Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/v9kymc31

AN EXPRESSION SYSTEM BASED ON BACULOVIRUS WAS UTILIZED IN ORDER TO EXPRESS THE HEMAGGLUTININ HA1 SUBMIT OF THE INFLUENZA VIRUS

Hira Aslam[1*], Praveenkumar Periyasamy[2], Michel Bolis[3], Likowsky Desir[4]

[*1]Pharmacy Student, Department of Pharmacy, University of Sargodha, Pakistan
Email: hirahassan1000@gmail.com
[2]Lecturer, Department of Anatomy, Levy Mwanawasa Medical University, Lusaka, Zambia
Email: praveenkumar.periyasamy@lmmu.ac.zm
[3]MD, Department of Clinical Laboratory, USA, Email: MBolis@aol.com
[4]Designation: MPH, MSc, Department of Surgery, Wyckoff Heights Medical Center
Email: kkLikowsky@gmail.com

***Corresponding Author:** Hira Aslam
*Pharmacy Student, Department of Pharmacy, University of Sargodha, Pakistan
Email: hirahassan1000@gmail.com

ABSTRACT:
Background: Respiratory diseases in horses are predominantly caused by equine influenza virus (EIV) infection, with vaccination being the primary preventive measure using inactivated whole virus vaccines produced mainly in embryonated hen eggs.
Objective: This study explores an alternative approach to traditional egg-based vaccine production by investigating the feasibility of using a baculovirus expression system for recombinant horse influenza hemagglutinin production.
Methods: A baculovirus expression vector was employed to clone and express the hemagglutinin ectodomain (HA1 subunit). Expression was confirmed using SDS-PAGE and immunoblotting, with viral protein yield quantified at 20 μg/ml from recombinant baculovirus-infected cells. Immunological response was evaluated in BALB/c mice immunized with recombinant HA1.
Results: Recombinant hemagglutinin produced in baculovirus-infected cells elicited a robust antibody response in mice, indicating its potential as an antigen for subunit vaccinations and diagnostic applications.
Conclusion: Baculovirus-mediated expression of recombinant horse influenza hemagglutinin shows promise as an effective alternative to egg-based vaccine production, offering potential advantages such as scalability, cost-effectiveness, and reduced dependency on egg supply.

KEYWORDS: Equine influenza virus, Vaccination, Inactivated whole virus vaccines, Baculovirus expression system, Recombinant hemagglutinin, Horse influenza, Subunit vaccines.

INTRODUCTION:

Infectious disorders that afflict horses, donkeys, and mules are referred to as equine influenza. This disease is currently recognized as one of the primary causes of respiratory ailments that affect horses worldwide (Atwa et al., 2024). The agent responsible for this condition is a virus member of the

Orthomyxoviridae family (Trombetta et al., 2022). Conventional vaccinations for equine influenza contain inactivated entire viruses, distributed either as an aqueous suspension or with adjuvants like aluminum hydroxide or aluminum phosphate (Gao et al., 2021). For vaccine manufacture, the influenza virus is often cultured in the amniotic cavity of fertile hens' eggs or in a cell culture maintained in a medium containing trypsin. After being extracted from egg allantoic fluid or culture medium, virions are inactivated or treated with detergent once retrieved. Because of their unpredictability and the variety of their biological constituents, these systems present a fundamental disadvantage that must be considered (Gao et al., 2021). For this reason, alternative approaches to manufacturing vaccines against equine influenza were devised (Pushko & Tretyakova, 2020). These include a vaccine that is vectored for canarypox (ProteqFlu, manufactured by Merial Inc (Kumar et al., 2024). in the United States), a subunit vaccine that contains purified hemagglutinin and neuraminidase proteins that have been adjuvant with ISCOMatrix (Equilis Prequenza), an attenuated live vaccine (Flu AvertTM I.N., manufactured by Intervet), and numerous inactivated influenza vaccines, with the latter being the one that is used commercially the most frequently. An examination of vaccination against equine influenza was carried out not too long ago (Ramirez, 2019). Protein Sciences Corporation announced in 2008 that they had developed a human influenza vaccine called FluBlok®. This vaccine was created using the insect cell/baculovirus technique (Mashudu, 2019). Clinical testing demonstrated that this vaccination is safe and efficacious (Yu et al., 2020). Regarding the production of recombinant proteins, insect cell systems have been extensively used (Tsai et al., 2019). Because of the high yield that can be produced from baculovirus-infected insect cells, these cells are potentially valuable instruments for the manufacture of therapeutic proteins (Keresztes et al., 2022). A recognized significant antigen in the host response to influenza virus in both natural infection and vaccination, hemagglutinin is the predominant glycoprotein on the surface of the influenza virus (Keresztes et al., 2022). It is also a critical antigen in the immune response to influenza virus. The initial translation of the protein, encoded by segment 4 of the viral genome, forms a preprotein (HA0) (Chen et al., 2021).

