Isolation and Identification of Avian Influenza Virus H9N2 Subtype

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Summary

In August 1998, the avian influenza (AI) outbreak appeared in one of the layer farms in Tehran province. Birds on the affected farm exhibited respiratory infection and reduction of egg production with very low mortality. An agar gel precipitation (AGP) test using AIV antiserum revealed the presence of the virus in the samples from the affected birds. The sera collected from the surviving birds of the affected flocks also showed the presence of antibodies against AIV by AGP test. An avian influenza virus H9N2 subtype was isolated in embryonated eggs from the tracheal and clocal swabs as well as pooled visceral organs of the affected birds and coded A/chicken/Iran/259/1998/H9N2. Virulence testing of the low passage virus was undertaken at the time of the outbreak in specific pathogen free (SPF) chickens. The experimentally infected chickens showed significant clinical signs and recovered gradually without any death. The isolated virus was characterized as nHPAI on the basis of pathotyping studies in SPF chickens. It is the first report on isolation of AIV from the chicken flocks in Iran.

Key words: isolation, identification, avian influenza, H9N2 subtype

Introduction

Avian Influenza (AI) is a syndrome of poultry caused by type A influenza virus, with multiple manifestations ranging from respiratory signs to severe generalized septicemia. The disease assumes different epidemic forms in various poultry species. Highly pathogenic avian influenza (HPAI) takes the form of an acute hemorrhagic infection with extremely high mortality, causing massive death in chickens, turkeys,

etc., and LPAI is typically a respiratory infection with very low mortality resulting in no or very few deaths or only subclinical infection. AI is clinically characterized mainly by listlessness, anorexia, short-term reduction of egg production and hatchability, frontal sinusitis, diarrhea, and swollen heads (Jordan 1990, Easterday & Hinshaw 1991).

AI appeared first in Italy in 1878 and was historically termed Fowl Plague. Later it appeared in other European countries, Egypt, South America, parts of Southeast Asia, the United States of America, and the former USSR. Now the disease is distributed almost everywhere in the world. AI has a wide spectrum of infectivity and affects most species of wild birds or aquatic birds, but chicken, turkey and some species of wild birds are the most susceptible. Ducks, geese, and other waterbirds may have subclinical infection and shed the virus, but sometimes, losses may be great (Alexander 1993, Jordan 1990).

Influenza viruses belong to the family Orthomyxoviridae, and are classified into types A, B, and C on the basis of the antigenic character of their internal nucleoprotein (NP) and matrix protein. Avian influenza viruses (AIV) are belonging to type A and further subclassified on the basis of two surface glycoproteins, hemagglutinin and the neuraminidase. They have fifteen hemagglutinin (H) and nine neuraminidase(N) subtypes. No cross-reaction takes place between different H antigens or N antigens. The subtypes of AI have different pathogenicity to birds. Historically, H5 and H7 subtypes of AIV have caused outbreaks of HPAI. However, not all H5 and H7 subtypes are highly virulent strains (Yingjie 1997, Alexander 1993, Jordan 1990, Easterday & Hinshaw 1991, Bread 1989).

AI has been reported from other countries in the central Asia. In 1995 the outbreak of highly pathogenic avian virus subtype H7N3 occurred in Pakistan (Naeem & Hussain 1995). The flock-level morbidity rates ranged from 13.9 to 86.7% and within flock mortality ranged from 51 to 100%. Serological analysis has indicated the emergence of non-pathogenic subtypes out of the original highly pathogenic virus (Naeem 1997). The presence of AIV was identified by immunodiffusion in turkey sera in Iran, but isolation of the causative agent was failed (Samadieh *et al* 1975). In the present study, we described the isolation and identification of avian influenza virus H9N2 subtype for the first time, during the avian influenza outbreak in the chicken flocks in Iran.

Materials and Methods

Specimens. Live or dead birds were received from several layer and broiler farms in Tehran province. For isolation of AIV, specimens that included cloacal swab and/or tissues such as the lung, trachea, spleen, kidney and large intestine were placed in brain-heart infusion broth (Difco). Blood samples were taken and the sera were tested for AGP and HI antibodies against the virus.

