Journal of Animal Research: v.12 n.3, p. 331-335. June 2022

DOI: 10.30954/2277-940X.03.2022.3

Detection of HA Stalk Specific Antibodies in Chicken Serum Immunized with Different Subtypes of Inactivated Avian Influenza Viruses

Sivasankar Panickan[1,2], Sandeep Bhatia[2*], Nisha Bhandari[3], Shanmuga Sundaram Nagarajan[2], Semmannan Kalaiyarasu[2], Richa Sood[2], Atul Kumar Pateriya[2] and Meghna Tripathi[2]

[1]Immunology Section, ICAR-Indian Veterinary Research Institute, Bareilly, INDIA
[2]ICAR- National Institute of High Security Animal Diseases, Bhopal, INDIA
[3]Molecular Biophysics Unit, Indian Institute of Science, Bangalore, INDIA

**Corresponding author: S. Bhatia; E-mail: sbhatia1967@gmail.com*

Received: 30 April, 2022 **Revised:** 24 May, 2022 **Accepted:** 28 May, 2022

ABSTRACT

To control avian influenza infection in chickens, whole inactivated influenza viruses (IIV) are used as vaccine. These vaccines provide immunity only to antigenically matched strains or homologous viruses. Influenza viruses circulating in field undergo spontaneous antigenic changes in head domain, thereby escape from the preexisting vaccine immunity. Therefore, annual reformulation of vaccines are needed to compensate the antigenic change. To overcome this issue, universal flu vaccines are preferred which may protect against antigenically unmatched strains or heterologous viruses. Apart from natural infection, IIV also may induce stalk reactive antibodies but in meager level. Stalk domain of haemagglutinin is one of the target for the development of universal vaccine as it is conserved among influenza viruses. The antigenic changes are rare in stalk rather than head domain. Designing of stalk is challenging in prefusion conformation as it requires skilled knowledge. In the present study, we have tested the reactivity of *in vitro* expressed HA stalk protein with sera of chickens immunized with different subtypes of inactivated influenza virus vaccines. Our result showed that the HA stalk is reactive with sera against group I viruses mostly because our stalk belongs to the same group I. Hence, we concluded that in-vitro expressed HA stalk protein used in this study is conformationally stable and mimics the HA stalk as present in the intact viruses. This study provides hope for development of universal influenza vaccine in chicken is possible if the stalk immunogen is designed better.

HIGHLIGHTS

- Recombinant HA stalk protein is cross reactive with sera raised against group I influenza A viruses
- Inactivated influenza viruses in chicken induces HA stalk specific antibodies

Keywords: Chicken, ELISA, HA stalk, Stalk reactive antibodies, Vaccine

Haemagglutinin (HA) is a surface glycoprotein of avian influenza viruses which projects as spikes (Chen *et al*., 2011). Based on this glycoprotein, influenza A viruses are divided into 18 HA subtypes (H1-H18) which are further divided into two groups. Group I contains subtypes of H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, H18 and group II contains H3, H4, H7, H10, H14, H15 (Krammer *et al*., 2013; Wu *et al*., 2014). HA protein is a trimer, 225 kDa, 13.5 nm long, and essential for attachment of host cell and fusion (Chen *et al*., 2011). The haemagglutinin consist of two domains HA1 and HA2 (Kim *et al*., 2017). The HA1 is globular in shape induces neutralizing antibodies, having immunodominant epitopes but antigenically highly variable or less conserved among influenza subtypes. Whereas the HA2 is a dimer, and conserved among group I or II; and less immunogenic

How to cite this article: Panickan, S., Bhatia, S., Bhandari, N., Nagarajan, S.S., Kalaiyarasu, S., Sood, R., Pateriya, A.K. and Tripathi, M. (2022). Detection of HA Stalk Specific Antibodies in Chicken Serum Immunized with Different Subtypes of Inactivated Avian Influenza Viruses. *J. Anim. Res.*, **12**(03): 331-335.

