N subtyping of AIV isolates were carried out kindly by Dr. Alexander at Central Veterinary Laboratory (OIE and FAO, AI International Reference Laboratory; Weybridge, UK), using 15 H and 9 N subtype specific antisera.

Intravenous pathogenicity test for pathotype detection of isolates. One-day-old chickens (Behparvar Co., Iran) were housed in controlled-access laboratory animal rooms. The pathogenicity of the three challenge viruses strains (ZMT-101, 102 and 103) was carried out in 4-6 wk-old chickens by the standard intravenous pathogenicity test described by Swayne *et al.* [16]. For each strain, eight chickens were inoculated with 0.2 ml of 1:10 dilution of bacteria-free first infectious allantoic stock virus (Log 2, 9 HAU). Chickens were observed daily for signs of illness until 10 days post inoculation.

RESULTS

The HI test for antibody titration of flocks. In HI test, antibodies against ZMT-101 strain of AIV antigen were increased from zero to 9 log 2 titers in sera prepared at 1, 2, 3, 4, 5 and 6 weeks after acute phase of disease occurred in 32-wk-old layer and 38-wk-old breeder flocks; Whereas HI antibodies against La Sota strain of ND virus antigen were not increased (Fig. 1).

Virus isolation in ECE. All inoculated ECE with tracheal, lung and swab samples of 32-wk-old layer flock survived within seven days. Whereas; those of

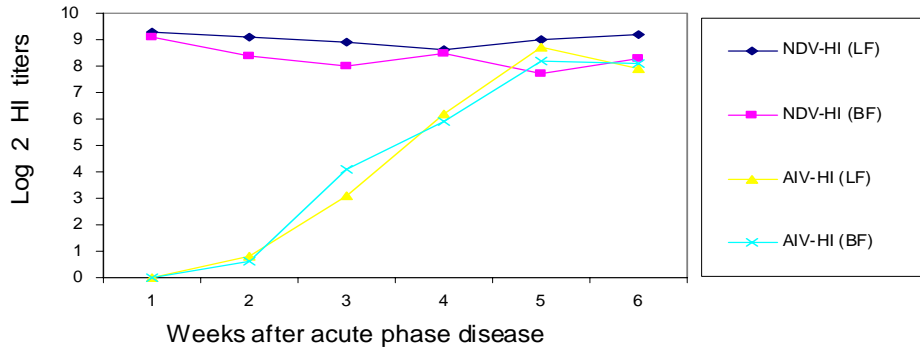


Fig. 1. This figure shows the mean of H9N2 avian influenza virus (AIV) and Newcastle disease virus (NDV) hemagglutination inhibition (HI) antibodies titers in 32-week-old layer flock (LF) and 38-week old breeder flock after 1, 2, 3, 4, 5 and 6 weeks of acute phase of avian influenza (AI) disease.

6-wk-old broiler and 38-wk-old breeder flocks died within 2-3 days. The ECE inoculated with liver, spleen, kidney, brain tissue samples prepared from layer, breeder and broiler flocks survived within 7 days. The harvested allantoic fluids from ECE inoculated with lung, tracheal and swab samples of all three flocks agglutinated chicken's RBC and their HA activities were inhibited by the sera obtained from infected flocks but not by specific antiserum against NDV. The harvested allantoic fluids from ECE inoculated with liver, spleen, kidney and brain tissue samples of layer, breeder and broiler flocks were unable to agglutinate chicken's RBC.

Type, subtype and pathotype detection of AIV isolates. In IIF assay, a type A specific mAb recognized embryo cells prepared from allantoic fluids, showing HA activities (Fig. 2). All isolates from layer, breeder and broiler flocks were belonged to H9N2 subtype, in HI and NI tests. After intravenous inoculation of the infectious allantoic fluid of H9N2 isolates in 4-6-wk-old chickens, the feed consumption was decreased. The chickens were depressed for 3-4 days. Sneezing and rales were also observed in some birds. However, no chickens died with 10 days after inoculation (Table 1).

DISCUSSION

Samadieh *et al.* [14], demonstrated the presence of type A specific antibodies of AIV in back yard turkey sera samples in agar gel precipitation test, but not in chicken serum samples. They failed to isolate the AIV isolates from both chicken and

turkey flocks in Iran [14]. Aghakhan *et al.* [17] used three different H subtype antigens in HI test in order to demonstrate H subtype specific antibodies in sera prepared from commercial chicken flocks. A considerable number of the sera tested, showed low to moderate HI titers. However, the same sera showed negative reaction in HI test when the sera treated with the receptor destroying enzyme. Besides, no positive reaction was observed in AGP test. According to the sensitivity and specificity of the test used and their AI survey between 1974-1994, they suggested that AIV were not exit in Iran.



Fig. 2. The reactivity of type A influenza nucleoprotein monoclonal antibody with embryo cells presenting avian influenza virus antigens. The goat anti-mouse FITC IgG antibodies were used to visualize AIV antigen in indirect Immunofluorescence assay.

Based on the results obtained in this study such as (i) isolation of viruses with HA activity from tracheal, lung and cloacal swab samples of birds from layer, breeder and broiler flocks with clinical

Table 1. Isolation and identification of avian influenza viruses (AIV) from layer, breeder and broiler farms following avian influenza outbreak in Iran in 1998.