Virus isolation and identification. Isolation of the virus was done according to Pearson *et al* (1992) description. A suspension of swab contents or tissues was inoculated into five 9- to 11-day-old SPF (Valo, Lohmann, Germany) chicken embryos via allantoic sac route. The allantoic fluid (AF) was collected from embryos that died between 24 and 96h of inoculation. The AF from dead as well as live embryos was tested for the presence of hemagglutinating virus by the hemagglutination (HA) test. A hemagglutinating virus was assayed by hemagglutination-inhibition (HI) test using an antiserum specific for Newcastle disease virus (NDV). If the hemagglutinating activity of the virus was not inhibited by the NDV-specific antiserum, the AIV HI test was performed using antiserum against each of the hemagglutinin subtypes of the AIV. Initially the isolated AI-virus was sent to the Central Veterinary Laboratory (CVL, Weybridge, Surrey, UK) for confirmation of serotyping and pathotyping.

Hemagglutination test. The test was performed by conventional technique in microtitre plates using 1% chicken erythrocytes and incubating at room temprature. Briefly, twofold dilutions of 25µl amounts of infectious AF were made in PBS, pH7.2, from initial dilutions covering a close rang. An equal volume of PBS was added to each well followed by a volume of 1% chicken red blood cells (RBCs). After gentle mixing the cells were allowed to settle for 45 minutes.

Electron microscope. The AF was examined by transmission electron microscopy after negative staining with sodium phosphotungstate acid as previously described with slight modifications (McFerran *et al* 1971). Briefly, a drop of the AF was placed on a 200-mesh Formvar-coated carbon electron microscope grid and then allowed to air dry. The grids were then floated on a drop of 4% (w/v) sodium phosphotungstate (pH7.2). After 1min, excess liquid was removed by touching the edge of the grids with a piece of filter paper. The grids were then allowed to air dry. Grids were examined on a Philips 400 electron microscope operating at 100-kV, 50-nA emission.

Haemolysis test. The AF was diluted (1:20) in 0.1M PBS (pH7.2). The diluted AF was mixed with an equal volume of 1% chicken RBCs. Controls of RBCs alone and mixed with a known influenza virus and a known NDV were included in this test. Mixtures were reacted for 1h at 37°C and the cells pelleted by gentle centrifugation and haemolytic activity was noted.

Serology. A total of 2536 sera were collected from chicken flocks in and around the affected area soon after the outbreak. Sera were initially tested for antibodies to AIV by the agar gel precipitation (AGP) and further tested by the HI test to determine subtype specificity.

Antisera. A panel of 8 antisera against various subtype of AIV (H1, H2, H4, H5, H6, H7, H9 and H10) employed in HI test and type A avian influenza specific positive serum for AGP test were obtained from CVL. The AIV antibody positive and negative sera were obtained from KPL (Kirkegaard and Perry Laboratories, OR Outside, USA) and used in AGP test.

Hemagglutination-inhibition test. The HI test was a standard beta test (Beard *et al* 1989), using four hemagglutinin units with the first dilution beginning 1:10. The antigen used for the HI tests was prepared from a-propiolactone inactivated (0.01%) (Sigma Chemical Co. St-Louis, USA) AF harvested from SPF emberyonated chicken eggs incubated with the AI H7 and H9 subtypes.

Agar gel precipitation test. The persence of type A nucleocapsid-specific antibodies was detected by AGP test described by Beard (1970). Briefly, the agar gel was prepared with 1% Noble agar (Difco Laboratories, Detroit. Michigan, USA) containing 8% NaCl in PBS (pH7.4, without calcium and magnesium). The mixture was autoclaved at 121°C for 5min, stored at room temperature, and melted again as needed. Ten ml of dissolved agar were poured into 100×15 mm Petri dishes. Wells were punched into the agar such that one central and six peripheral 5.3mm diameter wells were formed, 2.4mm apart. Four of them were filled with test serum samples and two were filled with known positive and negative serum. The central well was filled with AGP antigen. Precipitin lines of identity were recorded after 24 or 48h incubation at room temperature. The type A influenza specific antigen was purchased from SPAFAS (Norwhich, Connecticut, USA) and used in AGP test.