Source of Support: None; **Conflict of Interest:** None

(de Vries *et al.*, 2015). Current avian influenza vaccines (whole inactivated virus with adjuvant) primarily produces neutralizing antibodies against HA1 domain which correlates with protection against challenge viruses (Kang *et al*., 2020) and these vaccines are strain specific or provides immunity only to antigenically matched viruses in chickens; whereas antibodies produced against HA2 are broadly reactive against different subtypes of influenza viruses (Corti *et al.*, 2011; Ekiert and Wilson, 2012). In vaccination, primarily HA1 specific antibodies are produced rather HA2 specific antibodies. If there is antigenic change in HA1 of circulating strains, the vaccine strain has to be updated. This is because HA1 frequently undergoes mutation due to error prone RNA polymerase. So, annual reformulation of vaccines are essential to antigenically match the circulating strains (Tricco *et al.*, 2013). To overcome this issue, universal influenza vaccines are the preferred choice, which are protective against antigenically different strains. The HA2 is conserved among antigenically different strains and hence, *in-vitro* expressed HA2 fragment can be used as universal vaccine (Corti *et al.*, 2010; Sui *et al.*, 2011; Ekiert and Wilson, 2012). Such vaccines are currently under development for human influenza viruses. HA2 based universal flu vaccine are moderately successful in mice (Steel *et al.*, 2010) and HA2 specific antibodies or stalk reactive antibodies are reported to be broadly reactive against heterologous viruses in mice. The protectiveness of stalk reactive antibodies in chicken are unclear. Conventional IIV elicit insufficient stalk reactive antibodies (Corti *et al*., 2010; Moody *et al.*, 2011; Margine *et al.*, 2013). There is question of whether the natural infection or immunization of inactivated whole virus vaccine induces stalk reactive antibodies in chickens?. If, so then is it possible to detect the stalk reactive antibodies using *in-vitro* expressed HA2 protein or not? The present study was designed to address these questions using in-vitro expressed HA stalk (as vaccine) which protected mice against lethal challenge of H5N1 (Valkenburg *et al.*, 2016). Although, expression of stalk protein in tertiary structure as present in intact virus is challenging because of meta stable nature of HA2 (stalk), it has been successfully expressed and the same was used as coating antigen for the testing of reactivity of sera samples received from chickens immunized with inactivated vaccines. Hence, this study may lay foundation or insights for development of universal flu vaccines in chickens.

MATERIALS AND METHODS

Serum samples

Immunized SPF chicken reference serum samples were received from Avian Influenza repository of ICAR-NIHSAD, Bhopal, India. These are reference sera against (H1-H16) procured from NLVS, Ames, Lowa. H5-HA stalk (current study), rgH5N2 (reverse genetics virus) specific serum and M2e-HA2 (Kalaiyarasu *et al*., 2021) specific chicken serum were also included in the study.

Recombinant proteins

M2e-HA2 is a recombinant protein expressed in *E. Coli*. The conserved HA2 and M2e sequences were collected from common circulating H5N1 viruses in India and expressed together with His-tag sequence (Kalaiyarasu *et al.*, 2021). H5-HA stalk purified recombinant protein received from Indian Institute of Science (IISc), Bangalore, India. The protein was expressed in *E. coli* using gene sequence derived from HA1 and HA2 of H5N1 (A/VietNam/1203/04, GenBank Acc. AAW80717.1). This protein consist of three stem derived fragments containing amino acid sequences of HA1: 14–37, 286–319 and HA2: 41–113 (Valkenburg *et al.*, 2016).

ELISA Procedure

96 well ELISA plate (Nunc) was coated with H5-HA stalk (1 µg in 50 µl/well) at 37°C for 1 hr in carbonate bicarbonate buffer (pH=9.4). Following washing once with PBS-tween (0.05%) (PBST), wells were blocked with 200 µl of 2% bovine serum albumin (Proliant, USA) and incubated at 37°C for 1 hr. Subsequently after discarding blocking buffer, wells were incubated with 50 µl of diluted test serum samples (in duplicates) along with positive control (immune sera against HA stalk), negative control (unimmunized SPF chicken sera) and blank (PBS) at 37°C for 1hr. The test serum samples initially diluted to two fold serial dilution starting from 1:100 and up to 1: 6400 for optimization and finalized at 1:400 with PBST. Following incubation of test serum samples, wells were washed thrice with 200 µl of PBST and then, 50 µl of Anti- chicken HRPO in PBS (1:10,000) was added and incubated at 37°C for 1hr and washed thrice with PBST. Later, developed with 50 µl of substrate TMB (3,3',5,5'-Tetramethylbenzidine)

and the reaction was stopped with 50 µl of 1M H_2SO_4. The OD was measured at 450 nm using TriStar^{2}S LB942 Micro plate reader (BERTHOLD Technologies, Germany).

