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Comparative evaluation of factors affecting hemagglutinating activity of avian influenza (H9) virus

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Abstract: Influenza virus H9 was standardized for hemagglutination assay using different factors such as red blood cell (RBC) types, concentrations, diluent types, and storage times. Avian influenza virus H9 was grown in embryonated chicken eggs and confirmed by spot agglutination. A significant ($P < 0.05$) difference with the highest mean titer (9.00 ± 0.00) was observed using RBCs from different species. Nonsignificant differences ($P > 0.05$) were found between human blood type O, chicken, and dog RBCs, as well as among rabbit, pigeon, sheep, and parrot. The highest titer (9.00 ± 0.00) with a nonsignificant difference was found using virus stored at 4 °C and –20 °C while 37 °C showed the lowest significant mean hemagglutinin (HA) titer (11.08 ± 188.21). Nonsignificant differences were observed in HA titers against H9 virus stored for 3, 4, 5, and 6 months. Nonsignificant differences were found between the use of normal saline and 0.5% peptone water with the lowest HA titers of 7.83 ± 0.40 and 8.00 ± 0.00, respectively, while the highest HA titer (9.00 ± 0.00) with nonsignificant difference was observed by using HA-HI buffer and phosphate buffer saline as diluents. RBCs with 0.5% and 1% concentrations showed nonsignificant difference in HA titer but significant difference with 0.1% RBCs.

Key words: Avian influenza, hemagglutination, chicken red blood cells, diluents, temperature

1. Introduction

The influenza virus is a single-stranded, eight-segmented RNA-based enveloped virus belonging to the family *Orthomyxoviridae*. On the basis of core protein influenza viruses are divided into three types, A, B, and C (1). Type A viruses are the cause of a highly contagious disease, avian influenza, in birds and are subdivided into subtypes on the basis of hemagglutinin (HA) and neuraminidase (NA) proteins (2), which are glycoproteins and are present as peplomers. There are 16 HA and 9 NA known subtypes of influenza viruses. These proteins are the main targets of protective immunity (3). HA protein gives the virus its property of hemagglutination. On the basis of pathogenicity avian influenza virus is divided into "low path" avian influenza and "high path" avian influenza, which poses a great threat due to the high death rate.

In Pakistan H7 and H9 have been reported. In 1994 and 1995 an outbreak of H7 with more than 85% mortality was recorded in the vicinity of Islamabad and infection due to H9 continues to occur in Karachi and adjoining areas to date (4,5).

HA protein plays a key role not only in the spread of infection but also in the detection of virus in the host system (embryonated eggs or cell line) and its purification, in the diagnosis of avian influenza virus outbreaks, and in checking the efficacy of vaccines. In the process of infection HA protein plays a major role in assisting the virus to attach to sialic acids receptors present on carbohydrate side chains of glycolipid and glycoprotein on target cells (3).

Biological changes occur in the virus due to degradation and degeneration even at very low storage temperatures; amnio-allantoic fluid containing avian influenza virus needs to be studied under various storage conditions to check the stability of HA virus protein (4).

Avian influenza virus can agglutinate red blood cells (RBCs) of different species of birds and mammals (5). The hemagglutinating activity of avian influenza virus is affected by various factors like source of RBCs, concentration, temperature, and buffers used as diluents (6). Therefore, the present study was designed to explore the effect of different sources and concentrations of RBCs, different diluents, and different storage conditions on HA activity of avian influenza (H9 type).