Table 1: Overview of Equine Influenza

Reference	Description
Atwa et al., 2024	Overview of equine influenza
Trombetta et al., 2022	Virus responsible for equine influenza
Gao et al., 2021	Conventional vaccines and their components
Pushko & Tretyakova, 2020	Alternative vaccine approaches
Ramirez, 2019	Examination of equine influenza vaccination

Table 2: Vaccine Types for Equine Influenza

Reference	Description
Kumar et al., 2024	Vectored vaccine (ProteqFlu)
Gao et al., 2021	Inactivated and subunit vaccines
Ramirez, 2019	Attenuated live vaccine

Table 3: Production Techniques and Systems

Reference	Description
Mashudu, 2019	Insect cell/baculovirus technique (FluBlok®)
Tsai et al., 2019	Insect cell systems for recombinant proteins
Keresztes et al., 2022	Baculovirus-infected insect cells for protein yield

Table 4: Hemagglutinin Structure and Function

Reference	Description
Chen et al., 2021	Structure and antigenicity of hemagglutinin
Keresztes et al., 2022	Role of hemagglutinin in immune response
Zhang et al., 2021	Baculovirus system for recombinant hemagglutinin

This preprotein is then divided into two subunits (HA1 and HA2) to form a trimeric structure (HA1-HA2). Only the HA1 subunit is visible in the spatial configuration that mature hemagglutinin assumes, and as a result, this is where most of its antigenic determinants are located (Chen et al., 2021). The current investigation assessed the efficacy of a baculovirus expression system as a possible approach to producing recombinant equine influenza hemagglutinin (Zhang et al., 2021).

VIRUS AND CELLS:

The allantoic cavity of eleven-day-old embryonated hen's eggs was used to propagate an Argentine equine influenza virus strain previously discovered in our laboratory. This strain has a high degree of homology to the strain A/equine/Argentina/1999 (H3N8), which is more than 99% similar and closely connected to the strains that belong to the Floride clade. (Zhang et al., 2021). For transfection and propagation of recombinant viruses, Spodoptera frugiperda (Sf9 and Sf21) cells (Gibco et al.) were cultured at a temperature of 27 degrees Celsius in TC-100 media (US Biological, Swampscott, Massachusetts, United States) supplemented with 10% Fetal Bovine Serum (FBS) (PAA Laboratories GmbH, Pasching, Austria). The recombinant HA1 subunit was expressed using Sf9, Sf21, and Trichoplusia ni High Five cells, all of which were obtained from Invitrogen in Carlsbad, California, United States of America. Grace's (Gibco), TC-100, and SF900II (Gibco) were the three specific culture media evaluated for effectiveness.

RT-PCR:AN AMPLIFIATION TECHNIQUE: Total RNA was extracted from the sample using the Trizol reagent (Invitrogen).In order to do reverse transcription, a total of 1 µg of RNA was utilized, along with the HA1R primer, which was 5µ CACTCGAGTTGCTTTTCTGGTACATTCCTC 3´, and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega et al.) (Gao et al., 2023). The supplier provided the parameters for this reverse transcription. A reaction mixture was prepared by adding 5 µl of the cDNA to a reaction mixture that contained a final concentration of 200 µM of each of the dNTPs, 1.5 mM of MgCl2, 0.25 U of Taq DNA polymerase (Fermentas et al., MD, USA), and 10 pmol of each of the primers: HA1R and HA1F: 5´

TACCATGGTCTACAGTCAAAACCCAACCAG 3´. After this, the PCR reaction was carried out. An Eppendorf Mastercycler gradient was utilized for the amplification process, which was carried out in Hamburg, Germany. After being denaturated at 94 degrees Celsius for two minutes, the reactions were cycled thirty times at 94 degrees Celsius for thirty seconds, 63 degrees Celsius for thirty seconds, and 72 degrees Celsius for one minute. After this, a final elongation step was performed at 72 degrees Celsius for ten meters. A 1.5% agarose gel was stained with ethidium bromide, and the PCR product was run on that.

THE DEVELOPMENT OF A VECTOR FOR THW TRANSMISSION OF BACULOVIRUS:

Following the ligation of the PCR-amplified HA1 region to a pCR2.1TOPO vector (Invitrogen), the cells were converted into One Shot TOP10 Competent Cells (Invitrogen) (Deb Nath, 2021). The recombinant clones that included the insert were subjected to endonuclease restriction with XhoI (Promega) and DNA sequence analysis (Biotechnology Resource Center – Cornell University – Ithaca – New York, USA) in order to guarantee that the insert was oriented correctly (Gao et al., 2023). Digestion with NcoI and BamHI (Promega) was performed on the HA1 coding sequence to facilitate subcloning. A recombinant baculovirus was produced using the plasmid that was produced, which was given the designation pFastBac-HA.