RESULTS AND DISCUSSION

Stem-directed broadly neutralising antibodies are powerful probes to evaluate HA stalk conformation. Antibody response against the stalk domain in post infection or vaccination are relatively weaker than head domain. So, eliciting cross-protective, stem-directed broadly neutralising antibodies (bnAbs) remains challenging (Rathore *et al.*, 2014). Different strategies, such as prime-boost with a chimeric HA protein (Wei *et al.*, 2010; Krammer *et al.*, 2013), polypeptide mimics to the HA stem, headless HA virus (Steel *et al.*, 2010; Mallajosyula *et al.*, 2014) or sequential infection with different influenza subtypes (Krammer *et al.*, 2012) have been employed to elicit bnAbs antibodies towards the stalk domain. The anti-stalk broadly neutralizing antibodies (bnAbs) are detectable in humans at a low level with natural infection suggesting the possibilities of induction in other species too by either natural infection and or vaccination. In present study, we have evaluated the reactivity of H5-HA stalk in ELISA with reference sera (Ames, Lowa) derived from chickens immunised with different subtypes of inactivated influenza viruses such as H1, H2, H3, H4, H6, H7, H8, H10, H11, H12, H13, H14, H15, H16, H5N1, H5N8, H9N2, rgH5N2 and recombinant proteins namely, H5-HA stalk and HA2-M2e apart from negative unimmunized SPF chicken sera. The chicken sera samples that showed reactivity with HA stalk only, were mentioned in figures (Fig. 1 & 2). H5-HA stalk immunised chicken sera used as positive control. Sera from unimmunized chickens used as negative control. HA stalk immunised sera showed maximum OD value of 1.3 and negative control serum showed maximum of 0.29 (Fig. 1). Serum produced against HA stalk designed from H5N1 subtype (M2e-HA2) (Kalaiyarasu *et al.*, 2021) which belongs to group I, reacted with IISc HA stalk protein in ELISA and the OD varied from 0.6 to 1.08. Serum against rgH5N2 also reacted well (OD ranges from 0.14 to 0.73) with H5-HA stalk, which indicate the sharing of conformational epitopes among them. Among the subtype specific reference sera, H2 subtype specific sera, which belongs to group I reacted well (OD value of 0.59) (Fig. 2). Among the tested serum samples, sera against HA stalk (homologus) was highly reactive followed by M2e-HA2. In conclusion, these results showed that *in vitro* expressed HA stalk broadly reacts with HA specific sera against group I viruses.

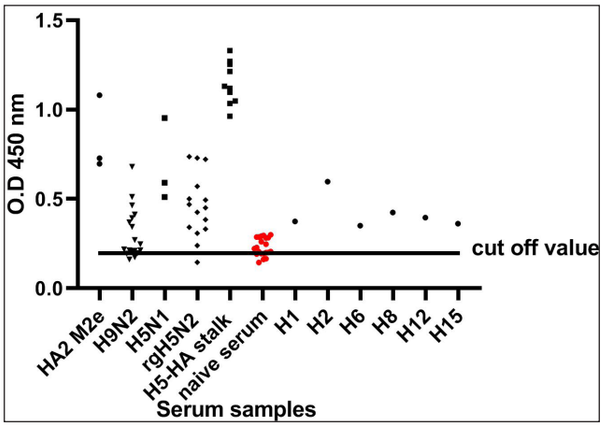

Fig. 1: ELISA. Reactivity of immunised chicken sera (1:400 dilution) with H5-HA stalk protein coated on ELISA plate. Number of sera samples used in this study were M2e-HA2 (n=3), H9N2(n=18), H5N1(n=3), rgH5N2 (n=15), HA stalk (n=10), naive serum (n=21), H1 (pooled sera), H2 (pooled sera), H6 (pooled sera), H8 (pooled sera), H12 (pooled sera), H15 (pooled sera). Average O.D value of naive serum samples considered as cut off value=0.230. HA stalk immunised sera used as positive control and naive serum samples used as negative control

