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In Silico Prediction and Evaluation of *E. coli* Expressed Recombinant HA Protein of Avian Influenza Virus

Sana Shakoor, Tahir Rehman Samiullah, Naila Shahid, Abdul Qayyum Rao*, Aneela Yasmeen, Sana Tahir, Ayesha Latif, Saira Azam, Ahmad Ali Shahid and Tayyab Husnain

Centre of Excellence in Molecular Biology, University of the Punjab, 87-West Canal Bank Road Lahore-53700, Pakistan.

Sana Shakoor and Tahir Rehman Samiullah equally contributed to this article.

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Authors' Contribution
AQR presented the idea. SS carried out research work. NS and TRS interpreted the data and wrote manuscript. ST did bioinformatics analysis. AY, AL and SA planned methodology and compiled results. AAS evaluated the animal study. TH reviewed and approved the article for submission.

Key words
Protein, Avian influenza virus, Poultry, *E. Coli*, Vaccines

ABSTRACT

The H9N2 strain of the avian influenza virus has emerged to infect poultry during the last few years. Besides antiviral treatment development of an effective vaccine to combat a high mortality rate is the need of time. As a step towards the development of the vaccine for the H9N2 strain of avian influenza virus *HA* gene was chosen as the immunogenic antigen because it is highly conserved among H9N2 strain of avian influenza virus. Moreover, immunogenic regions within *HA* gene may contain potential immunogenic epitopes. The specificity of HA protein produced from *E. coli* was confirmed through an antibody-antigen reaction on the nitrocellulose membrane. The appearance of 67 KDa protein on the nitrocellulose membrane confirmed its specificity. The intraperitoneal immunization of mice with HA protein along with enhancer (ferund adjuvants) was done and produced antibodies in serum was detected by immunodot blotting assay. The appearance of the dot on spotted position confirmed the specificity of anti-HA antibodies. The titer of antibody produced in immunogenic mice was determined through ELISA. The highest geometric mean titer of HA-specific serum IgG, which was greater as compared to the control.

INTRODUCTION

Pakistan's poultry sector is considered the most significant and vivacious section of agriculture that contributes 1.3% to the national GDP, but despite its importance, this industry has to face many challenges, among all disease outbreaks due to avian influenza virus (AIV) are more prevalent. Many subtypes of H9N2 were reported to be epidemic in Asian and middle east countries since late 1990 (Munir *et al*., 2013a). The avian disease like avian influenza has raised some serious concerns due to the closed food chain linkages of poultry with human health. The H9N2, the main cause of low pathogenic avian influenza is widespread all around the world. It has been isolated from the terrestrial poultry throughout the world (Davidson *et al*., 2013). This has attacted attention of researchers to avian influenza because the infected chickens are a source of secondary infection with pathogenic microbes, act as a reservoir host and can transmit the virus to the mammals. In 1878 causal agent of avian influenza was first defined by the Perroncito and in 1901 it was reported that disease was caused by a filterable agent. AIV is the member of family Orthomyxoviridae and genus Influenza A, having a lipid membrane and single-stranded, segmented negative-sense RNA genome, which is further divided into eight gene segments (Garten and Klenk, 2008; Monne *et al*., 2013).

The significant economic losses due to higher mortality of chicks and reduced egg production have been caused by a low pathogenic avian influenza virus (LPAIV) H9N2 in Pakistan. The H9N2 LPAIV was initially reported during 1998 in Pakistan and isolated strains were found genetically similar to the Honk Kong virus during 1997. Now a days, a new genotype of H9N2 A/Chicken/Pakistan/UDL-01/06 (H9N2) is widely spread in Pakistan which has evolved through recombination with H5N1 and H7N3 (Liu *et al*., 2013; Lee *et al*., 2016). The vaccination is a cost-effective approach that exists in live, inactivated, subunit as well as incomplete virus vaccines form to control the emergence of influenza pandemics. These vaccines are usually propagated in embryonated chickens or virus treated tissue culture and, in most cases, found to be linked with problems such as allergic reactions against egg protein, antigenic drift, incomplete protection against influenza strains at the time of

* Corresponding author: qayyumabdul77@yahoo.com
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an outbreak. These antibodies are usually subtype-specific and cannot induce the required level of immunity (Kim *et al*., 2013; Golchin *et al*., 2018).

The routine cure of both human and avian Influenza is through the application of vaccine and most of these vaccines are based on the hemagglutinin (HA) the surface glycoprotein, the main immunogenic protein of the influenza virus. Influenza is considered as viral surface antigen and can be visualized through an electron microscope. Its spikes radiate out from the spherical lipid envelope. A total of 90% of HA represents ectodomain while the remaining 10% sequence of HA comprises of transmembrane and cytosolic domains. The head, stalk and stem domain of HA ectodomain are mostly involved in binding of receptors and membrane fusion to induce a specific immune response (Garten and Klenk, 2008; Munir *et al*., 2013b). The HA molecule is produced as a precursor molecule HA0 of size 75KDa that cleaved up in two monomeric polypeptide chains HA1 and HA2 in trimeric form. The hemagglutinin of H9N2 (LPAI) is activated when the secreted protease from the epithelial cells of the respiratory or intestinal tissues activates the arginine or sometime lysine at the monobasic cleavage site. Therefore, the infection is only limited to these organs (Rajão and Pérez, 2018).