CREATING A RECOMBINANT BACULOVIRUS THROUGH AN EXPERIMENT:
It was necessary to transform the pFastBac-HA vector into Escherichia coli DH10Bac (Invitrogen) in order to induce transposition between the recombinant transfer vector and the bacmid of the Autographa californica nuclear polyhedrosis virus (bAcNPV). White recombinant and blue non-recombinant colonies could be distinguished from one another after the incubation period had concluded. There were a total of twelve white colonies and one blue colony that were subjected to colony PCR with M13 universal primers in accordance with the technique that was supplied in the Bac to Bac kit handbook (Invitrogen). This was done to confirm that the HA1 segment had been transposed into the bAcNPV (Gromadzka et al., 2022).The recombinant bacmid DNA that was recovered from white colonies that had been evaluated in the past was utilized in the process of transfecting Sf21 cells with the help of Insect GeneJuice® Transfection Reagent (Novagen et al.) (Bodle et al., 2023). At 27 degrees Celsius, the transfected cells were grown in TC-100 medium supplemented with 10% fetal bovine serum. After 96 hours had passed since the transfection, a primary viral stock was gathered and utilized to deliver two additional rounds of infections. After 72 hours of the second infection, the supernatant of the culture medium was preserved at a temperature of -70 degrees Celsius to be utilized as viral stock in subsequent protein expression tests (Sączyńska et al., 2021).

AN ANALYSIS OF THE PURIFICATION OF THE HA1 PROTEIN: Utilizing the nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen et al.) by the established protocols allowed for producing a homogenous and high-yield preparation of the HA1 subunit. CellStar – Greiner cell culture flasks were used to create a fresh culture with a more significant volume (25 ml of culture medium with 106 cells/ml) once the best expression conditions were discovered (see Results). This culture was carried out in a cell culture flask with a surface area of 175 cm 2 (Narayan et al., 2020). Following the infection, the cells were extracted and washed three times with sterile PBS. This process was repeated three times. The entire pellet was sonicated to disrupt the cells using a Binding Buffer (50 mM NaH2 PO4, 500 mM NaCl, pH 8.0). A Ni-NTA column that had been pre-balanced with Binding Buffer was loaded with the total protein solution that was present in the supernatant after the cell lysate was pelleted at a rate of 5000 g per 10 m (Alqazlan et al., 2022). The bound protein was washed with a solution of 50 mM sodium hydroxide, 500 mM sodium chloride, and 20 mM imidazole at a pH of 8.0 until the absorbance at an optical density of 280 nm returned to the baseline value. Following the stabilization of the baseline, the elution of the bound recombinant protein was performed with 20 milliliters of 50 millimolar sodium hydroxide phosphate, 500 millimolar sodium chloride, 250 millimolar imidazole (Merck), and a pH of eight. Through the process of precipitation with 100% ethanol, the recombinant protein that had been eluted was concentrated, and then it was resuspended in 10 milliliters of sterile PBS (Rockman et al., 2022).

HA1 PROTEIN ADMINISTERED TO MICE FOR THE PURPOSE OF MINIMIZATION:
Sixteen female BALB/c mice were six weeks old and were randomly assigned to one of four groups. Every animal procedure was carried out in a manner that conformed with the authorized protocols and the recommendations regarding the appropriate utilization and care of laboratory animals. In Group I, the intranasal (i.n.) administration of 1μg of pure protein was administered, with the Bradford assay being used to quantify the amount of protein. The second group was administered intramuscularly (i.m.) with a mixture consisting of 25 microliters of HA1 protein solution, which included 500 nanograms of pure protein, and 25 microliters of Specol adjuvant, which was manufactured by ID-DLO, Lelystad, and The Nether (Bullard & Weaver, 2021). (territories). Group III was administered intramuscularly (i.m.) with 50 microliters (μl) of HA1 protein solution equal to one microgram (μg) of pure protein. Group IV served as the control group for those who had not been inoculated (table 1). On the 15th and 30th day after the priming, two booster immunizations were administered under the same conditions, but in Group II, no adjuvant was administered.In order to conduct the Haemagglutination Inhibition Test (HI), serum samples from each group were collected,

pooled, and analyzed prior to each inoculation. On the fifteenth day after the last immunization, all mice were slaughtered, and the level of anti-HA1 antibodies was determined using Western blot (WB) and high-sensitivity (HI) samples, respectively, from the pooled and individual samples. The antigen that was utilized was the pure virus that was taken from the allantoic fluid of embryonated hen eggs that were 11 days after their conception.

HEMAGGLUTINATION INHIBITION TEST MUST BE PERFORMED:

According to the protocols that are generally accepted, the HI test was carried out in microtiter plates with a V-bottom using four hemagglutinating units (HAU) of the antigen for the equine influenza virus (H3N8) and 0.5 percent of chicken red blood cells. Following the analysis of variance (ANOVA), Tukey's post-hoc test was utilized to evaluate the differences in log-transformed data derived from HI titers across the experimental groups. In order to establish the level of statistical significance, the P Value was set at less than 0.05.