This study indicates the preservation of common conformational epitopes in the expressed HA stalk as present in the virion. Stabilization of HA stalk molecule is reported to be difficult because HA stalk is a metastable in nature but our HA stalk (H5) successfully designed in a way with incorporation of site specific mutation and GSA linkers to make it stable (Valkenburg *et al.*, 2016). All sera used in this study produced against group I viral subtypes except H15 which belongs to group II. Among all sera of whole viruses, serum against rgH5N2 showed better reactivity since HA stalk of rgH5N2 and H5-HA stalk are phylogenetically closer (Fig. 2). The serum dilutions up to 1:800 showed reactivity with H5-HA stalk which indicates the presence of sufficient concentration of stalk reactive antibodies (Fig. 2). The stalk reactive antibodies are not so far demonstrated in chickens with inactivated influenza vaccine but in mice it has been reported (Krammer *et al.*, 2013). These stalk antibodies are protective in mice (Impagliazzo *et al.*, 2015; Valkenburg *et al.*, 2016) against heterologous viruses but its efficacy in chicken is unclear and poorly studied. Since our stalk is broadly reactive

with many subtypes of IIV vaccine sera, there is hope for development of universal flu vaccine in chickens in near future.

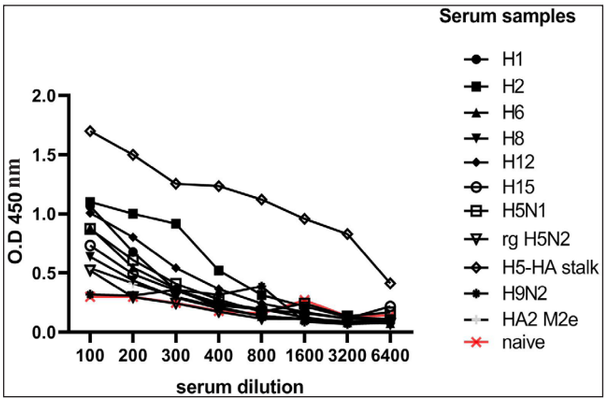

Fig. 2: ELISA. Reactivity of immunised chicken sera (pooled samples) at each serial dilution (1:100 to 1:6400) with H5-HA stalk protein coated ELISA plate

ACKNOWLEDGEMENTS

We thank Dr. Raghavan Varadarajan (IISc, Bangalore) for providing HA stalk, Dr. Chakradhar Tosh (ICAR-NIHSAD) for providing serum samples and Director, ICAR-NIHSAD for arranging necessary facilities to carry out this work.

REFERENCES

Chen, J.R., Ma, C. and Wong, C.H., 2011. Vaccine design of hemagglutinin glycoprotein against influenza. *Trends Biotechnol*., **29**(9): 426–434.

Corti, D., Suguitan, A.L., Pinna, D., Silacci, C., Fernandez-Rodriguez, B.M., Vanzetta, F., Santos, C., Luke, C.J., Torres-Velez, F.J. and Temperton, N.J. 2010. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *J. Clin. Invest*., **120**(5): 1663–1673.

Corti, D., Voss, J., Gamblin, S.J., Codoni, G., Macagno, A., Jarrossay, D., Vachieri, S.G., Pinna, D., Minola, A. and Vanzetta, F. 2011. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*, **333**(6044): 850–856.

de Vries, R.D., Altenburg, A.F. and Rimmelzwaan, G.F. 2015. Universal influenza vaccines, science fiction or soon reality? Expert Rev. *Vaccines*, **14**(10): 1299-301.

Ekiert, D.C. and Wilson, I.A. 2012. Broadly neutralizing antibodies against influenza virus and prospects for universal therapies. *Curr. Opin. Virol*., **2**(2): 134–141.

Impagliazzo, A., Milder, F., Kuipers, H., Wagner, M. V, Zhu, X., Hoffman, R.M.B., van Meersbergen, R., Huizingh, J., Wanningen, P. and Verspuij, J. 2015. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science,* **349**(6254): 1301–1306.

Kalaiyarasu, S., Bhatia, S., Mishra, N., Senthil Kumar, D., Kumar, M., Sood, R., Rajukumar, K., Ponnusamy, B., Desai, D. and Singh, V.P. 2021. Elicitation of Highly Pathogenic Avian Influenza H5N1 M2e and HA2-Specific Humoral and Cell-Mediated Immune Response in Chicken Following Immunization With Recombinant M2e-HA2 Fusion Protein. *Front. Vet. Sci*., pp. 1223.

Kang, Y.M., Cho, H.K., Kim, H.M., Lee, C.H., Kim, D.Y., Choi, S.H., Lee, M.H. and Kang, H.M. 2020. Protection of layers and breeders against homologous or heterologous HPAIv by vaccines from Korean national antigen bank. *Sci. Rep*., **10**(1): 1–9.