In this study, the efficacy of HA protein H9N2 strain expressed in *Escherichia coli* was tested. Mice immunization experiments demonstrated that expressed HA protein was active and produced antibodies which were immunogenic against HA protein as determined by dot blot and ELISA.

MATERIALS AND METHODS

Prediction of immunogenic HA protein epitopes via bioinformatics tools

Bioinformatics tools were used for the in-silico identification of the immunogenic nature of the protein. The prediction analysis of HA protein was done through various tools from the immune epitope database analysis resource (http://toolsimmuneepitope.org/main/). PHYRE 2 (http://www.sbg.bio.ic.ac.uk/phyre2/phyre2_output/2f61c22539708c42/summary.html), an online automated tool, was used for the homology modeling of HA protein. ProSA web server was used for the evaluation and validation of the HA protein model. Linear continuous epitopes as well as discontinuous epitopes were identified with Ellipro site and software. The frequency of occurrence of each residue in the predicted epitopes of HA protein was plotted graphically.

Construction of a prokaryote expression vector

The synthetic haemagglutinin gene (taken from H9N2 strain via NCBI) was cloned in PUC57 vector and amplified by using gene-specific primers at 60 °C annealing to be used for TA cloning. TA cloning of *HA* gene was done with TA vector (pTZ57/RT) using T4 DNA ligase. *E. coli* TOP10 competent cells were transformed with the ligation mixture through the heat shock method. The mixture was homogenized with 50µl *E. coli* TOP 10 competent cells and was subjected to heat shock for 90 seconds at 42 °C followed by immediate incubation at ice for two minutes. The mixture was then spread on LB plates and incubated at 37 °C for 16–18h. Restriction digestion was done to confirm the positive clones with *Sal*1 and *Xho*1. Further TA clone was subjected to restriction digestion with *Sal*1 and *Xho*1 along with pET30a followed by ligation of *HA* with pET30a and its transfer into *E. coli* TOP10 competent cells with an overnight incubation at 37 °C. Confirmation of plasmid was done by restriction digestion with *Sal*1 and *Xho*1 enzymes as well as through amplification by using gene-specific primers.

Expression of immunogenic HA protein

For expression, *E. coli* Rosetta Strain BL21DE3 was transformed with HA-pET30a vector. The crude protein was extracted from the BL21DE3 culture at OD 0.6-0.8 and induced with 20% glucose and IPTG at 30 °C for 6-7 h. Harvesting was done through centrifugation at 4 °C. The pellet was resuspended in 1x phosphate saline buffer (1:10 W/V) and centrifuged again at 14000xg for 10 min. Cell lysis was done through the addition of lysozyme (4mg/ml) in lysis buffer and keeping it at room temperature for 20-30 min. Sonication was done after lysis for 10-12 seconds with a continuous pulse on ice followed by final centrifugation at 14000 x g for 15min at 4°C. The procedure was repeated in order to take the 4-5 sample fractions after different treatments. The protein samples were prepared for SDS through heat shock with addition of 5X loading dye (65µl + 35µl) at 95 °C for 8 minutes. Protein fractions were resolved on SDS-PAGE by using 12% polyacrylamide gel according to the procedures described by the Laemmli system (1970). The gel was stained with Coomassie blue (Thermo Scientific). The protein prestained markers (cat#926-98000 Western Sure®) were used for determination of protein size. Mini protean II tetra cell (cat# 1658004; BioRad) was used for fractionation of recombinant protein by SDS-PAGE. Polyvinylidenediflouride membrane (PVDF, cat # RPN303E; Amersham) was used to transfer resolved recombinant protein using BIO-RAD Mini Trans-Blot® Electrophoretic Transfer Cell (Cat# 170-3930) blocked with 5% non-fat dry milk with His-tag specific antibodies. Recombinant *HA* gene was detected through rabbit polyclonal anti-His-tag serum (1: 5000 dilution; cat # sc-

804; Santa Cruz).

Polyclonal antibodies production and immunogenicity confirmation

Experiment for vaccine production was conducted using mouse model according to the ethical standards, in the animal house facility of National Centre of Excellence in Molecular Biology, University of the Punjab.

