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Full Article

Monitoring virus harvesting time in embryonated chicken eggs inoculated with avian influenza H9N2 vaccine strain

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ABSTRACT

Knowledge of virus and replication kinetic is one of the most important issues in the vaccine production. The present study aimed to evaluate the best harvesting time of H9N2 avian influenza virus (AIV) vaccine strain inoculated in specific pathogen free (SPF) embryonated chicken eggs (ECE)s. For this purpose, 10^{-5} dilution of AIV (A/Chicken/Iran/99/H9N2) was inoculated into 336, 11-day old ECEs at the rate of 0.1 ml/ECE via intera-allantoic. Amnio-allantoic fluid (AAF) and chorioalantoic membranes were collected at 2 hours intervals up to 96 post inoculations from 7 eggs in each trial. The presence of virus in the harvested suspensions was evaluated by Hemagglutination assay (HA) and Egg infective dose 50 (EID_{50}) assays. Our results showed that, the best virus harvesting time for vaccine production is between 50 – 60 hours post inoculation (hpi) in ECEs. This finding may provide possibility of the sufficient and increasing rate of production, immunogenicity and economic factors in H9N2 influenza vaccine production.

Keywords: Virus growth, Incubation, Influenza, Vaccine

INTRODUCTION

Influenza A viruses infects a wide range of animal species and human (Nazir *et al* 2010). Among the avian influenza virus (AIV) subtypes, H9N2 virus has the potential to cause an influenza pandemic because of its prevalency in avian species and the ability to induce human infection (Fedorko *et al* 2006). H9N2 viruses have been responsible for outbreaks of disease in poultry in various parts of the world, including European countries (Germany, Italy and Ireland), Middle Eastern countries (Iran, Pakistan and Saudi Arabia), South Africa and the United States (Alexander 2000, Alexander 2006). In Iran the first outbreak of H9N2 infection in poultry farms was reported in 1998 (Alizadeh *et al* 2009). The virus is now endemic in Asia and vaccination against this subtype is routinely practiced. Several studies have demonstrated that (Alizadeh *et al* 2009) inactivated oil emulsion H9N2 vaccines of induce antibody and provide protection against the virus (Vasfi-Marandi *et al* 2002, Sun *et al* 2012, Choi *et al* 2008, Lee *et al* 2011). The licensed

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inactivated influenza vaccines are developed by propagation of the virus in embryonated chicken eggs (ECE)s (Reisinger *et al* 2009). Like all other viruses, influenza viruses rely on the host cellular machinery to support their life cycle (Watanabe *et al* 2010). On the other hand, for the vaccines production, knowledge of the growth characteristics of the vaccine strains in host cells are essentially needed (Rimmelzwaan & Osterhaus 2001). So, undrestand of viral behavior and replication in chorioallantoic membranes and free extracellular fluids (amnio-allantoic fluids) (AAF) are one of most important vaccine production indexes (Finter *et al* 1954). In this study, we evaluated the best harvesting time of H9N2 virus during the growth period by collecting and assay AAF and membranes from groups of ECEs following replication.

MATERIALS AND METHODS

Viral stock preparations. Standard vaccine strain AIV (A/Chicken/Iran/99/H9N2) was inoculated into 11-day old SPF ECEs. The eggs were observed for 24-72 hour post inoculation according to the OIE Terrestrial Manual (2008). The AAFs of the inoculated eggs were collected and centrifuged at 1200 rpm for 30 minute. The 50 percent of egg infective dose (EID_{50}) was calculated by Reed and Muench (1938) method in 11-day-old embryonic SPF eggs. Hemagglutination assay (HA) was performed in V-bottom 96-well plates with 1% chicken red blood cell as described by Burleson et al (1992).

Design study. The 10^{-5} dilution of the H9N2 virus work seed (EID_{50} = 9.8 log10 and HA titer = 10 log2) was intra allantoically inoculated into 336 SPF ECEs at the rate of 0.1 ml/ECE. All eggs were sealed and incubated at 37°C with 60% humidity. The AAF and chorioalantoic membranes of inoculated eggs, were collected from 7 eggs at 2 h intervals up to 96 hours post inoculation (hpi). In case, after removing the shell and shell membranes at the blunt end of the eggs, the AAF samples were withdrawn into the small measuring cylinders. Then, the chorioalantoic membranes were sampled and washed tree times in Petri dishes containing sterile phosphate buffer saline (PBS). Suspensions of the membranes were made by blending in chilled Ultra-Turrax blender for 5 minutes in 10000 rpm, using 1 ml of PBS/membrane. The AAF and membrane suspensions were clarified by using the 0.22 µm filters (Schleicher & schuell, Germany). Prepared samples from each group was tested for HA activity and EID_{50}. Assays were performed in three replicates.

