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The Effect of Egg-Derived Antibody on Prevention of Avian Influenza Subtype H₉N₂ in Layer Chicken

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Abstract: Avian Influenza (AI) is a contagious disease of poultry which caused by type A influenza virus. In the present outbreak of AI in Iran, the isolates (H₉N₂) were characterized as a low pathogenic form of the virus. Outbreaks due to H₉N₂ subtypes have been reported in many countries. Because the common methods of control of outbreaks of AI may not be very effective to prevent and control of this disease, we decided to study the effect of egg derived antibody on reducing morbidity and spread of virus in a population of chickens by using so-called transmission experiments. Birds in test group (which received antibody in drinking water) had lower morbidity ($p < 0.05$) and virus shedding compared to the control group. Our experiments demonstrate that administration of egg derived antibody (Ab) to chickens, not only may protect birds against this disease but is also an effective strategy to reduce transmission of AI virus. Therefore, egg derived specific antibody can be an attractive tool to prevent outbreaks of AI viruses in poultry, thereby achieving the aim of eliminating the source of human infections.

Key words: Avian influenza, antibody, egg, layer chicken

Introduction

Avian Influenza (AI) is caused by viruses of the influenza A genus of the family Orthomyxoviridae (Saif *et al.*, 2003). At present sixteen hemagglutinin subtype (H1-H16) and nine neuraminidase subtype (N1-N9) of influenza A viruses have been recognized (Britta *et al.*, 2005). Highly Pathogenic Avian Influenza (HPAI) is a viral disease of poultry caused by H5 or H7 AI A strains, with mortality that ranges up to 100%. The number of outbreaks in the last few years has been unprecedented: Hong Kong (Claas *et al.*, 1998), Italy (Capua and Marangon, 2000), Chile (Suarez *et al.*, 2004), the Netherlands (Stegeman *et al.*, 2004), Canada (Bowes *et al.*, 2004) and the continuing outbreaks in Southeast Asia (Li *et al.*, 2004). Aside from causing havoc in poultry, it is becoming an increasing concern that certain HPAI viruses have the potential to directly cross the human-bird species barrier and may become a pandemic threat (Li *et al.*, 2004; Fouchier *et al.*, 2004). In the present outbreak of H₉N₂ AI in Iran, the isolates were characterized in the laboratory as of low pathogenic (Vasfi Marandi and Bozorgmehrifard, 1999). While investigating the transfer of H5N1 influenza virus from chicken to man in the Hong Kong outbreak of 1997, it was revealed that high homology exists between the internal gene of H₉N₂ and H6N1 and furthermore, these subtypes were found to exchange their internal genes and are therefore a potential source of new pathogenic influenza virus strains capable of infecting humans (Zhou *et al.*, 1999). To reduce the primary risk of human HPAI infection, it is

crucial to prevent infection of poultry. Common methods to control outbreaks of HPAI are the depopulation of infected poultry, preemptive culling, biosecurity measures and vaccination.

An ideal way to control AI should reduce the spread of virus between animals in a flock and, subsequently, the spread of virus between flocks to such an extent that a major outbreak will not occur. We studied the effect of egg derived antibody on the spread of virus in a population of chickens by using so-called transmission experiments. The purpose of this research, was 1) Production and isolation of avian influenza antibodies from the egg yolk, 2) to determine if infection by avian influenza can be prevented in layer chicken using oral egg-derived (Ab) recovered from hyper immunized chickens.

Materials and Methods

Experiment 1

Animals: Twenty Commercial Single Comb White Leghorn chickens were divided in two groups. Birds in the test group were hyper immunized with 0.5 mL of an inactivated oil emulsion vaccine (H₉N₂) for four times at interval of 2 weeks in pectoral muscle (the vaccine was obtained from Razi Research Institute of Vaccine and Serum Production in Iran). The control group also received 0.5 mL of sterile PBS as the same way.

Hi assay: The antibody was tested by Hemagglutination Inhibition test (HI) in serum and egg yolk in both groups.

