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Short communication

SEROLOGICAL AND RT-PCR ASSAYS FOR DETECTION OF AVIAN INFLUENZA OF DOMESTIC PIGEONS IN KAVAR AREA (FARS PROVINCE, IRAN)

A. MOHAMMADI[1], M. MASOUDIAN[2], Y. NEMATI[3] & S. SEIFI[4]

[1]Department of Pathobiology; [2]Department of Biotechnology; [3]DVM Student; School of Veterinary Medicine, Shiraz University, Shiraz; [4]Department of Veterinary Science, Azad University of Chalus, Chalus; Iran

Summary

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Avian influenza (AI) is one of the most common and disastrous diseases in industrial poultry farms of Fars province, Iran. Based on the relatively high prevalence of the disease in Kavar area, 50 domestic pigeons were selected for serological analysis and virus shedding into the area. Blood and faecal samples were collected and evaluated using HI and RT-PCR methods respectively. The results showed that 17 serum samples (34%) had antibody titres $\geq 2^{-5}$ against the H_9N_2 AI virus, but the virus genome was not detected in any of faecal samples. Although the results demonstrated that a considerable percentage of domestic pigeons dwelling the Kavar area were seropositive for AIV, no evidence was provided for transmission of the virus from domestic pigeons to the poultry farms of Kavar.

Key words: avian influenza, HI test, pigeons, RT-PCR test

Viruses of orthomyxoviridae family include 5 distinct genera in which influenza virus A genus (type) is considered as a cause of the avian influenza disease. They are categorized to several subtypes, according to antigenic features of its surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). At present, 16 AH (H_1-H_{16}) and 9 NA (N_1-N_9) subtypes are recognized, and their combinations make different AIV subtypes (Alexander, 2000; 2007).

Up to now, all causes of peracute pandemic AI or fowl plague, have been recognized to be caused by H_5 and H_7 (Swayne & Halvorson, 2003).

According to recent investigations, AIV are isolated from 105 wild bird species belonging to 26 different families. However, the real number of virus-susceptible bird species is higher.

The H_9N_2 AIV is isolated from domestic fowls, ducks, geese, quails and pigeons (Bano *et al.*, 2002; Fang *et al.*, 2006). This serotype has spreaded among domestic birds throughout the world since 1990s. Coast waterfowl are considered to be a natural reservoir of H_9 viruses.

The H_9N_2 AIV causes respiratory pathology, reduced egg production, and occasionally mortality if accompanied with opportunistic or immunosuppressive pathogens (Chalmers, 2005). Experimental infections in specific pathogen-free chicks revealed that the virus is not able to cause pathological lesions and severe mortality by itself (Fang *et al.,* 2006). As pigeons are able to transfer the virus from one location to another by flying, and because of the frequent occurrence of the disease in industrial poultry farms in different areas of Iran such as Kavar, in this study we investigated seropositivity and virus shedding in local domestic pigeons in order to evaluate the potential of these birds to transmit AIV.