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2. Materials and methods

Avian influenza virus (H9), a known characterized avian influenza virus, was taken from Olympia Laboratory in Sheikhupura, Pakistan, and propagated (7) in 9-day-old embryonated chicken eggs (n = 48) via chorioallantoic route (@ 10^3 to 10^4 EID_{50}.) All eggs were incubated at 37 °C at a humidity level of 40%. After incubation for 48–72 h, all the embryonated eggs were kept at 4 °C for chilling. Amnio-allantoic fluid was harvested aseptically from each egg and separately harvested fluid was tested by spot agglutination for the presence of HA virus, while the agar gel precipitation test was used for the confirmation of the hemagglutinating virus as avian influenza H9 type (AI H9 type) virus (8) using control sera (AHVLA, UK). All the positive harvested fluid confirmed as avian influenza H9 type was pooled. Hemagglutinating activity of the harvested fluid containing virus was evaluated using different parameters like 1% washed RBCs locally prepared that originated from various species including human blood group type O (null blood group), chicken, pigeon, parrot, duck, rabbit, dog, sheep, horse, buffalo, and guinea pig (6,7). To observe the effect of diluents on HA activity of influenza virus, various diluents (normal saline: NS, phosphate buffer saline: PBS, hemagglutination-hemagglutination inhibition buffer: HA-HI buffer, and 0.5% peptone) were used for making serial two-fold dilutions of virus, RBC concentrations (0.1%, 0.5%, and 1%), and storage temperature. These factors affect not only the hemagglutinating activity but also the immunogenicity of the virus. For comparison of temperature's effect on HA activity, harvested fluid containing the virus was divided into 120 aliquots, each containing 1.5 mL of fluid containing virus. These aliquots were divided into four groups, A, B, C, and D, each comprising 30 aliquots. Group A was stored at room temperature (25 ± 3 °C), group B at 37 °C, Group C at –4 °C, and group D at –20 °C. All these groups were tested for their HA activity at intervals of 7 days for up to 6 months to determine the effect of storage temperature on HA receptors. To observe the effect on the immunogenicity of virus, amnio-allantoic fluids stored at different temperatures for 6 months together with positive and negative controls were used for the preparation of oil-based vaccine (9). These oil-based vaccines were used in birds of 4 weeks of age for developing hyperimmune sera. Hyperimmune sera were stored at 37 °C, 25–30 °C, 4 °C, and –20 °C and were then tested against hemagglutinating positive AI (H9) virus by hemagglutination inhibition test (7).

3. Results

The avian influenza H9 subtype virus induced death in 9-day-old embryonated chicken eggs within 48–72 h. Dead embryos showed lack of movement and collapse of blood vessels in chorioallantoic membranes on candling. Amnio-allantoic fluid from dead embryos was subjected to a spot agglutination test for confirmation of hemagglutination. An agar gel precipitation test was performed using known antiserum of avian influenza (H9) virus as a positive control and Newcastle disease virus as a negative control. A clear precipitin line between the antigen of avian influenza (H9) virus and its standard antiserum was formed, which confirmed positive results for avian influenza (H9) virus. Titer of hemagglutinating activity of positive pooled amnio-allantoic fluid was checked by HA test, which was recorded up to 512 GMT titer.

Blood from different species was collected and used to prepare RBCs at a concentration of 1% in phosphate buffered saline to find the sensitivity of RBCs in titrations of H9 virus. A significant ($P < 0.05$) difference with the highest mean $\log_2$ titer (9.00 ± 0.00) was observed using RBCs from different species. The highest titer was observed using RBCs from human blood group O, chicken, and dog blood while in the case of RBCs from rabbit the lowest mean titer (1.33 ± 0.57) was observed, as shown in Figure 1. A nonsignificant difference ($P > 0.05$) was observed between human blood type O, chicken, and dog RBCs. Similar nonsignificant differences ($P > 0.05$) were also found between rabbit, pigeon, sheep, and parrot. Parrot showed nonsignificant results when compared with duck RBCs. In another experiment H9 virus was kept at different temperatures for different time intervals to find effects of storage temperature. A significant ($P < 0.05$) difference in HA titer was observed with virus stored at temperatures of 37 °C, 25–30 °C, 4 °C, and –20 °C. The highest titer (512.00 ± 0.00) with a nonsignificant difference was found in the case of virus stored at 4 and –20 °C while the lowest mean HA titer (11.08 ± 188.21) was observed with virus stored at 37 °C as shown in Figure 2. Effect of storage time was also noted on HA titer with a significant difference ($P < 0.05$) and the highest mean HA titer (512.00 ± 0.00) was observed with the virus after storage for 1 month. A nonsignificant difference was observed in HA titer against H9 virus stored for 3, 4, 5, and 6 months. There was a nonsignificant difference between HA titer against virus stored for 1 and 2 months as shown in Figure 3. A significant difference ($P < 0.05$) was examined in HA titer by using different diluents including normal saline, phosphate buffered saline, HA-HI buffer, and 0.5% peptone water, while a nonsignificant ($P > 0.05$) difference was found in log HA titer against RBCs with different concentrations of 0.5% and 1%. A nonsignificant difference was found between using normal saline and 0.5% peptone water with lowest HA mean titers of 7.83 ± 0.40 and 8.00 ± 1.09, respectively, while the highest HA titer (9.00 ± 0.00) with a nonsignificant difference was observed by using HA-HI buffer and phosphate buffered saline as diluents as shown in the Table.