Experimental infection. Pathogenicity testing of isolates was conducted according to guidelines published by the Office International des Epizooties (OIE 1992). Isolates were tested for pathogenicity in chickens by inoculating 0.2ml of a 1:10 dilution of bacterial-free infectious egg AF intravenously into eight 4-week-old SFP chickens. All chickens were examined during an observation period of 10 days for signs of illness, disease and death after which specimens and blood samples were taken for viral isolation and serological examinations. Isolates that did not produce disease or that killed only 1 to 5 of 8 chickens were classified as low pathogenic. Isolates that killed 6, 7, or 8 of 8 chickens were classified as highly pathogenic.

Results

The clinical signs of chickens observed were depression, diarrhea, edema of the face and/or head, depression with cyanosis of comb, respiratory involvement, gasping, difficult breathing, tears from eyes and swelling of the sinuses. The range of decrease in egg production in infected flocks was around 30-70%, and the infected flocks returned to egg production within four weeks but they never reached the same level as before the infection. Examination of birds, which died following natural infections, have revealed lesions similar to those reported by other investigators. The most prominent lesions observed were swollen kidneys with urate deposit and atrophic and ruptured ova. Other lesions such as mild congestion of the trachea, mild catarrhal tracheitis and airsacculitis were also found but not significant. Based on the field reports, the disease was earlier confused with Newcastle disease (ND), fowl cholera and infectious laryngotracheitis (ILT), however, laboratory investigation nullified the presence of the diseases.

The sera collected from the surviving birds of the affected flocks also showed the presence of antibodies against AIV by AGP. Hemagglutinating viruses were isolated from the tracheal and cloacal swabs and also from pooled visceral organs of the affected birds using through *in ovo* inoculation. Hemagglutinating virus isolates were not neutralized by NDV positive serum but partially neutralized by positive serum of AIV. These isolated viruses were completely neutralized by antiserum against H9 but not H7 subtype. These isolated viruses were sent to CVL for both serotyping and pathogenicity test. All three viruses were confirmed as H9N2 subtype AIV and coded

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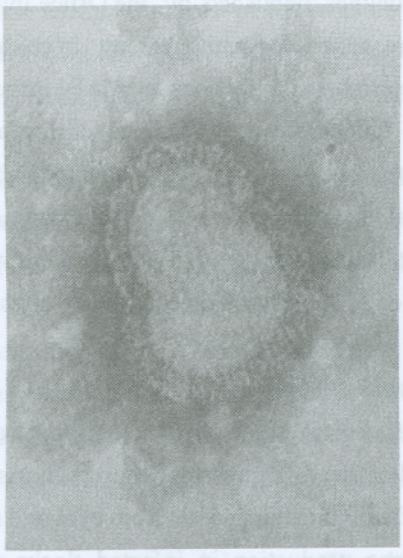

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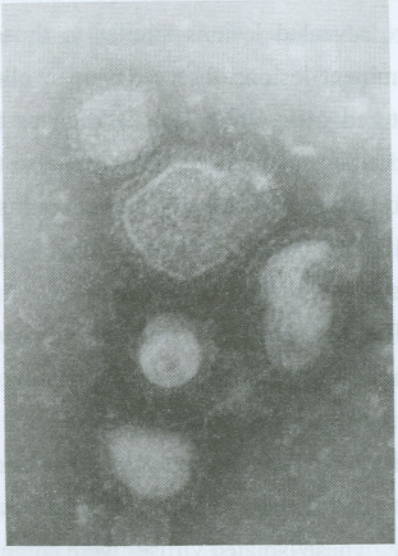