The mice were divided into two groups. Group one was considered as a control group (A) and the second group was considered as an experimental group (B). The mice were immunized with 15µg/dose rHA-*E. coli* along with ferund adjuvant. During the period of immunization, blood samples from both experimental and control mice were taken for immunogenic analysis. The Immunogenic response of HA protein was evaluated through immuno-dot blotting assay. Serum (2µl) of each group was blotted on nitrocellulose membrane, dried, and washed with 1X PBS. The extracted protein was spotted on this nitrocellulose membrane and incubated at 37°C for an hour. Then enzyme-conjugated secondary antibody was added and incubated at 37°C for an hour. After incubation BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/nitrotetrazoline blue), was added and the change in color was observed to define the specificity of an immune response. The titer of antibodies was further determined through enzyme linked immuno sorbant assay (ELISA). The diluted serum in phosphate buffer (1:500, 1;1000, 1:1500) from both experimental and control groups was used to coat the microtiter plate. After coating, the plate was washed with 1X PBS (phosphate buffer saline) followed by the addition of 20µg/ml protein as an antigen. The incubation at 37°C for an hour was done followed by three-time washing with 1XPBS. The secondary antibody (Anti-mouse IgG enzyme-conjugated) was added and incubation was done for one hour. The washing was done with 100µl 1X PBS after incubation. Finally, the substrate (BCIP/NBT) was added and the change in color was observed that stopped the reaction with 1N solution of HCL. The Optical density (O.D) was taken at 450 nm to check the immune response.

RESULTS

Prediction of HA protein antigenicity

PHYRE 2 predicted three models for HA proteins based on the principle of structure homology modeling as shown in (Fig. 1A). The chosen model for the HA protein was found to have a maximum amino acid sequence coverage of over 85% and 497 residues. Total 89% of sequences have been modelled with 100% confidence by the single highest scoring template. ProSA-web provides an easy-to-use interface to the program ProSA (Sippl, 1993) which is frequently employed in protein structure validation.

ProSA calculates an overall quality score for a specific input structure. If this score is outside a range characteristic for native proteins the structure probably contains errors. A plot of local quality scores points to problematic parts of the model which are also highlighted in a 3D molecule viewer to facilitate their detection in (Fig. 1B). A plot of residue scores showed local model quality by plotting energies as a function of amino acid sequence position i. In general, positive values correspond to problematic or erroneous parts of the input structure.

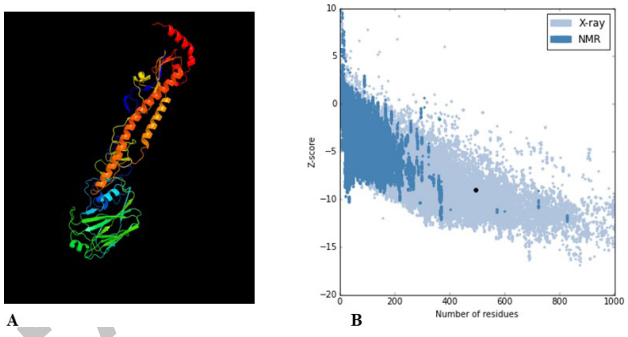

Fig. 1. Prediction of immunogenic HA protein epitopes via bioinformatics tools. A, Image coloured by rainbow N → C terminus model dimensions (Å):X:46.119 Y:134.537 Z:45.524; B, Plot of residue scores.

Elliopro connects every predicted epitope with Protrusion Index (PI) as a score having averaged value over epitope residue. In This method, the protein's 3D shape is closely related to 13 ellipsoids listed in Table I. Ellipsoid with PI = 0.9 would means that 90% of the protein residues are being within, while 10% of the protein residues being outside of the ellipsoid. Residues with high scores are associated with greater solvent accessibility. Discontinuous epitopes are defined on the bases of PI values, and they are clustered on the bases of distance R (in Å– between residue's centers of mass) (Table II). The frequency of occurrence of each residue in the predicted epitopes of HA protein was graphically plotted based on their score (Y-axis) and residue position (X-axis) as shown in Figure 2. In Figure 3, JSmol-rendered 3D structures of continuous antigenic epitopes of the HA protein along with their PI values, as predicted by ElliPro.

Confirmation of r-HA expression cassette

The recombinant HA-protein gene in the TA vector was confirmed through amplification with gene-specific primers. Amplification of 1683bp product confirmed the successful ligation of the *HA* gene in the TA vector. The HA-TA plasmid was also evaluated through restriction

Table I. Predicted linear epitopes of the HA protein.

No.	Chain	Start	End	Peptide	Number of residues	
1	_	455	516	NLYNKVKRALGSNAMEDGKGCFELYHKCDDQCMETIRNGTYNR-RKYTEESRLERQKIEGVKL	62	0.812
2	_	163	183	TQKNGNYPIQDAQYTNNRGKD	21	0.765
3	_	192	237	HPPTDTAQTNLYTRTDTTTSVTTENLDRTFKPLIGPRPLNGLIGR	45	0.758
4	_	245	258	LKPGQTLRVRSNGN	14	0.738
5	_	363	373	QHSNDQGVGMA	11	0.735
6	_	130	155	QRIQIFPDAIWNVTYDGTSKSCSNSF	26	0.7
7	_	344	352	IAGFIEGGW	9	0.696
8	_	36	42	TLTESNI	7	0.655
9	_	409	418	SEIETRLNMI	10	0.642
10	_	279	286	TDLKSGNC	8	0.594
11	_	100	106	RPSAVNG	7	0.59
12	_	331	340	NVPAKSSRGL	10	0.561
13	_	82	93	NPSCDLLLGGRE	12	0.529

Table II. Predicted discontinuous epitopes of HA protein.