Fifty domestic pigeons from the Kavar area in Fars province, Iran were studied. In order to examine seropositivity, 1–2 mL blood samples were taken from each pigeon and the serum separated. The haemagglutination inhibition (HI) test was performed for antibody detection against H_9N_2 virus. Briefly, two-fold dilutions of heat treated (at 56 ^oC for 30 min) sera were made and 4HA avian influenza virus with equal volume (50 µl) of diluted sera was used in each well of V-type 96 well micro plate. After 40 min incubation at room temperature, 50 µl 1% chicken RBC was added and after 30 min incubation at room temperature, the last well which had a complete inhibition, was considered as the antibody titre. In order to evaluate virus shedding, faecal samples were taken and stored at –70 ^oC until used for RT-PCR test. Prior to RNA extraction, a w/v suspension of faecal samples was prepared in 10% PBS and after centrifugation at 3000 rpm for 5 min., 100 µL supernatant was transfered to another 1.5 mL tube. For RNA extraction, commercial RNA extraction kit was used (CinnaGen). Briefly, 1 mL of RNX solution was added to 100 µL of each supernatant. After 5 min incubation at room temperature, 200 µL of chloroform were added to the mixture centrifuged. RNA was precipitated with equal volume of isopropanol to the separated supernatant and centrifugation. After washing the RNA pellet with 75% ethanol, it was dissolved in 50 µL of sterile double distilled water and stored at –70^o C until used. For cDNA synthesis, random hexamer and AIMCD primers were used in Accupower RT premix kit (Bioneer, South Korea). Briefly, 5 µL of RNA template and 20 pmols of each primers (1 µL) were incubated at 70 ^oC for 5 min. Then the mixture was transferred to the 0.2 mL tube containing the lyophilized master mix provided in the kit and the tube was incubated at 42 ^oC for 1 h with the total volume of 20 µL. Inactivation of reverse transcriptase was performed at 94 ^oC for 5 min. PCR was performed using M protein genes primers (CN1, CN2) (Table 1) designed at the Laboratory of Virology, School of Veterinary Medicine, Shiraz University, Iran. Accupower PCR premix kit (Bioneer, South Korea) was subjected for PCR using 5 µL of cDNA and 20 pmol of the primers CN_1 and CN_2 with the total volume 20 µL. The reaction mixture was subjected to 94^o C for 5 min and 35 cycles of 94 ^oC for 35 s, 54.9 ^oC for 45 s and 72 ^oC for 45 s, followed by a final extension at 72 ^oC for 5 min.

Table 1. Primers used for RT-PCR

Primer	Nucleotide sequence
AIMCD	5' TCT AAC CGA GGT CGA AAC GTA 3'
CN1	5' GGG AAG AAC ACA GAT CTT GAG 3'
CN2	5' TGC TGG CTA GCA CCA TTC TC 3'

Table 2. Avian influenza antibody titres of blood sera of pigeons from Kavar (Fars province, Iran), tested in the HI test

Antibody titre	$<2^{-5}$	2^{-5}	2^{-6}	2^{-7}
Number (percentage)	33 (66%)	12 (24%)	2 (4%)	3 (6%)
Total number (percentage)	33 (66%)		17 (34%)	

Note: Sera with titres $\geq 2^{-5}$ were considered positive.

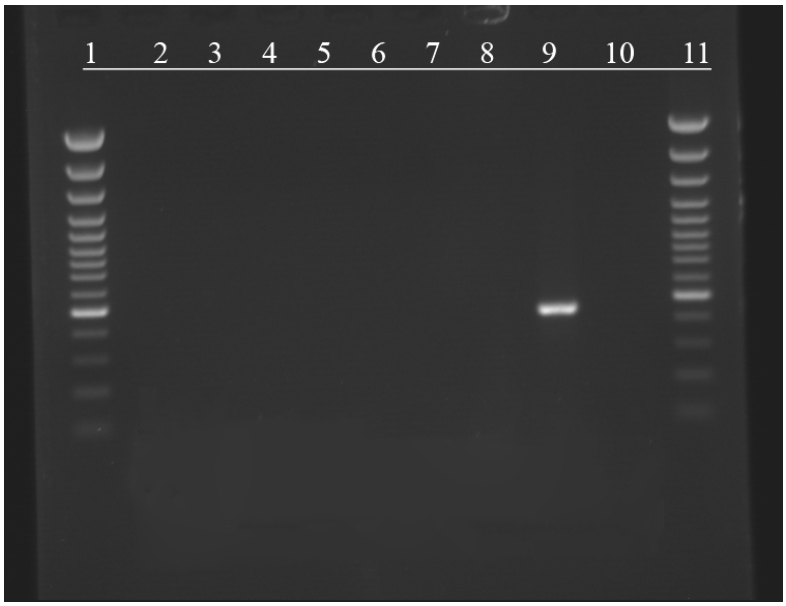

Fig. 1. Results of RT-PCR test of faecal samples. Column 1 and 11: 100 bp marker, columns 2–8: faecal samples, column 9: positive control; column 10: blank. PCR product size: 450 bp.