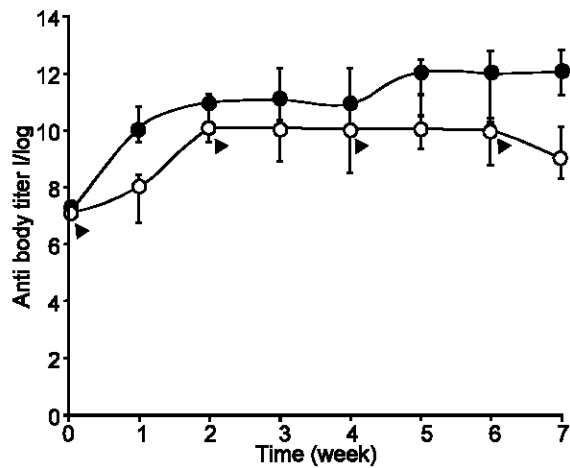


Fig. 1: Average of avian influenza antibody (GMT) in serum and yolk

This assay was performed by standard methods (Swayne *et al.*, 1988). Briefly, the test was performed in V-bottom 96-well micro titer plates with 4 hemagglutinating units of H₃N₂ virus and 0.5% specific pathogen-free chicken erythrocytes. Data was analyzed by student t-test and Fisher's exact test.

Antibody preparation: Eggs from both groups were collected 2 weeks after the first immunization. Then eggs were broken out and the yolk was collected in an equal volume of PBS. This mixture was mixed in a food blender until a uniform product was produced (approximately 3 min at high speed). The resulting mixture was centrifuged at 1.800×g for 15 min in a refrigerated centrifuge at 4°C. The superficial lipid layer was removed and the resulting supernatant was collected and stored at 4°C until it was used as the antibody source. The sediment was discarded (Fulton *et al.*, 2002; Carlander *et al.*, 2000). Immunoglobulin Y obtained from immunized hens was used for treatment.

Experiment 2

Animals: Fifty day old Specific Pathogen Free (SPF) white Lohmann chickens were divided in two groups and were housed in a high containment unit under biosafety level 3 conditions for 45 days. Strict biosecurity and precautions were taken not to allow the virus outside of the laboratory. Birds in the test group received 15 mL of Ab mixed per 3.84 L of drinking water beginning on day 17 and continuously until the end of the experiment. Birds in control group did not received any antibody (according to procedure of Fulton *et al.*, 2002). Both groups were inoculated intranasally with 0.1 mL of live virus with 10⁷ egg-infectious dose (EID50) per mL on day 22. For the AI virus challenge, chickens were

placed in negative pressure ventilated with HEPA-filtered air and provided with continuous lighting. Water and feed were provided *ad libitum*.

ELISA assay: Serum was collected from each bird on the day of challenge and in days 38, 42 and 45 post challenge. The antibody was tested by ELISA test. All birds were monitored twice daily for clinical signs of illness.

Virus shedding: To investigate virus shedding, 8 birds were separated randomly at day 45. Cloacal swabs were taken for virus isolation and titration in 10 day embryonating SPF chicken eggs. Swabs were put in 2 ml of 2.95% tryptose phosphate buffer with 5 to 10³ IU of penicillin-sodium and 5 mg of streptomycin per mL. The swabs were stored at -70°C until analyzed. Three embryonated chicken eggs incubated for 9 days were inoculated with 0.2 mL per egg. After 72 h, the allantoic fluid was harvested. A Hemagglutination Assay (HA) was performed following standard procedures (Swayne *et al.*, 1988).

Macroscopic lesions: The remaining birds were humanely euthanized by carbon dioxide asphyxiation and necropsied at the end of experiment. Gross lesions were recorded.

Results and Discussion

Experiment 1: Maximum titer of antibody in serums and egg yolks were detected in 14 and 35 days after the first immunization, respectively (Fig. 1). Levels of antibody in yolk were increased with levels of antibody in serum. Antibody level in yolk was higher than serum. There was a significant difference in levels of antibodies of yolk and serum between groups ($p < 0.05$) (Table 1).

Experiment 2: No clinical signs or mortality via AI were observed in any of the birds in experiment 2. Titer of antibody in both groups were determined by ELISA test. Only three birds in the test group had a positive titer, though 21 birds in the control group had positive titer (Table 2). The results were analyzed by Fisher's exact test. Birds in the test group had lower morbidity ($p < 0.05$). Virus shedding was significantly decreased in the test group ($p < 0.05$). In this study 7 birds in the test group had no virus shedding, though only 1 bird in the control group was negative (Table 3).