No.	Residues	Number of residues	
1	_:T413, _:R414, _:L415, _:M417, _:I418, _:K421	6	0.784
2	_:D19, _:K20, _:I21, _:N331, _:V332, _:P333, _:A334, _:S336, _:S337, _:R338, _:G339, _:L340, _:Q363, _:H364, _:S365, _:N366, _:D367, _:Q368, _:G369, _:V370, _:G371, _:M372, _:A373, _:N455, _:Y457, _:N458, _:K459, _:V460, _:K461, _:A463, _:L464, _:G465, _:S466, _:N467, _:A468, _:M469, _:E470, _:D471, _:G472, _:K473, _:G474, _:C475, _:F476, _:E477, _:L478, _:Y479, _:H480, _:K481, _:C482, _:D483, _:D484, _:Q485, _:C486, _:M487, _:E488, _:T489, _:I490, _:R491, _:N492, _:G493, _:T494, _:Y495, _:N496, _:R497, _:R498, _:K499, _:Y500, _:T501, _:E502, _:E503, _:S504, _:R505, _:L506, _:E507, _:R508, _:Q509, _:K510, _:I511, _:E512, _:G513, _:V514, _:K515, _:L516	83	0.772
3	_:I69, _:L70, _:D71, _:T72, _:C73, _:T74, _:G81, _:N82, _:P83, _:S84, _:C85, _:D86, _:L87, _:L88, _:L89, _:G90, _:G91, _:R92, _:R100, _:P101, _:S102, _:A103, _:V104, _:N105, _:G106, _:T107, _:C108, _:Y109, _:P110, _:Q130, _:R131, _:I132, _:Q133, _:I134, _:F135, _:P136, _:D137, _:A138, _:I139, _:W140, _:N141, _:V142, _:T143, _:Y144, _:D145, _:G146, _:T147, _:S148, _:K149, _:S150, _:C151, _:S152, _:N153, _:S154, _:F155, _:R157, _:N158, _:T163, _:Q164, _:K165, _:N166, _:G167, _:N168, _:Y169, _:P170, _:I171, _:Q172, _:D173, _:A174, _:Q175, _:Y176, _:T177, _:N178, _:N179, _:R180, _:G181, _:K182, _:D183, _:H192, _:P193, _:P194, _:T195, _:D196, _:T197, _:A198, _:Q199, _:T200, _:N201, _:L202, _:Y203, _:T204, _:R205, _:T206, _:D207, _:T208, _:T209, _:T210, _:S211, _:V212, _:T213, _:T214, _:E215, _:N216, _:L217, _:D218, _:R219, _:T220, _:F221, _:K222, _:P223, _:L224, _:I225, _:G226, _:P227, _:R228, _:P229, _:L230, _:N232, _:G233, _:L234, _:I235, _:G236, _:R237, _:L245, _:K246, _:P247, _:G248, _:Q249, _:T250, _:L251, _:R252, _:V253, _:R254, _:S255, _:N256, _:G257, _:N258, _:W263, _:Y264, _:V267	140	0.683
4	_:T33, _:D35, _:T36, _:L37, _:T38, _:E39, _:S40, _:N41, _:I42	9	0.605
5	_:T28, _:N29, _:S30, _:T31, _:E32, _:I344, _:A345, _:G346, _:F347, _:I348, _:E349, _:G350, _:G351, _:W352, _:P353	15	0.604
6	_:N56, _:G57, _:M58, _:L59, _:C60, _:T279, _:D280, _:L281, _:K282, _:S283, _:G284, _:N285, _:C286	13	0.506

digestion by using *Sal*1 and *Xho*1 enzymes and digested fragments were resolved on the agarose gel. The appearance of 1683bp band on agarose gel restricted from the TA pTZ57R/T vector further validates the successful ligation of the *HA* gene in the TA vector. The ligation of the *HA* gene into the pET30a vector was done by using the separated fragment of 1683bp from the TA vector through *Sal*1 and *Xho*1 sites of pET30a. The appearance of 1683bp through amplification as well as through restriction digestion confirmed the successful construction of rHA cassette (Figs. 4B, 4C).

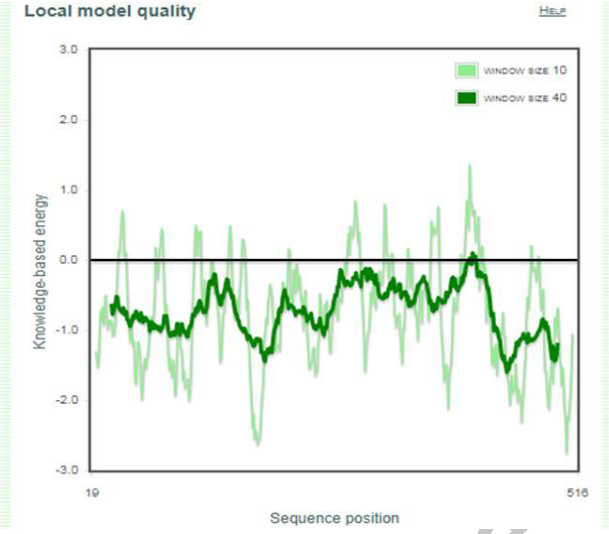

Fig. 2. Graphical representation of the occurrence frequency of HA protein residues based on individual score (Y-axis) and residue position (X-axis).