Statistical analyses. Data were examined using a commercially available statistical package (SPSS version 17 for Windows), and comparisons made using the descriptive statistics tests.

RESULTS

The results of HA titers of AAF and chorioalantoic membranes are shown in Figure 1 and Table 1. No virus was detected until 12 hpi in the AAF, whereas a 3 log 2 of viral titer detected in chorioalantoic membranes at the first phase. Figure 2 and Table 1 shows the results of EID_{50} titers of H9N2 virus during the 96 hour-growth period. There appropriate EID_{50} titers were evaluated in chorioalantoic membranes and AAF (for virus harvesting and antigen preparation) at 24 and 28 hpi, which was maintained in AAF until 58 hpi. However, the titer was continued until the end of experiment with mild decreasing in membranes The proper HA titers were obtained in AAF between hours of 38 – 66. This rate was started in chorioalantoic membranes at 26 hpi and maintained until end of experiment. The overlapping of optimum peak for HA and EID_{50} titers in AAF were occurred between 50 to 60 hpi.

DISCUSSION

The viral replication process could be divided in four distinct phases: 1) virus entry into the cells, 2) intracellular replication and production of the viral proteins, 3) virus assembly and budding at the cell membrane and 4) release of the progeny viruses (Emma & Kamen 2012). In the last stage, virus particles bud from the apical side of polarized host

Table 1. Mean titers of HA and EID_{50} in AAF and chorioalantoic membranes in various hpi.

Mean titers of EID_{50} in chorioalantoic membranes (Log10)	Mean titers of EID_{50} in AAF (Log10)	Mean titers of HA in chorioalantoic membranes (Log2)	Mean titers of HA in AAF (Log2)	Hours post inoculation
0	0	3	0	2
0	0	3	0	4
2.74±0.17	2.50±0.21	3	0	6
4.49±0.21	3.36±0.11	4	0	8
5.52±0.11	4.40±0.04	4	0	10
6.38±0.14	5.47±0.04	4	0	12
7.39±0.22	7.11±0.12	+4	1	14
7.48±0.23	8.64±0.14	+4	6	16
8.52±0.02	8.49±0.21	6	7	18
8.50±0.21	9.15±0.28	7	7	20
9.39±0.12	9.25±0.14	7	+7	22
9.51±0.24	9.62±0.16	7	8	24
9.50±.04	9.41±0.08	+10	8	26
9.50±0.18	9.50±0.14	+9	8	28
9.83±0.31	9.74±0.18	11	+8	30
10.37±0.08	9.40±0.16	+9	+8	32
10.16±0.27	9.62±0.19	+11	9	34
10.49±0.25	9.36±0.05	10	9	36
10.62±0.20	9.74±0.27	9	+9	38
10.34±0.27	9.74±0.31	11	+10	40
10.49±0.31	9.39±0.04	+10	10	42
10.24±0.04	9.60±0.08	10	10	44
10.50±0.07	9.82±0.02	+10	+9	46
9.83±0.24	9.74±0.24	9	+10	48
10.63±0.06	9.58±0.12	+11	10	50
10.49±0.18	9.50±0.11	+11	+10	52
10.34±0.24	9.74±0.07	+11	10	54
10.52±0.22	9.16±0.05	+11	+10	56
10.32±0.18	9.59±0.15	+11	10	58
10.50±0.25	9.35±0.04	+11	10	60
10.24±0.14	9.16±0.16	+9	+10	62
10.32±0.24	9.32±0.24	11	+10	64
10.32±0.09	9.15±0.18	10	+9	66
10.62±0.01	9.29±0.21	+11	9	68
10.49±0.30	9.59±0.04	+11	9	70
10.39±0.012	9.24±0.17	+11	9	72
10.39±0.07	9.24±0.29	+11	9	74
10.24±0.18	9.47±0.08	+10	10	76
10.16±0.24	8.62±0.12	+10	9	78
9.50±0.021	8.66±0.19	+11	9	80
9.83±0.07	9.15±0.15	11	10	82
9.76±0.18	8.56±0.18	9	+9	84
9.62±0.11	8.50±0.10	10	+9	86
9.24±0.14	8.83±0.18	11	8	88
9.59±0.18	8.24±0.24	+10	9	90
9.50±0.22	8.78±0.17	9	+9	92
9.74±0.30	8.50±0.15	10	9	94
9.62±0.14	8.53±0.12	+9	9	96

cells, then the viral particles are released from the plasma membrane (Samji 2009). Identification of quantity and viral particles release time after inoculation of vaccine strain virus in the host cells are important factors for antigen preperation used in vaccine industry. In the present study virus entry were detected with HA test in the chorioalantoic membranes only after 2 hour of inoculation. Also virus release in