The products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide. A/chicken/Iran/772/ 1998/ (H_9N_2) virus was used as a positive control in the RT-PCR test and as antigen in the HI test.

Seventeen out of the 50 collected pigeon sera were positive for antibodies against H_9N_2 AIV in the HI test (Table 2). No evidence of AIV genome was detected in faecal samples by RT-PCR (Fig. 1).

Perkins & Swayne (2002) have investigated the susceptibility of 4 species including emus, domestic geese, domestic ducks and pigeons to the H_5N_1 virus and showed that pigeons were resistant to the virus, no macroscopic lesions were seen and virus isolation was not successful too. They concluded that probably pigeons had the least role in the epidemiology of H_5N_1 virus (Perkins & Swayne, 2002; Swayne & Halvorson, 2003). In another investigation (Panigrahy *et al.*, 1996), several groups of pigeons inoculated with 2HP or NHP influenza strains, remained healthy 21 days after inoculation and did not shed the virus. No antibody was detected in their sera, and it was therefore assumed

that pigeons were not involved in disease distribution. AIV was isolated from faecal samples of domestic fowls, ducks, quails and pigeons over a period of 16 months with the least isolation rates of H_9N_2 virus in pigeons (domestic fowls 1.3%, ducks 1.2%, quails 0.8% and pigeons 0.5%) (Liu *et al.*, 2003).

Kaleta & Hönicke (2004) reviewed fowl plague in pigeons and stated that infection of pigeons with H7 viruses resulted only in some of them in signs, virus shedding and seroconversion but pigeons appeared to be even less susceptible to infection with influenza A viruses of the H5 subtype. It was suggested that H7-infected pigeons could multiply and excrete H7 viruses and develop circulating antibodies.

Resistance to AIV infection was evaluated in healthy and immunosuppressed pigeons by Fang *et al.* (2006). Two subtypes of LPAIV was inoculated occularly and virus was detected by nested RT-PCR. Both groups (healthy and immunosuppressed) did not shed the virus and antibody presence test was negative for a period of 21 days. Samples from trachea, lung, pancreas, spleen, kidney and rectum were negative. Negative results were seen in domestic fowls in contact with them. Thus, pigeons were resistant to the two tested AIV and did not act as carriers of the viruses, even when their immune system was suppressed. Contrary to observed resistance of pigeons to AIV and the lack of virus in their excreta, a hypothesis is proposed that freely flying pigeons can act as mechanical carriers and transport the virus to far distances, if their plumage and feet are contaminated (Fang *et al.,* 2006).

During a H_5N_2 outbreak in north-eastern America in 1983–1984, Chalmers (2005) attempted to establish the potential for distribution of the disease by wild birds and rodents. Swabs were taken from trachea and cloaca of birds and also from lungs of mice and rats and evaluated for virus tracing. Concurrently samples were taken from nails of birds and rodents. Results showed that none of pigeon samples were positive and virus detection in pigeons' nails was not successful

In another investigation performed in Pakistan, AIV surveillance in wild birds was evaluated. It was shown that antibodies against H_9N_2 subtype of AIV was present in 10% of wild birds, while the virus was detected only in 6.72% of specimens (Khawaja *et al.*, 2005). It was also reported that the antibody titre against AIV (H_7 & H_9) was negative in doves; in addition no virus was isolated from body tissues (Khawaja *et al.*, 2005).

To conclude, the evidence obtained in the present study indicated that pigeons were least likely to transmit AIV to domestic birds in the investigated region in Iran.

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Correspondence:

A. Mohammadi
Department of Pathobiology
School of Veterinary Medicine,
Shiraz University, Shiraz, Iran
cell phone: 0098917 718 8264;
fax: 0098711 228 6940
e-mail: mohammad@shirazu.ac.ir