Egg derived antibody as a source of antibody has been used in many studies for therapeutics purpose. Maximum titer of antibody in egg yolks were detected in 42 days after the first immunization with hyper immunized leghorn hens by *Salmonella enteritidis* (Gurtler *et al.*, 2004). With injection of *Helicobacter pylori* antigen in brown leghorn intramuscularly in femur maximum titer of antibody in egg yolk were detected in

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Table 1: Experiment 1. Average of avian influenza antibody (GMT) in serum and yolk

Group	Average of AI antibody (GMT) in serum	Group	Average of AI antibody (GMT) in yolk
Control	7.43±1.19 ^b	Control	8.37±1.34 ^b
Treatment	9.37±1.57 ^a	Treatment	10.47±1.67 ^a

Different lower case superscripts denote significant difference between treatments groups

Table 2: Experiment 2. The results from ELISA test in layer chicken

Group	Number with positive titer	Number with negative titer
Control	21/22 (95.6%) ^a	1/22 (4.5%) ^a
Treatment	5/8 (62.5%) ^b	3/8 (37.5%) ^b

Different lower case superscripts denote significant difference between treatments groups

Table 3: Experiment 2. Attempted virus isolation from cloacal swabs

Group	Birds excreting virus	Birds not excreting virus
Control	7/8 (87.5%) ^a	1/8 (12.5%) ^a
Treatment	1/8 (12.5%) ^b	7/8 (87.5%) ^b

Different lower case superscripts denote significant difference between treatments groups

42 days after the first immunization by ELISA test (Shin *et al.*, 2002a). Maximum titer of antibody in serum and egg yolk were detected in 28 and 42 days after the first immunization, respectively, with injection antigens *Pasteurella multocida*, *Boedetella brochiseptica* and *Actinobacillus pleuropneumoniae* in white leghorn in pectoral muscle (Shin *et al.*, 2002b). The results from many studies indicate that in all studies, level of antibody in yolk is higher than serum. Factors such as SPF chicken or inbred chickens, kind of antigen, injection way, number of injection, adjuvant and the test for detection antibody have an important roles in the results obtained (Gurtler *et al.*, 2004; Shin *et al.*, 2002a; Shin *et al.*, 2002b; Carlander *et al.*, 2002; Coleman, 1996; Kruger, 2004; Narat, 2003). In all studies ELISA test was more sensitive than other methods to detect antibody.

Influenza is a zoonotic virus that occurs in lower animals and birds as well as in humans and can result in economic loss (Saif *et al.*, 2003). Outbreaks due to H₉N₂ subtypes have been reported in Germany, Italy, Ireland, South Africa, USA, Korea, China, the Middle East, Saudi Arabia and Pakistan (Alexander, 2000; Banks *et al.*, 2000). These outbreaks indicates that common methods to control outbreaks of AI such as depopulation of infected poultry, preemptive culling, biosecurity measures, and vaccination might not be very effective in prevention and control of AI. In the past, immunotherapy was carried out via the systemic or intravenous administration of specific antibodies, for such applications as a targeting agent for cancer diagnosis and therapy, the inactivation of toxic

substances including drugs and passive immunotherapy for neoplastic or infectious diseases. However, there has been increasing interest in the oral administration of specific antibodies for localized treatment of infections in the gastrointestinal tract (Carlander *et al.*, 2000; Reilly *et al.*, 1997). The increase in antiviral-resistant virus, such as viral pathogens, has prompted much research into the oral administration of antibodies as an alternative to treat infections. Orally administered antibodies would provide the advantage of reduced cost and ease of administration, as well as the potential for localized treatment and prevention and reduce the spreading the pathogen (most important factor for widespread disease). Our experiments demonstrate that egg derived antibody not only protects chickens against disease but is also an effective strategy to reduce transmission. Specifically, with use of egg derived antibody, transmission of the virus is decreased, and a major outbreak may be prevented. The egg derived antibody could be an attractive tool to prevent outbreaks of AI viruses in poultry, thereby possibly contributing to the aim of eliminating the source of human infections.

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