THE EXAMINATION OF IMMUNOBLOTS:

Using a semi-dry transfer blotter (BioRAD et al.) at a current of 150 mA for a duration of 30 meters, recombinant and viral proteins that SDS-PAGE separated were electrotransferred onto a Nitrocellulose membrane with a pore size of 0.22 μm of Sigma. The membrane was blocked for 24 hours at a temperature of 4 degrees Celsius in PBS containing 5% skim milk and 0.1% Tween-20. Following this, it was reacted with a 1:100 dilution of both sera pools. This was followed by incubation with horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (Sigma) at a dilution of 1:1000 for 1.5 hours at room temperature. The membrane was washed three times with washing buffer (PBS containing 0.1% Tween-20) after 1.5 hours had passed. Following the completion of the final washing phase, the membrane was developed in phosphate-buffered saline (PBS) containing 0.05% diaminobenzidine tetrahydrochloride (Wako pure) in the presence of hydrogen peroxide.

RESULT:

Using HA1F and HA1R primers, the RT-PCR amplification assay was used to construct the baculovirus transfer vector. This resulted in the generation of a unique fragment that was visible on agarose gel and had the expected molecular size of 1002 base pairs. More than one hundred clones were obtained by cloning the PCR product into the pCR2.1-TOPO vector and then transforming it into TOP10 bacteria. Endonuclease restriction with XhoI was used to evaluate twelve of them randomly chosen to guarantee that they were correctly oriented. A single clone that was oriented correctly was selected during the sequencing analysis. The right open reading frame of the cloned segment was authenticated through sequencing analysis. In conclusion, the HA1 fragment was digested using the NcoI and BamHI enzymes. Then, it was ligated into the pFastBac HTB vector to generate the plasmid pFastBac-HA.

Table 1: Diagram of the immunization

Ensemble	IV inoculation through the	The amount of protein	The volume of the Special	Volume used for inoculation
1	i.n.	50μl	-	50μl
2	i.m.	25μl	25μl	50μl
3	i.m.	50μl	-	50μl
4	unvaccinated group serving as the control			

A RECOMBINANT BACULOVIRUS WAS PRODUCED SUCCESSFULLY:

When pFastBac-HA was transformed into E. coli DH10Bac, recombinant baculoviruses were produced as a result. Many colonies were found in the Luria broth plates after being incubated for one and a half days. Colony-PCR was performed with M13 universal primers to examine twelve white colonies and one blue colony that was determined to be a non-recombinant control. In eleven of the

colonies that appeared to be positive, the hypothesis that the HA1 segment had been transposed into the bAcNPV vector was confirmed. A decrease in cell growth, an increase in cell width, and the separation of cells from the monolayer were seen four days following the infection of Sf21 cells with the Sf21 virus. Seventy-two hours following the re-infection of fresh Sf21, signs of cell infection were confirmed to have been present.

EXPRESSION OF THE RECOMBINANT HA1 PROTEIN DURING THE PROCESS:
Using SDS-PAGE, a recombinant protein with a molecular weight of about 40 kDa was identified following the infection of Sf9, Sf21, and High Five cells. In addition to being comparable to the size of the genuine viral HA1 subunit, the size of the recombinant protein was estimated to be approximately equivalent to the molecular weight expected for the HA1 that had the N terminal His tag. After 96 hours of infection, the highest level of recombinant protein expression was reached in High Five cells cultivated in TC-100 media supplemented with 10% of FBS, as demonstrated by the analysis of cell lysate and cell culture media (figure 1). During the 96-hour examination, neither Sf-9 nor Sf-21 cells exhibited significant expression levels (note that the data are not included here).

AN ANALYSIS OF THE PURIFICATION OF THE HA1 PROTEIN: By using Immobilized Metal-ion Affinity Chromatography (IMAC) using NTA-Ni2+ resin (QIAGEN), the recombinant HA1 protein was purified to a level that was very close to being homogeneous. Using SDS-PAGE, the purity of the protein was examined and validated (figure 2). The quantity of proteins present in the Bradford assay, the standard method, was used to determine it. Upon completion of the purification process, a final concentration of 20 micrograms per milliliter of protein was obtained. The quantity of protein that was assessed using the Bradford assay method corresponded fully to the recombinant HA1 protein, and the existence of a single band on the polyacrylamide gel (Fig. 2) showed that the protein had been purified by IMAC practically to the point of homogeneity.