Kim, S.M., Kim, Y.I., Park, S.J., Kim, E.H., Kwon, H., Si, Y.J., Lee, I.W., Song, M.S. and Choi, Y.K. 2017. Vaccine efficacy of inactivated, chimeric hemagglutinin H9/H5N2 avian influenza virus and its suitability for the marker vaccine strategy. *J. Virol*., **91**(6): e01693-16.

Krammer, F., Pica, N., Hai, R., Margine, I. and Palese, P. 2013. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. *J. Virol*., **87**(12): 6542–6550.

Krammer, F., Pica, N., Hai, R., Tan, G.S. and Palese, P. 2012. Hemagglutinin stalk-reactive antibodies are boosted following sequential infection with seasonal and pandemic H1N1 influenza virus in mice. *J. Virol*., **86**(19): 10302–10307.

Mallajosyula, V.V.A., Citron, M., Ferrara, F., Lu, X., Callahan, C., Heidecker, G.J., Sarma, S.P., Flynn, J.A., Temperton, N.J. and Liang, X. 2014. Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. *Proc. Natl. Acad. Sci*., **111**(25): E2514–E2523.

Margine, I., Hai, R., Albrecht, R.A., Obermoser, G., Harrod, A.C., Banchereau, J., Palucka, K., García-Sastre, A., Palese, P., Treanor, J.J. and Krammer, F. 2013. H3N2 influenza virus Infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. *J. Virol*., **87**(8): 4728–4737.

Moody, M.A., Zhang, R., Walter, E.B., Woods, C.W., Ginsburg, G.S., McClain, M.T., Denny, T.N., Chen, X., Munshaw, S., Marshall, D.J., Whitesides, J.F., Drinker, M.S., Amos, J.D., Gurley, T.C., Eudailey, J.A., Foulger, A., DeRosa, K.R., Parks, R., Meyerhoff, R.R., Yu, J.S., Kozink, D.M., Barefoot, B.E., Ramsburg, E.A., Khurana, S., Golding, H., Vandergrift,

N.A., Alam, S.M., Tomaras, G.D., Kepler, T.B., Kelsoe, G., Liao, H.X. and Haynes, B.F. 2011. H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. *PLoS One*, **6**(10).

Rathore, U., Kesavardhana, S., Mallajosyula, V.V.A. and Varadarajan, R. 2014. Immunogen design for HIV-1 and influenza. Biochim. Biophys. *Acta (BBA)-Proteins Proteomics*, **1844**(11): 1891–1906.

Steel, J., Lowen, A.C., Wang, T.T., Yondola, M., Gao, Q., Haye, K., Garcia-Sastre, A. and Palese, P. 2010. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio*., **1**(1): e00018-10.

Sui, J., Sheehan, J., Hwang, W.C., Bankston, L.A., Burchett, S.K., Huang, C.Y., Liddington, R.C., Beigel, J.H. and Marasco, W.A. 2011. Wide prevalence of heterosubtypic broadly neutralizing human anti influenza A antibodies. *Clin. Infect. Dis*., **52**(8): 1003–1009.

Tricco, A.C., Chit, A., Soobiah, C., Hallett, D., Meier, G., Chen, M.H., Tashkandi, M., Bauch, C.T. and Loeb, M. 2013. Comparing influenza vaccine efficacy against mismatched and matched strains: a systematic review and meta-analysis. *BMC Med*., **11**(1): 1–19.

Valkenburg, S.A., Mallajosyula, V., Li, O.T.W., Chin, A.W.H., Carnell, G., Temperton, N., Varadarajan, R. and Poon, L.L.M. 2016. Stalking influenza by vaccination with pre-fusion headless HA mini-stem. *Sci. Rep*., **6**(1): 1–11.

Wei, C.J., Boyington, J.C., McTamney, P.M., Kong, W.P., Pearce, M.B., Xu, L., Andersen, H., Rao, S., Tumpey, T.M. and Yang, Z.Y. 2010. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science*, **329**(5995): 1060–1064.

Wu, Y., Wu, Y., Tefsen, B., Shi, Y. and Gao, G.F. 2014. Bat-derived influenza-like viruses H17N10 and H18N11. *Trends Microbiol*., **22**(4): 183–191.