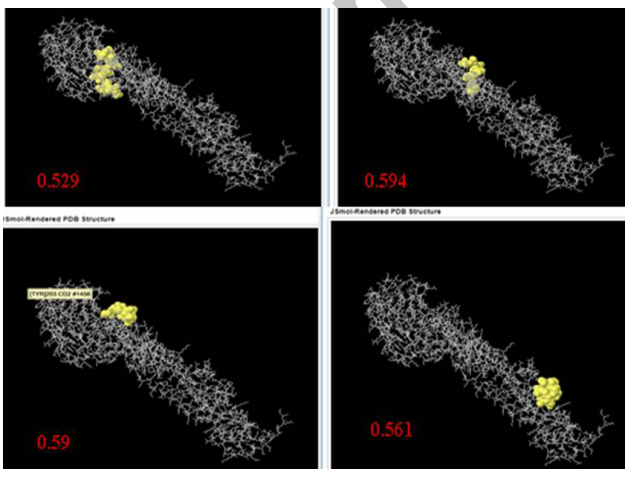

Fig. 3. JSmol-rendered 3D structures of continuous antigenic epitopes of the HA protein along with their PI values, as predicted by ElliPro.

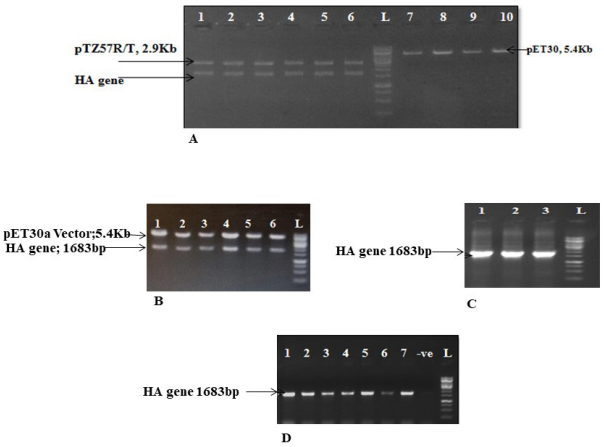

Fig. 4. Confirmation of cloning of *HA* gene in pET30a expression vector and *E. coli* BL21 (D3). A, Confirmation of successful digestion of TA vector containing *HA* gene and pET30a vector; B, Confirmation of *HA* gene-pET30a Ligation through restriction digestions; Lane 1-12, Restriction digestion of pET30a clones, Lane L, 1Kb DNA ladder; C, Confirmation of *HA* gene ligated in pET30a through PCR through PCR; Lane 1-3, Amplification of 1663bp of *HA* gene in pET30a; D, Confirmation of presence of *HA* gene ligated in pET30a transformed into Rosetta by PCR; Lane 1-7, Amplification of 1683bp *HA* gene ligated in pET30a plasmid; Lane 8, -ve control; Lane L, 1 Kb DNA Ladder.

r-HA expression analysis in E. coli

Recombinant plasmid r-HA was transformed in the BL21DE3 expression host. The amplification of the 1683bp *HA* gene confirmed the successful transformation of Rosetta BL21DE3 with recombinant r-HA (Fig. 4D). Whole-cell lysate induced with the 1mM IPTG shown a sharp expressed protein band of 67kDa of *HA* gene after staining as shown in Figure 5A. The high protein expression was observed at ImM IPTG induction after 5-8 h of incubation at 30°C. The identification of His-tagged HA protein was successfully done through antibody-antigen reaction by using western blot analysis. The appearance of 67kDa protein on SDS PAGE confirmed the successful expression of HA recombinant protein.

Determination of HA antibody specificity through antigen-antibody reaction

The serum from immunized mice was examined for the determination of IgG. After complete immunization, the serum from mice's blood was taken to be evaluated for its response against HA protein and was confirmed through immune dot blotting assay (Fig. 6A). The appearance of the dot on the spotted position confirmed the specificity of anti-HA antibodies produced (Fig. 6A). The titer of

antibody produced in immunogenic mice was determined through ELISA. It was found that diluted serum in 1/500 and 1/1500 effectively work as antibody as compared to 1:1000 dilutions (Fig. 7).