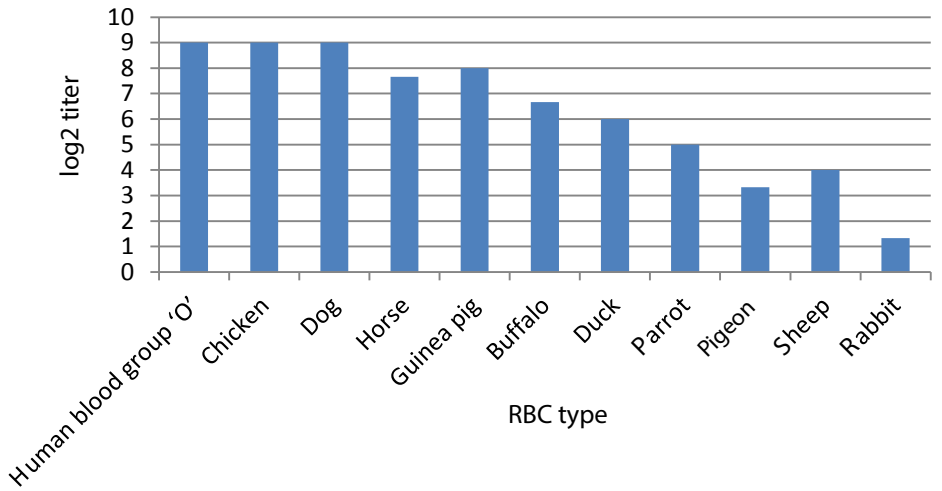

Figure 1. Mean log titer of H9 virus against red blood cells from different species.

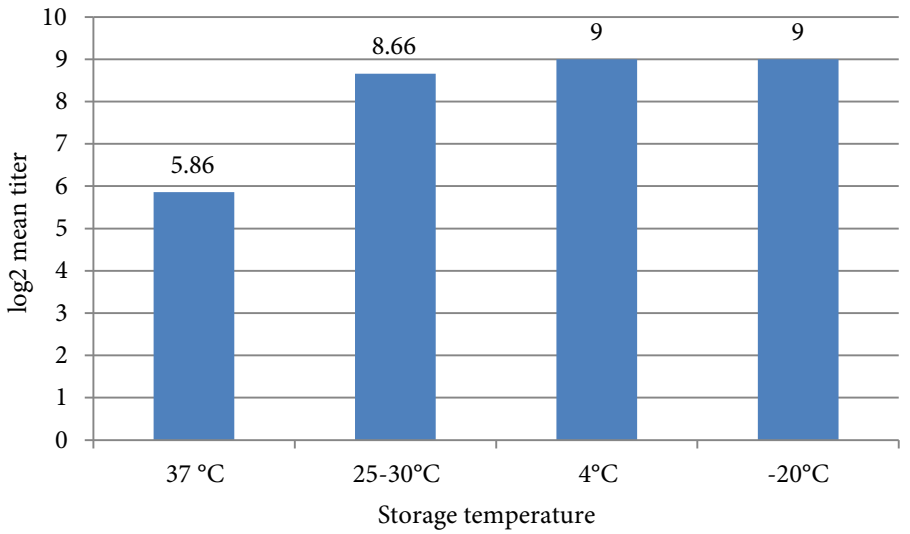

Figure 2. Mean HA titer against H9 virus stored at different temperatures.

4. Discussion

In the present study, results showed that although influenza virus H9 has a uniform ability of hemagglutinating RBCs from different species, the virus still showed difference in receptor specificity. Avian influenza virus H9 showed similar specificity against RBCs from human blood group O, chicken, and dog blood. RBCs from these species can be used in HA assay for diagnostic purposes, but as chicken RBCs are economical and easily available, they are more preferable as compared to human blood group O and dog blood. Similar results were also observed in prior studies (8,9). According to another study (10), H9N2 has proper HA test activity with guinea pig, turkey, and fowl RBCs. Current results also emphasized that RBCs from rabbit, pigeon, sheep, and parrot showed similar specificity and lowest mean HA titer as compared to human blood group O, chicken, and dog blood. There was no difference found in HA activity while using parrot and duck RBCs. It was examined recently (10) that amino acids at the 221st position in the influenza virus play an important role in determining receptor specificity in HA assay and minor changes in amino acids change avian receptor specificity. Hemagglutinating activity of the virus depends on the interaction of sialic acid receptors on RBCs and HA of the virus. This interaction mediates the attachment of the virus in the specific sialic acid glycoprotein receptors and agglutinates the erythrocyte (9,11).

