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Factors Affecting Hemagglutination Activity of Avian Influenza Viruses (H7N3, H9N2 Subtypes)

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Abstract: Hemagglutination (HA) and hemagglutination inhibition (HI) tests for avian influenza (AIV) viruses (H7N3 and H9N2) were standardized varying different factors like erythrocytes from different species, type of diluents, incubation temperature and incubation period. The virus was propagated in embryonated chicken eggs (9-11 days). The allantoic fluid (AF) was harvested 36 hours post incubation and was confirmed by spot agglutination test and agar gel precipitation test. The maximum HA titres were obtained using 1% RBCs of chicken, human blood (O^+) and dog at 22-37°C for 30-40 minutes. Both the avian influenza virus subtypes that eluted rapidly with higher temperature and maximum elution was observed within 8 hours.

Key words: Avian influenza virus (AIV) · Hemagglutination (HA) · Hemagglutination inhibition (HI)

INTRODUCTION

Avian influenza is caused by an orthomyxovirus. It is a single stranded, negative sense RNA virus which has eight segments of its genome surrounded by a lipid envelope. A peculiar characteristic of the virus is that it contains rod-shaped and mushroom-shaped glycoproteins called hemagglutinin and neuraminidase respectively [1,2] Both structural proteins are also important antigenic components of virus [3] Avian influenza viruses are capable of agglutinating red blood cells of various animal species [3] following the observation that influenza viruses agglutinate chicken erythrocytes, it was found that several other viruses are capable of agglutinating erythrocytes from certain animal species [4] Consequently, the hemagglutination reaction became a much widely used technique for measuring either viral antigen or antibody concentrations. Both HA and HI tests are reliable, economical and time saving test for initial diagnosis and monitoring of AI outbreaks as both also needs ordinary used chemicals.

In Pakistan, first outbreak was in 1994 by AI virus subtype H7N3 that caused serious economic losses [5,6] In subsequent years, different subtype of AI virus (H9N2) was diagnosed in breeder and broiler flocks in different regions of Pakistan [7] Recently, another subtype of AI virus (H5N1) has been reported from various poultry regions in Pakistan during 2005-06 and 2008.

Keeping in view the importance of the virus, it became imperative to diagnose the infection early so that effective measures against the spread of the virus may be implemented. The present study, therefore, aimed to determine the influence of various factors like source of red blood cells (RBC), type of diluent, incubation time, temperature, elution time of AI viruses (H7N3 and H9N2) in an attempt to standardize both serological tests under present prevailing conditions.

MATERIALS AND METHODS

Propagation of Antigen: The AI virus subtypes H7N3 and H9N2 were obtained from the University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan that were isolated during the outbreak. The embryonated eggs (n = 30: 9-11 days old), Big Bird Hatchery, Lahore, were inoculated in chorio-allantoic sac with 0.1ml of each of viral suspension [8] Following 36 hours incubation, the allantoic fluid was aseptically harvested, tested for its hemagglutinating activity using spot agglutination test [9] and was stored at –20°C until further test. The AIV subtypes were further confirmed by agar gel precipitation test (AGPT) as described earlier [10].

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Standardization of Hemagglutination Test

Diluents: Different diluents like normal saline (NS), phosphate buffer saline (PBS) and hemagglutination-hemagglutination inhibition buffer (HA-HI buffer) were prepared at pH of 7.2 [11] and were sterilized by autoclaving. The HA test was carried out using these buffers as diluents for erythrocytes preparations and diluting the antigen

Erythrocytes: The blood from chicken, duck, pigeon, horse, dog, sheep and human (blood group O^+) each species was collected aseptically using ethylenediaminetetra acetic acid. The erythrocytes were washed thrice and working suspensions of 1% RBCs was prepared from the stock suspensions using the same diluents.

Influence of Temperature: The influence of incubation temperature on HA activity was studied at four different temperatures (4°C, 22°C, 37°C and 42°C).

Influence of Incubation Period: The HA activity was determined at three different incubation time (20, 30 and 40 minutes).

Determination of Elution Time: The elution time for AI virus was determined after observing HA activity with tear drop method. The AIV were detached from the RBC surface and appeared as button.

Statistical Analysis: The titers were expressed as geometric mean titers (GMT) at various treatments.

RESULTS AND DISCUSSION

Standardization of Hemagglutination Test

Effect of Source of RBCs on HA activity: Hemagglutination activity of AI virus was checked by using 1 % RBCs from different animal species and diluents like P.B.S, N.S and HA-HI buffer keeping other factors like temperature and incubation time constant. The PBS (pH 7.2) was used for erythrocyte washing, diluting agent and suspension vehicle. The GMT HA titers for H7N3 were found to be 256, 64, 64, 64, 32, 64 and 32 respectively with RBCs of chicken, human blood group O^{+ve}, horse, dog, duck, pigeon and sheep respectively. AIV H9 gave HA titers with RBCs from chicken, human blood group O^{+ve}, dog, horse, pigeon, duck and sheep 512, 256, 256, 128, 64, 32 and 16 respectively.