Figure 1. *Negative contrast electron microscopy of AIV isolated from a case of infected chicken. Morphology of influenza virus particles in allantoic fluid from inoculated SPF chicken embryos.*

as A/chicken/Iran/259/1998/H9N2. The isolated viruses were characterized as nHPAI on the basis of pathotyping studies in SPF chickens. The clinical signs of experimentally infected SPF chickens were depression, respiratory involvement, and tears from eyes and recovered gradually without any deaths. The experimentally infected chickens showed mainly respiratory lesions, mild congestion of the trachea and sometimes with tracheal edema. No virus was isolated from the specimens that collected from the chickens two weeks after infection. Also, all sera showed moderate to high titers in HI test and had positive reaction in AGP test. These isolates had very good immunogenicity and conformity with influenza virus in morphological features. The diameter of the viral particles ranged between 80-120nm under the electron microscope (Figure 1). Influenza virus causes lysis of RBCs at pH5.5, whereas ND virus causes lysis at pH7.2 and the supernatant was redder than in the control of RBCs. The isolated viruses caused lysis at pH 5.5.

A total of 1450 (73%) sera tested developed a single well-defined precipitin line and was AGP positive. Of 145 chicken serum samples obtained from one farm, 135 (93%) had precipitin antibodies against AGP antigen. All positive sera for AIV antibodies had high HI titer ranged from 4-10 $\log_2$ antibody to H9 subtype. Also, no HI titer was observed to H7 subtype.

Discussion

In this study, we have identified for the first time the presence of avian influenza virus H9N2 subtype in Iran by virus isolation, serological techniques, negative contrast electron microscopic examination, gross histopathological investigations during the recent outbreak of avian influenza in the chicken flocks in Tehran province. In late 1997 and early 1998, veterinarians and poultry farmers in many of the Tehran's poultry producing areas began to report respiratory problems assignable to ND, infectious coryza (IC), infectious bronchitis (IB), swollen head syndrome (SHS) and infectious laryngotracheitis (ILT). Based on the signs of diseased chickens, clinical findings, gross lesion and laboratory diagnosis, we have nullified the presence of ND, IC, IB, SHS, or ILT, however, the presence of AIV was confirmed in the chicken flocks in Tehran province.

The AI outbreak was first reported at layer farm with 10% mortality and severe drop of egg production. Many virological and serological studies were carried out to evaluate all the farms around the ones that was originally affected in order to determine how far the problem had spread, and biosecurity measures were reinforced in the farms, while a diagnosis of the situation was undertaken. The viruses isolated from three farms identified as non-highly pathogenic H9N2 AI virus and confirmed by CVL.

The experimentally infected chickens showed significant clinical signs and recovered gradually without any deaths. However, the virus induced mild influenza in layers, and eliciting severe drops of egg production. The mortality varied according to the bird's age, with a higher rate among young birds than in adults. Another influence on mortality was the level of circulating antibodies in the affected birds so, the higher antibody levels were seen in the lower mortality rate. Although, 20-60% mortality in broiler flocks were seen during the outbreak of AI, but the mortality was closely associated with the improper disease prevention practiced and was not entirely caused by AI. This is due to secondary infections and is related to the greater bird density, the poorer hygienic measures and air quality of confinement conditions. It should be pointed out that the number of AIV isolation and the number of pathogenicity tests were not the same as the number of viral isolation positive for AI. This is because many farmers sent more than one sample, and the virus was isolated in almost all of them. However, when pathogenicity tests were done only one sample was taken from each of these farms.

Investigations on the source of the outbreak were inconclusive, but again centered on movement of exotic birds. The extensive worldwide trade of the poultry meat and eggs, movement of exotic birds and long migration of wild birds represents international problem requiring extensive coordination for surveillance and control of AI. In contrast to the epidemiological links between turkeys and wild birds, outbreaks in chicken's flocks in Tehran province seem to be related to live bird markets. The epidemiological studies that related with AI outbreak in Iran should be undertaken to determine the source of the outbreak. Due to lack of biosecurity measures, the virus spread easily to many farms. To date, the presence of low pathogenicity AIV was confirmed in five provinces of Iran (data not shown). In order to cope with disease,

farms free of AI should be confirmed, diagnostic laboratories and veterinarians should be aweared for influenza and actions should be taken to promote biosecurity of farms, controlling poultry movements and poultry products. A mass vaccination as well as biosecurity program should be launched in and around the affected area, which eventually help to overcome the disease. It is suggested that only vaccination can not reduce the amount of virus circulating virus, biosecurity and other control measures are important in preventing spread of the virus.