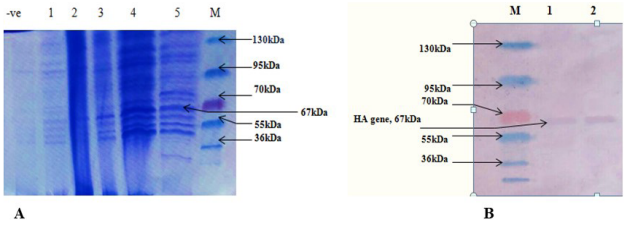

Fig. 5. Protein expression analysis by SDS-PAGE and western blotting.
A, Confirmation of HA protein expression resolved on SDS-PAGE gel stained with coomasie blue; Lane –ve, Rosetta culture as a –ve control; Lane 1-5, Total cell lysate with different treatments; Lane M, pre-stained protein marker (Fermentas); B, Confirmation of His-tagged HA protein on nitrocellulose membrane by western blotting; Lane M, pre-stained protein marker; Lane 1&2, Antigen-antibody reaction.

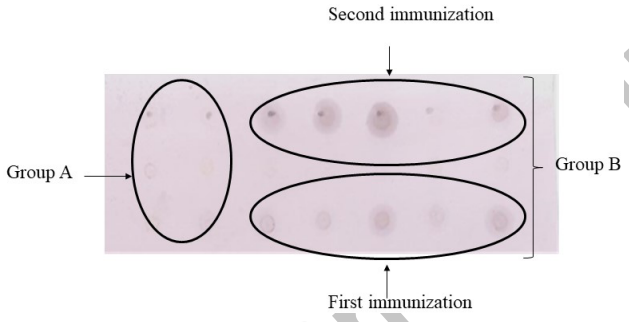

Fig. 6. Animal study: Confirmation of HA protein through immune dot blotting assay. The appearance of dot on spotted position confirmed the specificity of anti-HA antibodies produced. A, Blood from the control group; B, Blood from the experimental group after first and second immunization.

DISCUSSION

Avian influenza is a highly contagious respiratory disease of poultry caused by influenza A viruses, family Orthomyxoviridae. H9N2 avian influenza outbreaks are major problems in the poultry industry. It is challenging to control avian influenza through vaccines due to several reasons, such as genetic variation among endemic viral strains and mismatching of the AIV vaccine currently used in the field resulting in incomplete protection against the disease and a failure of the vaccine to recognize certain serological samples (Iram *et al*., 2014; Shabbir *et al*., 2016). The development and the aim of an efficacious poultry vaccine against the H9N2 strain of avian influenza were strongly encouraged by bioinformatics analysis. The antigenic epitopes were evaluated using the proSA web server and ElliPro. Further, the correlation was shown between *in vitro* results and *in silico* analysis; the predicted epitopes were producing immune responses in immunized mice. *E. coli* expressed HA epitopes can bestow to the development of an effective vaccine against H9N2 influenza virus. This idea was supported by a report on an epitope region, in which approximately 70% of the sequence had the potential to induce an immune response (Shahid *et al*., 2015).

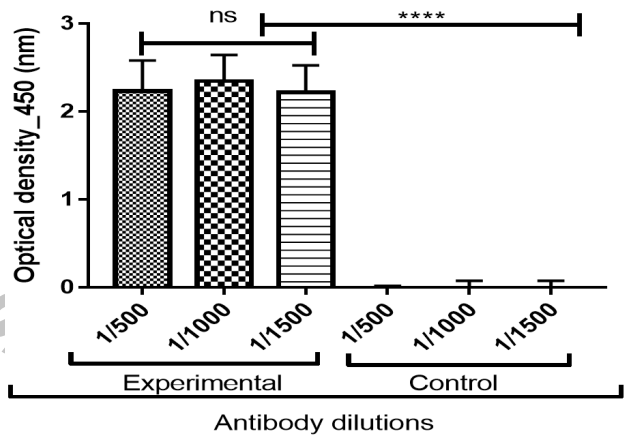

Fig. 7. ELISA assay. ELISA determined the titer of antibody produced in immunogenic mice, Diluted serum in 1/500 and 1/1500 effectively work as antibody as compared to 1:1000 dilution.

In this study, we explained the recombinant H9N2 HA protein with its immunogenic properties. The purified HA protein acted as the most significant antigen that can induce a strong immune response (Nagy *et al*., 2016; Hajam and Lee, 2017). The HA protein is a surface glycoprotein that attaches itself sturdily to the viral membrane, thus making it very difficult to separate and purify (Shahid *et al*., 2015). Immunization with recombinant HA protein has been chosen many times for immunization to study defensive immune responses in mice (laboratory model) (Boursnell *et al*., 1990; Sun *et al*., 2008). In the current study, an effort was made to evaluate the immune response against recombinant HA in mice at different time intervals; the induction of the immune response showed that recombinant HA protein had correctly folded. The efficacy of the obtained recombinant HA protein still needs comparison to the avian influenza vaccines already used in the field, including vaccine which is produced in embryonated chicken eggs. We used only purified HA antigen at a

concentration of 15 µg/dose in mice intramuscularly. Our results indicated that 15 µg of HA protein was safe for the mice and elicited a significant immune response that was stable for up to 50 days, but an immune response did not occur in the negative control group (Shen *et al*., 2013).