Effect of Diluent: The HA titers obtained by incubating the virus with erythrocytes from different species suspended in different diluents (NS, PBS, HA-HI buffer) at different temperatures (4°C, 22°C, 37°C and 42°C) for different time periods showed variations. The HA titers was higher using PBS as a diluent compared to the other diluents.

Effect of Incubation Temperature: As depicted from Table 2, the AI viruses H7 and H9 gave equally good titer when incubated at 4°C, 22°C or 37°C. However, the incubation of the virus at higher temperature (42°C) registered lower HA titre compared to other incubation temperatures (Table 2).

Effect of Incubation Period: The HA titer of the virus, which incubated at 37°C for 20, 30 and 40 minutes did not show any difference when RBCs from chicken, duck, pigeon are used. On the other hand, the RBCs from human, horse, dog and sheep required longer incubation period (40-60 minutes) showed higher HA titre (Table 3).

Determination of Elution Time: The results revealed that the virus was eluted rapidly with increase in temperature. The elution time for AIV H7 in PBS was 5, 5, 4 and 1 hours and AIV H9 was 8, 6 4 and 2 hours at the temperature of 4, 22, 37 and 42°C respectively.

This study was conducted to standardize the hemagglutination technique for higher HA activity of AIV H7N3 and H9N2. Various factors like source of erythrocytes, type of diluents, incubation temperature and incubation period were evaluated with their potential effects on HA activity. The viruses agglutinated RBCs Hemagglutinin of AI virus mediates the attachment in the specific sialic acid receptors on the surface of erythrocytes resulting in agglutination [12, 13] These hemagglutinins have been found in the cell surface of erythrocyes of different species like of human, chicken, pigeon, duck, horse, dog and sheep. The agglutination of erythrocytes depends on the nature of receptors [12,14]. From the present study, it has been shown that type of diluent affected the HA activity of the AIV. The higher HA titre was obtained with PBS over other diluents. The PBS seemed to have an edge over the HA-HI buffer as it is commercially cheaper, easy to prepare. Various workers like also used PBS in their studies and reported similar findings [15,16,11].

Table 1: Effect of different diluents on HA activity of avian influenza virus

		Titer in log_2		
AIV subtype	Diluents	Range	Average	GMT
H7	PBS	7-9	8.3	315
	HA-HI buffer	7-9	8.0	256
	NSS	6-7	6.5	90
H9	PBS	8-10	9.2	588
	HA-HI buffer	8-9	8.5	362
	NSS	7-8	8.0	256

Table 2: Effect of incubation temperature on HA activity of avian influenza virus

	HA titer							
	H7				H9			
Time (minutes)	4C°	22C°	37C°	42C°	4C°	22C°	37C°	42C°
20	64	64	64	2	128	512	512	64
30	64	64	64	2	256	512	512	64
40	64	64	64	2	256	512	512	32

Table 3: Effect of incubation period on HA activity of avian influenza virus from RBCs of different animals

	HA titre					
	H7			H9		
Source of RBCs	20 min	30 min	40 min	20 min	30 min	40 min
Chicken	128	256	256	64	512	512
Duck	32	64	64	16	32	32
Pigeon	16	32	32	32	64	64
Horse	00	16	32	00	32	64
Dog	00	32	64	00	64	128
Human blood group O^{+ve}	16	32	64	64	128	256
Sheep	00	4	16	00	8	16

A comparison of average titers obtained with four incubation temperatures 4°C, 22°C, 37°C and 42°C) used in the present study showed all the temperatures except 42°C had any pronounced effect on the HA titers of AV virus. It was found that incubating temperature of 42°C resulted in lower HA titer. Similar findings were observed by Expand and Expand (2002) who reported that the HA protein is largely in the monomeric form at 25°C and there is little change with temperature. There is a weakening of the quaternary structure of HA at acidic pH prior to heating. At the temperature at which the virus exhibits an increased fusogenicity at neutral pH, there is a loss of secondary structure and a beginning of a destabilization of the trimeric form of HA resulting in low HA activity at higher temperature.

The HA titer of AI viruses obtained by keeping the plates at 37°C for 20 minutes, 30 minutes and 40 minutes did not show any significant difference when RBCs from chicken, duck, pigeon were used. Whereas the RBCs from Human, horse, dog and sheep required longer incubation period showing maximum HA titer (Table 3). This difference may be due to differences between RBCs of avian and mammalian origin. The results are in agreement with the findings [1,15,16].

In the present study, the elution of the virus took much longer time if were incubated at 4°C, but the viruses eluted rapidly with increase in the incubation temperature. Workers like Giannecchini *et al.* [17] performed elution experiments on turkey influenza viruses at 4°C, a temperature at which NA activity is blocked, observed that none of the viruses were able to elute, even after an overnight incubation. Conversely, when the experiments were performed at 37°C in the presence of receptor-destroying enzyme, all viruses were started eluting from

30 minutes incubation to 8 hours. Similar findings were also recorded by [2,18,19] The HI titers obtained with 4 HA units were higher than those obtained with 1 or 8 HA units of AI virus antigen. A two-fold increase of HA units resulted in reduction in HI titer. The serum titers were hand to be influenced by the concentration of antigen [20, 21,] also used 4 HA in their studies and their findings also support findings of present work

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