Strains	Province	Farm	Sample	Type ^a	Subtype ^b	Pathotype ^c
ZMT-101	Tehran	Layer	Trachea	A	H9N2	NHPAI (LP)
ZMT-102	Tehran	Breeder	Trachea	A	H9N2	NHPAI (LP)
ZMT-103	Qazvin	Broiler	Lung	A	H9N2	NHPAI (LP)

(a), type of isolated viruses was detected by indirect Immunofluorescence (IIF) assay using monoclonal antibody against type A nucleoprotein; (b), Subtype of isolated viruses was detected by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests using 15 H and 9 N subtypes specific antisera; (c), Pathotype of avian influenza viruses (AIV) was detected in 4-6-wk-old broiler chickens following intravenous inoculation of 1:10 dilution of allantoic fluids.

signs and gross lesions of AI disease, (ii) the reaction of type A specific mAb with embryo cells in IIF assay and (iii) increasing of HI antibodies titers of flocks against the isolated AIV, it is suggested that AIV have been involved in respiratory disease outbreak of Iran. Since avian influenza H and N subtype specific antisera were not available in our laboratory, the AIV isolates from various farms were sent to AI laboratory in UK for H and N subtyping. All the isolates were subtyped as H9N2. Based upon the recognized procedure of AIV nomenclature, the AIV strains from layer, breeder and broiler flocks have been respectively identified as A/Chicken/Iran/ZMT-101(101)/1998 (H9N2), A/Chicken/Iran/ZMT-102(102)/1998 (H9N2) and A/Chicken/Iran/ZMT-103(103)/1998 (H9N2). The birds inoculated separately with the three different AIV isolates did not die but temporally depressed. Therefore, representative Iranian H9N2 subtype AIV classified as low pathogenic or nHPAI pathotype.

Isolation of the homologue subtype and pathotype of AIV, from layer, breeder and broiler flocks as well as the detection of HI antibodies of H9 subtype from the same flocks, suggested that, this H9N2 subtype is a common pathogen implicated in respiratory disease outbreak. Attempts to isolate AIV from liver, kidney, spleen and brain were not successful, indicating that H9N2 isolates were unable to induce viraemia, in infected birds. Based on the discrepancies between the high mortality in the field and low pathogenicity of H9N2 strains in the laboratory conditions, it is suggested that exacerbating conditions and concurrent bacterial and viral infections caused sever mortality (to 80 %) and egg drop (to 75 %) in respiratory outbreak. Such discrepancy has been described in the Korea nHPAI outbreak due to H9N2 in 1996 and other nHPAI outbreaks [3, 9-11]. Attempts have been made to find the origin of 1998 outbreak, to detect

the rate of H9N2 infection and to develop an oil-based vaccine against H9N2 AI.

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REFERENCES

1. Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y. (1992) Evolution and ecology of influenza A viruses. *Microbiol. Rev.* 56: 152-179.
2. Webster, R.G. and Kawaoka, Y. (1988) Avian influenza. *Crit. Rev. Poult. Biol.* 1: 211-246.
3. Alexander, D.J. (1995) The epidemiology and control of avian influenza and Newcastle disease. *J. Comp. Patho.* 112: 105-126.
4. Swayne, D.E., Perdus, M.L., Garcia, M., Rivera-Cruz, R. and Brugh, M. (1997) Pathogenicity and diagnosis of H5N2 Mexican avian influenza viruses in chickens. *Avian Dis.* 41: 335-346.
5. De Jong, J.C., Claas, E.C., Osterhaus, A.D., Webster, R.G. and Lim, L.A. (1997) Pandemic warning? *Nature* 389: 554 (Letter).
6. Guan, Y., Shortridge, K.F., Krauss, S. and Webster, R.G. (1999) Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? *Proc. Natl. Acad. Sci.* 96: 9363-9367.
7. Subbarao, K. and Katz, J. (2000) Avian influenza viruses infecting humans. *Cell Mol. Life Sci.* 57: 1770-1784.
8. Zanella, A., Dall' Ara, P. and Martino, P.A. (2001) Avian influenza epidemic in Italy due to serovar H7N1. *Avian Dis.* 45: 257-261.

9. Davison, S., Benson, C.E., Ziegler, A.F. and Eckroade, R.J. (1999) Evaluation of disinfectants with the addition of anti-freezing compounds against nonpathogenic H7N2 avian influenza virus. *Avian Dis.* 43: 533-537.
10. Shalaby, A.A., Slemons, R.D. and Swayne D.E. (1994) Pathological studies of A/chicken/ Alabama/ 7395/75 (H4N8) influenza virus in specific-pathogen-free laying hens. *Avian Dis.* 38: 22-32.
11. Brugh, M. and Beard, C.W. (1986) Influence of dietary calcium stress on lethality of avian influenza viruses for laying chickens. *Avian Dis.* 30: 672-678.
12. Alexander, D.J. (2000) A review of avian influenza in different bird species. *Vet. Microbiol.* 74: 3-13.
13. Peiris, M., Yam, W.C., Chan, K.H., Ghose, P. and Shortridge, K.F. (1999) Influenza A H9N2: Aspects of laboratory diagnosis. *J. Clin. Microbiol.* 37: 3426-3427.
14. Samadieh, B., Kargar Moaakhar, R. and Afnan, M. (1975) Demonstration of avian influenza A virus in Iran by immunodiffusion technique. *Avian Dis.* 19: 689-691.
15. Manvel, R.J., Jorgensen, P.H., Nielsen, O.L. and Alexander, D.J. (1998) Experimental assessment of the pathogenicity of two avian influenza A H5 viruses in ostrich chicks (*Struthio camelus*) and chickens. *Avian Pathol.* 27: 400-404.
16. Swayne, D.E., Senne, D.A. and Beard, C.W. (1998) Avian influenza. In: *A laboratory manual for the isolation and identification of avian pathogens.* (AAAP Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E. and Reed, W.M. eds.), Kendall-Hunt, Florida. Pp. 150-154.
17. Aghakhan, S.M., Abshar, N., Rasoul Nejad Fereidouni, S., Marunesi, C. and Khodashenas, M. (1994) Studies on avian viral infection in Iran. *Arch. Inst. Razi* 44/45: 1-10.