It is more appealing, even if the obtained recombinant HA in this study produces immunity comparable to that of avian influenza vaccines used in the field, as the recombinant technology described here can produce at least 1000 more doses than can egg-based vaccines in a parallel time interval. Moreover, a bacterial-expression-based vaccine production system is a cost-effective solution for most endemic and pandemic viral diseases (Song *et al*., 2008; Aguilar-Yáñez *et al*., 2010).

CONCLUSION

We conclude that the recombinant HA protein expressed in *E. coli* could potentially a substitute for commercial egg-based vaccines. Many potentially active recombinant vaccines against influenza have been produced in *E. coli*. As an example, recombinant HA63–286 produced in *E. coli* was immunogenic, produced neutralizing antibodies, and showed protection in a ferret model. Likewise, a recombinant 60-kDa HA protein from H5N1 produced in *E. coli* yielded a protective immune response in mice. These predicted epitopes will also be valued in the development of more immunogenic vaccines for avian influenza by targeting more-immunogenic epitopes.

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Statement of conflicts of interest

The autors have declared no conflicts of interest.

Research involving human participants and/or animals

All procedures in studies involving animals were performed in accordance with the ethical standards of the institution or practice at which the studies were conducted.

REFERENCES

Aguilar-Yáñez, J.M., Portillo-Lara, R., Mendoza-Ochoa, G.I., García-Echauri, S.A., López-Pacheco, F., Bulnes-Abundis, D., Salgado-Gallegos, J., Lara-Mayorga, I.M., Webb-Vargas, Y. and León-Angel, F.O., 2010. An influenza A/H1N1/2009 hemagglutinin vaccine produced in *Escherichia coli*. *PLoS One*, **5**: e11694. https://doi.org/10.1371/journal.pone.0011694

Boursnell, M., Green, P., Samson, A., Campbell, J., Deuter, A., Peters, R., Millar, N., Emmerson, P. and Binns, M., 1990. A recombinant fowlpox virus expressing the hemagglutinin-neuraminidase gene of Newcastle disease virus (NDV) protects chickens against challenge NDV. *J. Virol.*, **178**: 297-300. https://doi.org/10.1016/0042-6822(90)90408-J

Chiu, F.-F., Venkatesan, N., Wu, C.-R., Chou, A.-H., Chen, H.-W., Lian, S.-P., Liu, S.-J., Huang, C.-C., Lian, W.-C. and Chong, P., 2009. Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus. *Biochem. biophys. Res. Commun.*, **383**: 27-31. https://doi.org/10.1016/j.bbrc.2009.03.106

Davidson, I., Shkoda, I., Golender, N., Perk, S., Lapin, K., Khinich, Y. and Panshin, A., 2013. Genetic characterization of *HA* gene of low pathogenic H9N2 influenza viruses isolated in Israel during 2006–2012 periods. *Virus Genes*, **46**: 255-263. https://doi.org/10.1007/s11262-012-0852-4

Garten, W. and Klenk, H.-D., 2008. Cleavage activation of the influenza virus hemagglutinin and its role in pathogenesis. In: *Avian influenza* (eds. H-D. Klenk, M.N. Matrosovich and J. Stech), Monogr. Virol. Karger. Basel, pp. 156-167. https://doi.org/10.1159/000151618

Golchin, M., Moghadaszadeh, M., Tavakkoli, H., Ghanbarpour, R. and Dastmalchi, S., 2018. Recombinant M2e-HA2 fusion protein induced immunity responses against intranasally administered H9N2 influenza virus. *Microb. Pathog.*, **115**: 183-188. https://doi.org/10.1016/j.micpath.2017.12.050

Hajam, I.A. and Lee, J.H., 2017. Preexisting Salmonella-specific immunity interferes with the subsequent development of immune responses against the Salmonella strains delivering H9N2 hemagglutinin. *Vet. Microbiol.*, **205**: 117-123. https://doi.org/10.1016/j.vetmic.2017.05.021

Iram, N., Shah, M.S., Ismat, F., Habib, M., Iqbal, M., Hasnain, S.S. and Rahman, M., 2014. Heterologous expression, characterization and evaluation of the matrix protein from Newcastle disease virus as a target for antiviral therapies. *Appl. Microbiol. Biotechnol.*, **98**: 1691-1701. https://doi.org/10.1007/s00253-013-5043-2

Kim, E.-H., Lee, J.-H., Pascua, P.N.Q., Song, M.-S., Baek, Y.-H., Kwon, H.-i., Park, S.-J., Lim, G.-J., Decano, A. and Chowdhury, M.Y., 2013. Prokaryote-expressed M2e protein improves H9N2 influenza vaccine efficacy and protection against

lethal influenza A virus in mice. *J. Virol.*, **10**: 104. https://doi.org/10.1186/1743-422X-10-104

Lee, D.-H., Swayne, D.E., Sharma, P., Rehmani, S.F., Wajid, A., Suarez, D.L. and Afonso, C.L., 2016. H9N2 low pathogenic avian influenza in Pakistan (2012–2015). *Vet. Rec. Open.*, **3**: e000171. https://doi.org/10.1136/vetreco-2016-000171