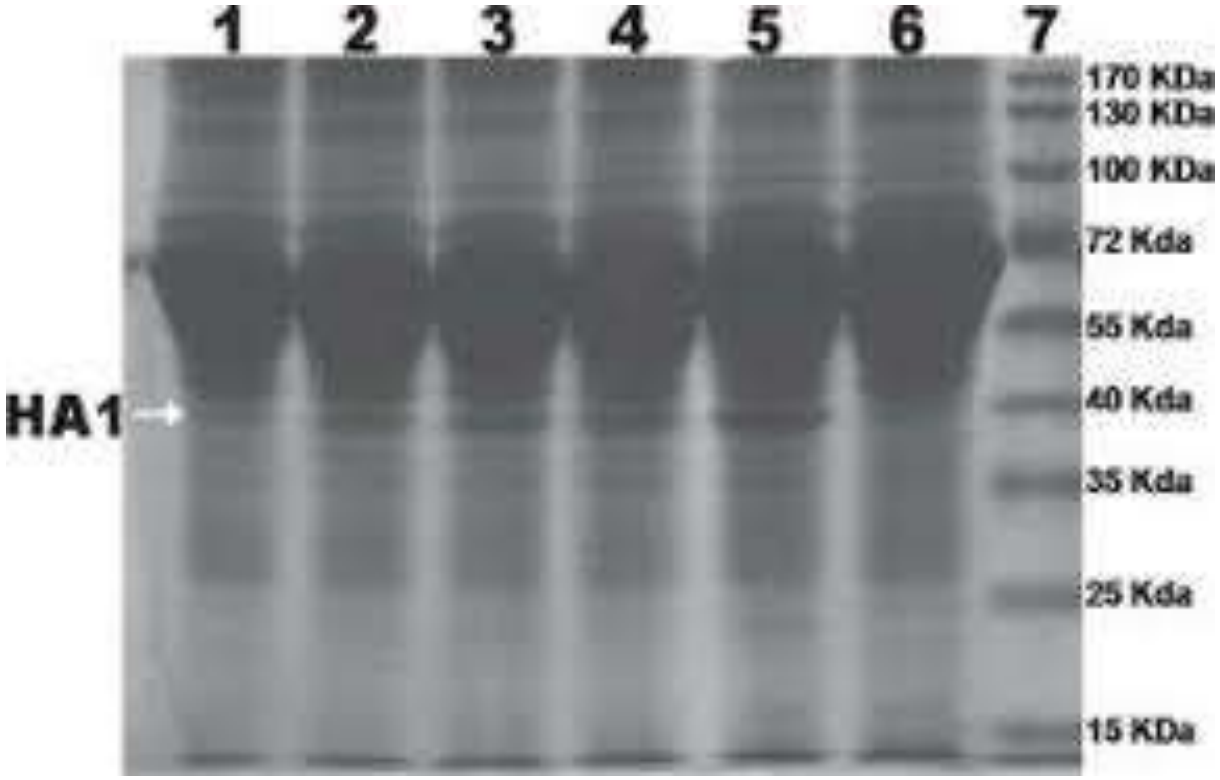

FIGURE 1: Recombinant protein was expressed by the use of SDS-PAGE analysis.
Lines 1 through 5 contain cell lysates collected 12, 24, 48, 72, and 96 hours after infection. Line 6 has a control consisting of High Five cells that were not infected. Line 7 contains a molecular weight marker called Fermentas.