In one study, a total of 1000 chicken serum samples (CSS) and 235 turkey serum samples (TSS) were tested by an immunodiffusion procedure against soluble antigen (S-antigen) prepared from avian influenza-A virus. None of the CSS tested developed any precipitin line, whereas 8.9% of the TSS tested developed well-defined precipitin lines against S-antigen (Samadieh *et al* 1975). In other study, a considerable number of the sera which covered a period about twenty years, showed low to moderate titers in HI test. However, these sera proved to be negative for specific antibodies following treatment with receptor destroying enzyme (RDE). Also, no positive reaction was observed in AGP tests (Aghakhan *et al* 1994). Although, the avian influenza was strongly suggested by the repeated demonstration of a single precipitin line between S-antigen and TSS, sign of diseased turkeys, the presence of AIV in Iran before this outbreak did not confirmed by isolation of the causative agent. In this study, the number of sera collected was very small in comparison to the population in Tehran province and, consequently, the level of infection would need to have been high for infected bird to be detected.

The only HPAI recorded during 1992-1997 was of an H7N3 subtype virus, which became widespread in chickens in Pakistan in 1995 (Naeem & Hussain 1995, Naeem 1997). The characterization of various field isolates by amino acid sequencing of HA cleavage site indicated some changes in the sequence over a period of four months. On the other hand, serological analysis has indicated the emergence of non-pathogenic subtypes out of the original highly pathogenic virus (Naeem 1997). In 1996, AI outbreak was reported in breeder broiler farm in Korea with 20-40% mortality and dramatic (80%) drops of egg production. The viruses isolated were identified as non-highly pathogenic H9N2 AI virus (Mo 1997). The range of mortality and decrease in egg production in infected flocks during the recent outbreak

of AI in Tehran province seem to be comparable to AI outbreak in Korea. Considering the antigenic variation among type A avian influenza viruses and the fact that it is rarely practicable to include a relatively large number of the recognized subtypes in a serological survey, two subtypes (H9, H7) were selected for the preparation of the antigens employed in HI tests. In our study, a considerable number of the sera showed moderate to high titers in HI test to H9 but not H7 subtype. Therefore, AI outbreaks in chicken's flocks in Tehran province do not seem to be related to AI outbreak in Pakistan.

The farmers and certain veterinarians did not agree with depopulation policy because the isolated viruses did not kill any SPF chickens in the laboratory pathogenicity test and the affected flock was gradually recovered in the mortality and egg production. Therefore, a control and surveillance system reflecting exact field situation such as mortality and egg drop is needed especially in the country with no experience of AI outbreak before. It is suggested that government authorities have to control the movements of poultry and poultry products from one infected province to another province free of AI.

Essentially the AI action plan focuses on three areas. 1) Early detection includes monitoring and surveillance; 2) rapid response includes quick definitive diagnosis, and 3) rapid control and eradication. Monitoring and surveillance are the keys to early detection. Serological surveillance for AIV of poultry and other birds is an essential part of early detection for prevention and eradication programs. Currently, routine surveillance and diagnosis rely on the AGP and the HI tests (Karen 1997). AGP and HI are the standard serological tests performed for the detection of specific serum antibody to AIV. AGP is performed for the detection of the group specific type A antibodies, whereas HI is performed for the detection of subtype specific antibodies. The AGP test has been used routinely in the USA for turkeys and chickens and has proven useful in confirming influenza infections when virus isolation has not been possible. Alexander and Allan (1982) compared the test against nucleocapsid antigen with HI titters to the known infecting viruses. They concluded that the AGP test was suitable for screening turkey's sera even when the H subtype was known, but that the correlation of positives and negatives in the two tests may vary with different infecting viruses. Some workers have used HI test for routine

diagnostic work, however, when a dominant H subtype is known to be present in the field, they may be useful in epizootiological studies (Alexander 1993).

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