Liu, D., Shi, W., Shi, Y., Wang, D., Xiao, H., Li, W., Bi, Y., Wu, Y., Li, X. and Yan, J., 2013. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: Phylogenetic, structural, and coalescent analyses. *Lancet*, **381**: 1926-1932. https://doi.org/10.1016/S0140-6736(13)60938-1

Monne, I., Yamage, M., Dauphin, G., Claes, F., Ahmed, G., Giasuddin, M., Salviato, A., Ormelli, S., Bonfante, F. and Schivo, A., 2013. Reassortant avian influenza A (H5N1) viruses with H9N2-PB1 gene in poultry, Bangladesh. *Emerg. Infect. Dis.*, **19**: 1630. https://doi.org/10.3201/eid1910.130534

Munir, M., Zohari, S., Abbas, M., Shabbir, M.Z., Zahid, M.N., Latif, M.S., Iqbal, M., Qureshi, Z.U.A. and Berg, M., 2013a. Isolation and characterization of low pathogenic H9N2 avian influenza: A viruses from a healthy flock and its comparison to other H9N2 isolates. *Indian J. Virol.*, **24**: 342-348. https://doi.org/10.1007/s13337-013-0144-1

Munir, M., Zohari, S., Iqbal, M., Abbas, M., Perez, D.R. and Berg, M., 2013b. The non-structural (NS) gene segment of H9N2 influenza virus isolated from backyard poultry in Pakistan reveals strong genetic and functional similarities to the NS gene of highly pathogenic H5N1. *Virulence*, **4**: 612-623. https://doi.org/10.4161/viru.26055

Nagy, A., Lee, J., Mena, I., Henningson, J., Li, Y., Ma, J., Duff, M., Li, Y., Lang, Y. and Yang, J., 2016. Recombinant Newcastle disease virus expressing H9 HA protects chickens against heterologous avian influenza H9N2 virus challenge. *Vaccine*, **34**: 2537-2545. https://doi.org/10.1016/j.vaccine.2016.04.022

Rajão, D.S. and Pérez, D.R., 2018. Universal vaccines and vaccine platforms to protect against influenza viruses in humans and agriculture. *Front. Microbiol.*, **9**: 123. https://doi.org/10.3389/fmicb.2018.00123

Shabbir, M.Z., Akhtar, S., Tang, Y., Yaqub, T., Ahmad, A., Mustafa, G., Alam, M.A., Santhakumar, D., Nair, V. and Munir, M., 2016. Infectivity of wild bird-origin avian paramyxovirus serotype 1 and vaccine effectiveness in chickens. *J. Gen. Virol.*, **97**: 3161-3173. https://doi.org/10.1099/jgv.0.000618

Shahid, N., Tahir, S., Rao, A.Q., Hassan, S., Khan, A., Latif, A., Khan, M.A., Tabassum, B., Shahid, A.A. and Zafar, A.U., 2015. *Escherichia coli* expression of NDV fusion protein gene and determination of its antigenic epitopes. *Biologia*, **70**: 1553-1564. https://doi.org/10.1515/biolog-2015-0191

Shen, H., Xue, C., Lv, L., Wang, W., Liu, Q., Liu, K., Chen, X., Zheng, J., Li, X. and Cao, Y., 2013. Assembly and immunological properties of a bivalent virus-like particle (VLP) for avian influenza and Newcastle disease. *Virus Res.*, **178**: 430-436. https://doi.org/10.1016/j.virusres.2013.09.009

Shen, S., Mahadevappa, G., Oh, H.L.J., Wee, B.Y., Choi, Y.W., Hwang, L.A., Lim, S.G., Hong, W., Lal, S.K. and Tan, Y.J., 2008. Comparing the antibody responses against recombinant hemagglutinin proteins of avian influenza A (H5N1) virus expressed in insect cells and bacteria. *J. med. Virol.*, **80**: 1972-1983. https://doi.org/10.1002/jmv.21298

Sippl, M.J., 1993. Recognition of errors in three dimensional structures of proteins. *Prot. Struct. Funct. Bioinfor.,* **17**: 355-362.

Song, L., Nakaar, V., Kavita, U., Price, A., Huleatt, J., Tang, J., Jacobs, A., Liu, G., Huang, Y. and Desai, P., 2008. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: A solution to global pandemic and seasonal needs. *PLoS One*, **3**: e2257. https://doi.org/10.1371/journal.pone.0002257

Sun, H.-L., Wang, Y.-F., Tong, G.-Z., Zhang, P.-J., Miao, D.-Y., Zhi, H.-D., Wang, M. and Wang, M., 2008. Protection of chickens from Newcastle disease and infectious laryngotracheitis with a recombinant fowlpox virus co-expressing the F, HN genes of Newcastle disease virus and gB gene of infectious laryngotracheitis virus. *Avian Dis.*, **52**: 111-117. https://doi.org/10.1637/7998-041807-Reg