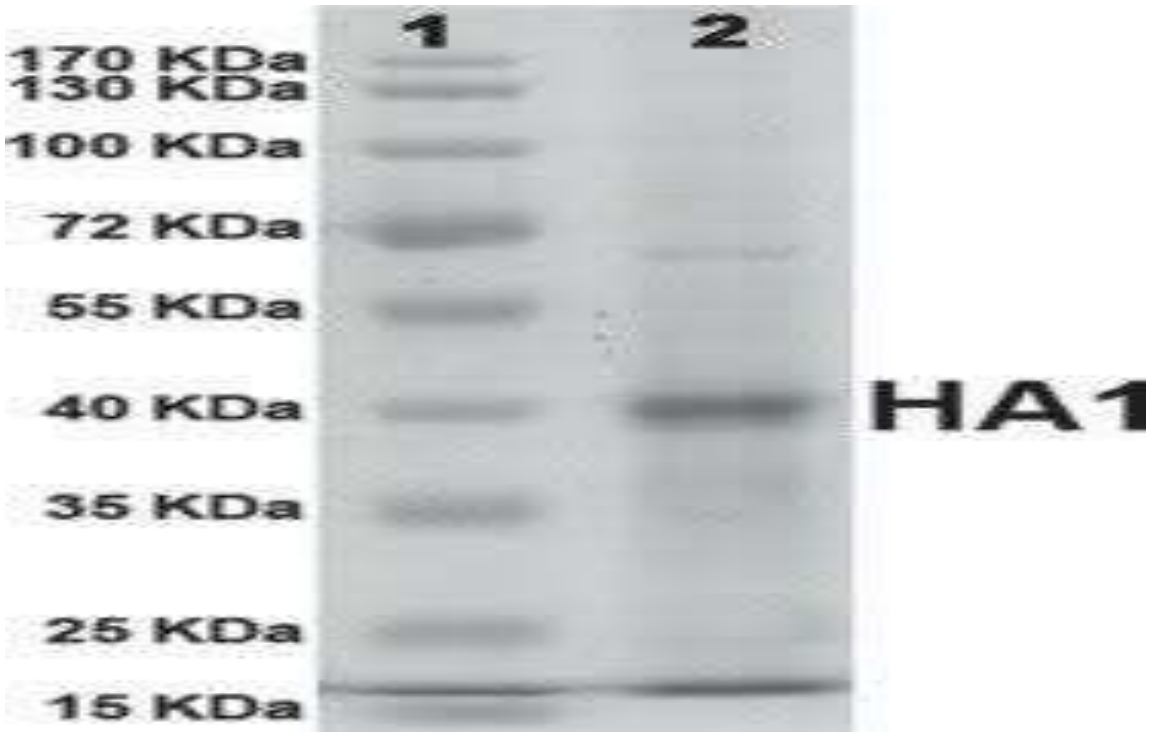

FIGURE 2: The purified recombinant HA1 protein was subjected to SDS-PAGE analysis. Lane 1 contains a molecular weight marker called Fermentas, while lane 2 contains a pure recombinant protein isolated using Ni2+ affinity.

HEMAGGLUTINATION INHIBITION TEST MUST BE PERFORMED: In the beginning, a logarithmic adjustment of the data was carried out to analyze the HI findings (table 2). According to Levene's test results, which were performed with a confidence level of 95.0% (P> 0, 42), no significant variations were identified between the standard deviations of any group. A one-way analysis of variance (ANOVA) was performed on the data, and the results showed significant differences between the cohorts of animals that had been injected (table 3). A Tukey's test was conducted to ascertain whether groups had remarkable differences. A comparison of Group II (animals infected intramuscularly with adjuvant) and other groups revealed significant differences between the two groups. In addition, between Group III (IM) and Group IV (control that had not been infected), significant changes were discovered; however, there were no discernible differences observed between Group I (IN) and the control. The subsequent qualitative confirmation of these results was accomplished by using western blot analysis, which was performed on a pool of sera from each group (figure 3).

Table 2: By group, the HI titer

	GROUP			
	1	2	3	4
Mouse1	4	64	4	2
Mouse2	8	64	16	4
Mouse3	4	128	16	2
Mouse4	4	64	8	2

Table 3: ANOVA using one-way analysis of variance: natural log of HI titer versus groups

Source	df	ss	ms	P	
Group	3	26.905	8.968	44.800	8.6*10
The error	12	2.402	0.200		
total	15	29.308			

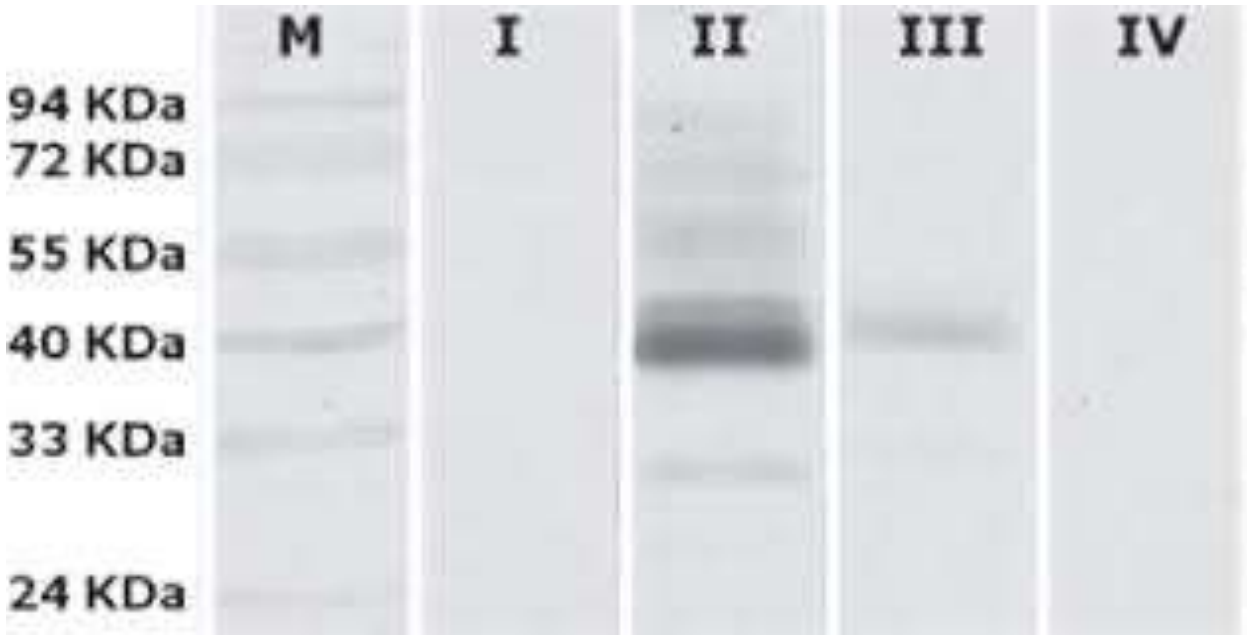

FIGURE 3: The Western blot analysis of sera pools is shown in Figure 3. M represents the PageRuler prestained marker (Fermentas), lane I represents the intranasal administration of the vaccine, lane II represents the intramuscular administration of the vaccine with adjuvant, and lane III represents the intramuscular administration of the vaccine without adjuvant. Lane IV represents the control group that was not inoculated.