AAF was seen in 14 hpi. Freymann *et al* (1950) have been detected the AIV (EID_{50}=$10^{6.15}$) in ECEs at 8 hpi also hemagglutinin was detected in AAF. Beale and Finter (1956) showed that HA titer of AAF significantly increased at 4 hpi. Differences in titer of AAF and membranes are in relation with replication and release time of the progeny viruses from host cells (Watanabe *et al* 2010).

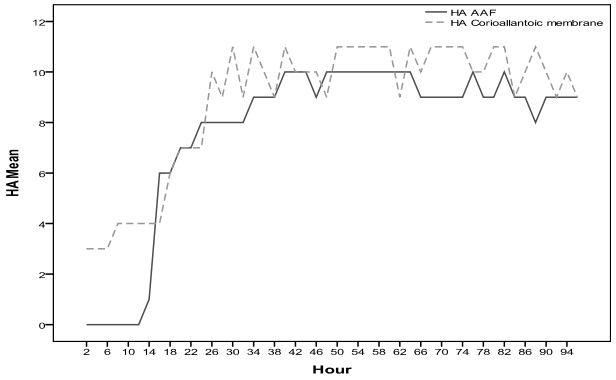

Figure 1. HA titers of AAF and chorioalantoic membranes in AIV H9N2 inoculated eggs, at 2 hour intervals up to 96 hpi.

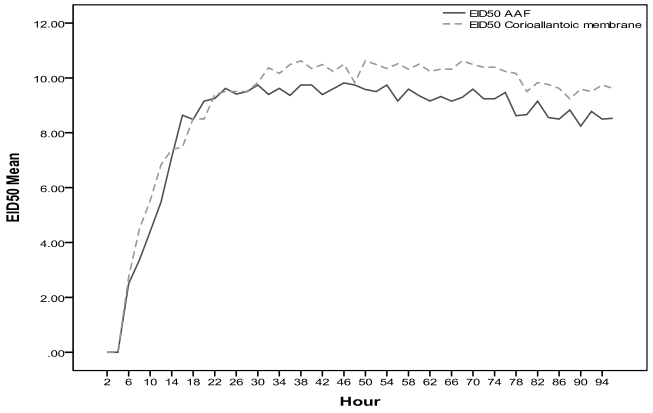

Figure 2. EID_{50} titers of AAF and chorioalantoic membranes in AIV H9N2 inoculated eggs, at 2 hour intervals up to 96 hpi.

It is also possible that the release in AAF affected by initial infection dose and viral volume. Likewise, when inoculum is insufficient to infect all the available cells, the remaining cells must be infected with progeny viruses at further infectious cycles (Henle *et al* 1954). Our results showed that the peak of EID_{50} titers in AAF and chorioalantoic membranes were occurred at 28 and 24 hpi respectively, while the maximum HA titers obtained at 38 and 26 hpi. According to Wu *et al* study (2010), the maximum titer of wild type H9N2 virus was detected in cell culture at 36 hpi. Ohta and co-workers (1981) have shown that when inoculums size was $10^{7.7}$ EID_{50}/ml, the peak of viral titer reached by 24 h. One important parameter for the viral vaccine production is the multiplicity of infection. This parameter is defined as the ratio of infectious viral particles to cells targeted for infection and could have a great impact on the final production level (Emma & Kamen 2012). Differences in results of various studies may be related to the initial infection dose and virus subtypes (Zarkov 2006). Our results showed that, the best virus harvesting time for vaccine production using H9N2 vaccine strain in ECEs is between 50 – 60 hpi.

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References

Alizadeh, E., Hosseini, S.M., Kheiri, M. T. (2009). Avian Influenza (H9N2) among poultry workers in Iran. *Iranian Journal of Microbiology* 1: 3-6.

Alexander, D. J. (2000). A review of avian influenza in different bird species. *Veterinary Microbiology* 74, 3-13.

Alexander, D. J. (2006). An overview of the epidemiology of avian influenza. *Vaccine* 6691: 1-8.

Beale, A. J., Finter, N. B. (1956). The Infectivity of Chorio-Allantoic Membrane Influenza Virus and Incomplete Influenza Virus By The Six-Hour Soluble Antigen Production Test. *Journal of Bacteriology* 55: 68-78.