In the present study, for dilution of H9 virus in HA tests, HA-HI buffer and phosphate buffered saline were found most preferable with the highest mean HA titer as

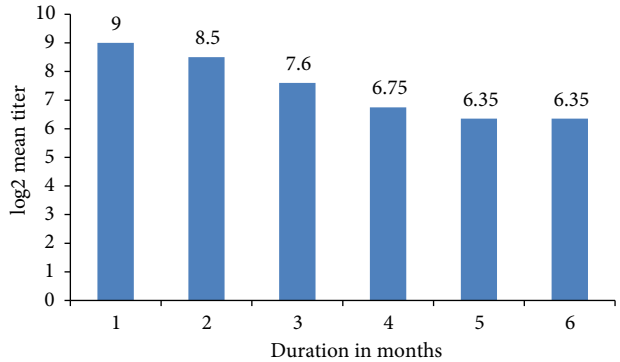

Figure 3. Mean HA titer against H9 virus stored for different time intervals.

Table. Effects of different diluents with different concentrations of chicken RBCs on HA activity of H9 virus.

RBC concentration (%)	NS	PBS	HA-HI buffer	0.5% peptone water
0.5	7.66 ± 0.57	9.00 ± 0.00	9.00 ± 0.00	7.66 ± 1.15
1	8.00 ± 0.00	9.00 ± 0.00	9.00 ± 0.00	8.33 ± 1.5
Total mean titer	7.83 ± 0.40 [a]	9.00 ± 0.00 [b]	9.00 ± 0.00 [b]	8.00 ± 1 .09 [a]

NS: normal saline, PBS: phosphate buffered saline, HA-HI: hemagglutination-hemagglutination inhibition.
Total mean titers with different superscripts showed significant difference ($P < 0.05$).

compared to normal saline and 0.5% peptone water. Results of the current study are in accordance with prior findings (6) and phosphate buffered saline has advantages over HA-HI titers due to being cheaper, available, and easy to prepare. The ionic concentrations of diluents influence macrohemagglutination of the virus to the cell receptors and these results are in agreement with those of previous studies (7,9,12).

RBCs with concentration of 0.5% and 1% showed respective mean HA titers of 8.33 ± 0.88 and 8.58 ± 0.66. There was no HA titer against 0.1% RBC concentration. Results of the current study were analogous to those of a past work (6). H9 virus was checked at different temperature for different time intervals to find the effects of storage temperature. A significant ($P < 0.05$) difference in HA titer was observed with virus stored at temperatures of 37 °C, 25–30 °C, 4 °C, and –20 °C. Influenza virus was found more stable in the case of virus stored at 4 and –20 °C and it showed nonsignificant HA activity, while the lowest mean HA titer (11.08 ± 188.21) was observed with virus stored at 37 °C. Previous results (11) indicated similar HA high titers at 4 °C and 22 °C but the HA titer at 37 °C was found higher than in the current study, which may be the effect of storage time. According to Epand and Epand (13), at high temperatures destabilization of the trimeric form of HA protein occurs and this results in low HA activity. Results of various storage temperatures are in accordance with prior results (4,14–16). Effects of storage time were also noted on HA titer with significant difference ($P < 0.05$) and the highest mean HA titer (512.00 ± 0.00) was observed with virus after storage for 1 month. A nonsignificant difference was observed in HA titer against H9 virus stored for 3, 4, 5, and 6 months. There was a nonsignificant difference between HA titers against virus stored for 1 and 2 months.

In conclusion, erythrocytes from chicken, dog, and human at 0.5% or 1% suspension in phosphate buffered saline or HA-HI buffer are equally good for monitoring the HA activity of avian influenza virus (H9 type). Long-term storage of the virus at room temperature and 37 °C should be avoided, while long-term storage at 4 °C and –20 °C preserves the hemagglutinating activity and immunogenicity of the virus.

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