DISCUSSION:

Large-scale recombinant protein production is the main benefit of the baculovirus expression technology. Using baculovirus-insect cell systems, previous research has revealed large quantities of viral proteins. Purified hemagglutinin protein from silkworm larvae infected with recombinant baculovirus14 was obtained by Sugiura from 0.4–4 μg/larvae, while Nwe et al. acquired 77 μg/106 cells of recombinant HA1 protein monolayer culture insect cells10. For this investigation, 20 μg/ml, or 200 μg of pure HA1 from 2.5x107 cells, were extracted from recombinant baculovirus-infected cells. Knowing whether the rHA produced in insect cells is appropriately glycosylated is critical because it may be crucial to HA18–20's biological activity. Though Wang et al. had shown that all N-linked oligosaccharide chains were present in the HA1 region when a baculovirus expression system was employed, suggesting that the rHA produced in insect cells was glycosylated with N-linked oligosaccharide side chains21, we did not analyze the HA1 glycosylation pattern during the current experiment. Our initial examination of mice that had received an immunization showed that the HA1 recombinant protein remained antigenic. Special served as an adjuvant in this investigation. Bokhout et al.2 described Specol. Two emulsifiers and a non-metabolizable mineral oil (Marcol 52) comprise its composition. IgG1 isotype 1 antibodies are often the ones that Specol primarily induces. An earlier investigation using mice7 found that Specol would make a good substitute for Freund's complete adjuvant (FCA). With fewer side effects than other water-oil adjuvants like FCA and potent immunostimulant qualities, specol is widely accepted as safe for usage in animals1, 2. Since the HI titer obtained was higher when 500 ng of the protein with the adjuvant (Group II) than when 1 μg of protein was inoculated in an aqueous solution (Group III), the use of Specol as an adjuvant significantly enhanced the immune response of immunized mice. HI and Western blot, on the other hand, did not reveal any appreciable antibody levels in blood samples from animals who received the injection intranasally (Group I) (fig. 3). According to earlier research, intranasal delivery of 1.5 μg of the HA recombinant baculovirus vaccine produced virus-specific antibodies, as shown by the enzyme-linked immunosorbent test (ELISA), but did not produce antibodies that neutralized the viral (VN)11. This shows that variables that affect vaccination efficacy, like dosage, mode of administration, and amount of protein injected, may significantly impact a subunit vaccine's efficacy. The collective

findings suggest that the HA1 component, expressed in insect cells as N-terminus fusion proteins containing a His-Tag sequence, may be synthesized in significant amounts in a biochemically active form from a tiny culture volume. Since egg ovalbumin is absent from the protein, it may be readily purified, removing the possibility of negative responses. Besides, there will not be any need for organic extraction or virus inactivation, so there will not be any denaturing impacts or further safety issues from the vaccine's leftover hazardous chemicals. One way to get around the drawbacks of the present egg-based production technique for the equine influenza vaccine is to use recombinant technology. Our findings highlight the value of HA1 subunit vaccination in conjunction with an adjuvant to improve antigen-specific immune response, even though more research is required to evaluate the viability of the HA1 recombinant protein for vaccine manufacturing. Future research may also assess a suitable noninflammatory adsorptive adjuvant, like carbomer polymer or aluminum hydroxide, to find horses' ideal formulation and inoculation route. The development of horse influenza subunit vaccines and diagnostic tests, as well as a viable replacement for the current egg-based inactivated vaccines, could be facilitated by the baculovirus system's easy adaptation to large-scale production of various HA antigens.

CONCLUSION:

Protection of subjects who are humans or animals. The authors affirm that the methods used complied with the guidelines set forth by the responsible Clinical Research Ethics Committee, the World Medical Association, and the Helsinki Declaration. Privacy of Information. The writers affirm that this article, entitled to informed consent and privacy, contains no patient data. According to the authors, this report contains no patient data.

REFERENCE.