Burleson, F. G., Chambers, T. M., Wiedbrauk, D. L. (1992). Virology: *A laboratory manual*. London: Academic press, 66-72.

Choi, J. G., Lee, Y. J., Kim, Y. J., Lee, E. K., Jeong, O. M., Sung, H. W., Kim, J. H. & Kwon, J. H. (2008). *Journal of veterinary science* 9, 67-74

Emma, P., Kamen, A., (2012). Real-time monitoring of influenza virus production kinetics in HEK293 cell cultures. *Biotechnology Progress* doi: 10.1002/btpr.601.

Fedorko, D. P., Nelson, N. A., McAuliffe, J. M. (2006). Performance of Rapid Tests for Detection of Avian

Influenza A Virus Types H5N1 and H9N2. *Journal of clinical microbiology* 44(4): 1596–7.

Finter, N. B., Liu, O. C., Liberman, M. (1954). Studies on host-virus interactions in the chick emberyo-influenza virus system. *Deembryonation Technics* 94(4): 305-22.

Food and Agriculture Organisation (FAO) (1989). *Animal production and health paper* (by Dr. V. Palya Phylaxia Veterinary Biologicals Company. Ltd., 1107 Budapest Szállás u. 5., Hungary).10-56.

Freymann, M. W., Tamm, I., Green, R. H. (1950). Growth Curves of Influenza Virus Based on Hemagglutinasion Titers in Individual Embryonated Eggs. *YALE Journal of Biology and Medicine* 23(4): 269-76.

Henle, W., Liu, O. C., Finter, N. B. (1954). Studies on host-virus interactions in the chick embryo-influenza virus system. IX. The period of liberation of virus from infected cells. *Journal of Experimental Medicine* 100(1): 53–70.

Lee, Y. N., Lee, D. H., Park, J. K., Lim, T. H., Youn, H. N., Yuk, S. S., Lee, Y. J., Mo, I. P., Sung, H. W., Lee, J. B., Park, S. Y., Choi, I. S. & Song, C. S. (2011). *Avian diseases* 55, 724-727.

Nazir, J., Haumacher, R., Abbas, M. D. (2010). Use of filter carrier technique to measure the persistence of avian influenza viruses in wet environmental conditions. *Journal of Virological Methods* 170: 99–105.

Office International des Epizooties (OIE) (2008). *Manual of diagnostic tests and vaccines for terrestrial animals*. Chapter 2.3.4, *Avian influenza* 465-481.

Ohta, C., Yanagawa, R., Kida, H., (1981). Growth of Avian and Human Influenza Viruses in Organ Cultures of Duck and Chicken Colons. *Archives of Virology*, 70: 137-45.

Reed, L. J., Muench, H. A. (1938). Simple method of estimating fifty percent endpoints. *American Journal of Hygiene* 27: 493-7.

Reisinger, K. S., Block, S. L., Izu, A. et al. (2009). Subunit Influenza Vaccines Produced from Cell Culture or in Embryonated Chicken Eggs: Comparison of Safety, Reactogenicity, and Immunogenicity. *The Journal of Infectious Diseases* 200, 849–57.

Rimmelzwaan, G. F., Osterhaus, A. D. (2001). Influenza vaccines: new developments. *Current Opinion in Pharmacology* 1: 491–6.

Samji, T. (2009). Influenza A: Understanding the Viral Life Cycle. *YALE Journal of Biology and Medicine* 82: 153-9.

Sun, Y., Pu, J., Fan, L., Sun, H., Wang, J., Zhang, Y., Liu, L., Liu, J. (2012). Evaluation of the protective efficacy of a commercial vaccine against different antigenic groups of H9N2 influenza viruses in chickens. *Veterinary Microbiology* 156: 193–199.

Vasfi-Marandi, M., Bozorgmehri-Fard, M. H., Hashemzadeh, M. (2002). Efficacy of inactivated H9N2 avian influenza vaccine against non-highly pathogenic A/Chicken/Iran/ZMT-173/1999 infection. *Archives of Razi Institute* 53: 23-6.

Watanabe, T., Watanabe, S., Kawaoka, Y. (2010). Cellular networks involved in the influenza virus life cycle. *Cell Host Microbe* 7(6): 427–39.

Wu, R., Guan, Y., Yang, Z. et al. (2010). A live bivalent influenza vaccine based on a H9N2 virus strain. *Vaccine* 28(3): 673–80.

Zarkov, I. S. (2006). Studies upon the possibilities of avian influenza viruses' cultivation in chick embryos at different age. *Bulgarian Journal of Veterinary Medicine* 9(1): 43-9.