1. Alqazlan, N., Astill, J., Raj, S., & Sharif, S. (2022). Strategies for enhancing immunity against avian influenza virus in chickens: A review. *Avian Pathology*, *51*(3), 211-235.
2. Atwa, A. S., Gomaa, L., Elmenofy, W., Amer, H. M., & Ahmed, B. M. (2024). Expression of recombinant Florida clade 2 hemagglutinin in baculovirus expression system: A step for subunit vaccine development against H3N8 equine influenza virus. *Open Veterinary Journal*, *14*(1), 350.
3. Bodle, J., Vandenberg, K., Laurie, K., Barr, I. G., Zhang, Y., & Rockman, S. (2023). An ELISA-based assay for determining haemagglutinin potency in egg, cell, or recombinant protein-derived influenza vaccines. *Frontiers in Immunology*, *14*, 1147028.
4. Bullard, B. L., & Weaver, E. A. (2021). Strategies targeting hemagglutinin as a universal influenza vaccine. *Vaccines*, *9*(3), 257.
5. Chen, J., Wang, J., Zhang, J., & Ly, H. (2021). Advances in the development and application of influenza vaccines. *Frontiers in immunology*, *12*, 711997.
6. Deb Nath, N. (2021). *Production of monoclonal antibody based on HA and NP protein of bat H18N11 influenza virus in mice*
7. Gao, J., Wan, H., Li, X., Rakic Martinez, M., Klenow, L., Gao, Y., Ye, Z., & Daniels, R. (2021). Balancing the influenza neuraminidase and hemagglutinin responses by exchanging the vaccine virus backbone. *PLoS Pathogens*, *17*(4), e1009171.
8. Gao, R., Wang, Z., Uprety, T., Sreenivasan, C. C., Sheng, Z., Hause, B. M., Brunick, C., Wu, H., Luke, T., & Bausch, C. L. (2023). A fully human monoclonal antibody possesses antibody-dependent cellular cytotoxicity (ADCC) activity against the H1 subtype of influenza A virus by targeting a conserved epitope at the HA1 protomer interface. *Journal of Medical Virology*, *95*(7), e28901.
9. Gromadzka, B., Chraniuk, M., Hovhannisyan, L., Uranowska, K., Szewczyk, B., Narajczyk, M., & Panasiuk, M. (2022). Characterization of the immune response towards the generation of universal anti-HA-stalk antibodies after immunization of broiler hens with triple H5N1/NA-HA-M1 VLPs. *Viruses*, *14*(4), 730.

10. Keresztes, G., Baer, M., Alfenito, M. R., Verwoerd, T. C., Kovalchuk, A., Wiebe, M. G., Andersen, T. K., Saloheimo, M., Tchelet, R., & Kensinger, R. (2022). The highly productive thermothelomyces heterothallic C1 expression system is a host for the rapid development of influenza vaccines. *Vaccines*, *10*(2), 148.
11. Kumar, R., Bera, B. C., Anand, T., Pavulraj, S., Kurian Mathew, M., Gupta, R., Tripathi, B. N., & Virmani, N. (2024). Evaluation of immunogenicity and protective efficacy of bacteriophage conjugated haemagglutinin based subunit vaccine against equine influenza virus in a murine model. *Veterinary Research Communications*, pp. 1–20.
12. Mashudu, N. (2019). *Heterologous expression of equine influenza virus major surface glycoproteins and determination of their immunogenicity in animal models*. University of Johannesburg (South Africa).
13. Narayan, C., Kwon, J., Kim, C., Kim, S.-J., & Jang, S. K. (2020). Virus-based SELEX (viro-SELEX) allows the development of aptamers targeting knotty proteins. *Analyst*, *145*(4), 1473-1482.
14. Pushko, P., & Tretyakova, I. (2020). Influenza virus-like particles (VLPs): opportunities for H7N9 vaccine development. *Viruses*, *12*(5), 518.
15. Ramirez, E. (2019). *Generation and Characterization of Dual-Fluorescent Influenza Virus-Like Particles (VLPs) in Insect Cells* University of Waterloo].
16. Rockman, S., Laurie, K., Ong, C., Rajaram, S., McGovern, I., Tran, V., & Youhanna, J. (2022). Cell-based manufacturing technology increases the antigenic match of the influenza vaccine and improves its effectiveness. *Vaccines*, *11*(1), 52.
17. Sączyńska, V., Florys-Jankowska, K., Porębska, A., & Cecuda-Adamczewska, V. (2021). A novel epitope-blocking ELISA for specific and sensitive detection of antibodies against H5-subtype influenza virus hemagglutinin. *Virology Journal*, *18*, 1-14.
18. Trombetta, C. M., Marchi, S., & Montomoli, E. (2022). The baculovirus expression vector system: A modern technology for future influenza vaccine manufacturing. *Expert Review of Vaccines*, *21*(9), 1233-1242.
19. Tsai, C.-H., Wei, S.-C., Jan, J.-T., Liao, L.-L., Chang, C.-J., & Chao, Y.-C. (2019). Generation of Stable Influenza Virus Hemagglutinin through Structure-Guided Recombination. *ACS Synthetic Biology*, *8*(11), 2472-2482.
20. Yu, L., Pan, J., Cao, G., Jiang, M., Zhang, Y., Zhu, M., Liang, Z., Zhang, X., Hu, X., & Xue, R. (2020). AIV poly antigen epitope expressed by recombinant baculovirus induces a systemic immune response in chicken and mouse models. *Virology Journal*, *17*, 1-13.
21. Zhang, J., Ma, K., Li, B., Chen, Y., Qiu, Z., Xing, J., Huang, J., Hu, C., Huang, Y., & Li, H. (2021). A risk marker of tribasic hemagglutinin cleavage site in influenza A (H9N2) virus. *Communications Biology*, *4*(1), 71.

22.