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Research Article

Expression and Characterization of the Hemagglutinin Gene of Avian Influenza Virus Subtype H9 in the Protozoan Host *Leishmania tarentolae*

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Abstract

During the last decades avian influenza viruses of subtype H9N2 became panzootic and have been isolated in multiple avian species throughout Asia, the Middle East, Europe and Africa. Co-circulation with other influenza viruses enhances the potential of reassortment events with the emergence of new viruses. Phylogenetic analyses showed that viruses of subtype H9N2 served as donor of internal gene segments for highly pathogenic influenza viruses of subtype H7 and H5. Therefore vaccination against influenza viruses of subtype H9N2 is of high relevance and economical importance. As an alternative to laborintensive and time consuming conventional egg-based influenza vaccine and mammalian cell culture propagation, recombinant H9 protein production using eukaryotic systems are being applied towards the development of influenza vaccines. Based on this concept, haemagglutinins (HA) from influenzavirus H9N2 strains were expressed in *Leishmania tarentolae*. This expression system, based on a parasite of lizards, yields proteins with animal like glycosylation pattern and can be readily propagated to high cell density (>10^8 cells/ml) at 26°C. The gene encoding HA was cloned from the influenza H9N2 strain named A/chicken/Bangladesh/VP01/2006(H9N2). Soluble HA proteins were secreted into the cell culture medium and were successfully purified via a His-Tag domain fused to the proteins. After purification of protein using Ni-NTA agarose, this system resulted in a yield of approximately 2–5 mg of HA per liter of culture. The expressed recombinant HA proteins were characterized by SDS-PAGE and western blotting using different antisera. Deglycosylation using Endo H proved that the recombinant HA was glycosylated. The produced recombinant glycosylated protein may act as a promising vaccine candidate for preventing H9N2 avian influenza associated disease.

ABBREVIATIONS

AI: Avian Influenza; HA: Hemagglutinin; Ni-NTA: Nickel-Nitrilotriacetic Acid; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Glectrophoresis; HRP: Horseradish Peroxidase

INTRODUCTION

Avian influenza (AI) is a highly contagious, acute viral respiratory disease of birds caused by influenza viruses resulting in significant morbidity and mortality in different bird species throughout the world [1,2]. Influenza viruses are single stranded RNA viruses belonging to the *Orthomyxoviridae* family, have been shown to infect a wide variety of wild and domestic birds such as turkey, chicken, quail, pheasant, chukar and other minor domestic poultry [3,4,5]. Avian influenza viruses of subtype H9N2 have been circulating in multiple avian species in Eurasia resulting in great economical losses especially in poultry holdings due to reduced egg production and moderate to high mortality [6,7]. In addition to infection of poultry, H9N2 infections have also been sporadically identified in pigs and human [8,9] which suggests that some of these have rapidly evolved and acquired mutations to facilitate their ability to replicate in mammalian hosts [10]. In Bangladesh the continued co-circulation of subtypes H9N2 and H5N1, and evolution of H9N2 viruses increase the possibility of generating new reassortant strains [11,12,13]. The ability of H9N2 viruses to acquire the human like receptor specificities [14] and their transmissibility to mammalian species including human raises public health concerns [10,15].

As there is no effective and specific treatment for AI in poultry, the precise diagnosis of AI, strict biosecurity measures and vaccination can help to control the disease. Protection against influenza virus is primarily mediated by antibodies to the

viral HA and NA [16,17]. Therefore, the HA glycoprotein has been recognized as key antigen of choice in the host response to avian influenza virus in both natural infection and vaccination [18,19]. In order to establish a promising vaccine or vaccine candidates against influenza infection recombinant protein-based approach has been used widely as an alternative to the established conventional egg-based and mammalian cell culture based influenza vaccines. A variety of recombinant protein expression system have been developed for HA gene expression using both prokaryotic and eukaryotic systems such as bacteria, yeast, mammals, algae, insects, transgenic animals and plants [20-28]. These provide advantages over current approaches, including well developed technologies for mass production within a limited time frame. These expression systems facilitate safe, quality-controlled and scalable conditions, and reduce biohazards due to lack of egg contaminant during manufacturing, and also allow an accelerated response to emerging influenza strains. Since the last decade recombinant HA expressed in baculovirus-based expression system has been evaluated as vaccine candidates and reported to provide considerable protection in animals [29,30]. Although *Leishmania tarentolae* has been proposed as a novel, alternative eukaryotic expression system for recombinant protein production [31], but very limited viral protein has been expressed using the expression system. It would facilitate the production of large quantities of glycosylated protein due to high growth rate of *L. tarentolae* [31].

To explore alternative approaches for the production of functional influenza antigens, this report investigates the expression and secretion of recombinant HA protein from Bangladeshi isolates of H9N2 virus using this novel eukaryotic cell based expression system. The integrated/inducible expression system (Jena Bioscience, Jena, Germany) and the *Leishmania tarentolae* T7-TR strain were used to produce recombinant HA (H9) protein.

MATERIALS AND METHODS

Influenza virus and vector

The influenza viruses isolate A/chicken/Bangladesh/VP01/2006 (H9N2) (Accession No. KC986294) characterized by Parvin et al., 2014 [12], was used for expression of recombinant HA protein. The inducible LEXSY expression system (Jena BioScience, Jena, Germany) which includes the pLEXSY_I-neo3 expression vector and *Leishmania tarentolae* T7-TR expression host was chosen for the expression of the HA protein. In this system the HA gene is inserted into the expression cassette under the control of T7 promoter with TET operator (TRE) and integrated into the chromosomal ornithindecarboylase (odc) locus of the *Leishmania tarentolae* T7-TR strain that constitutively express bacteriophage T7 RNA polymerase and TET repressor under the control of host RNA polymerase I. Induction of the expressed protein was carried out via the T7 promoter inducible by tetracyclin addition as per the manufacturer's instructions (user's guide EGE-245, Jena Bioscience, Jena, Germany). The pLEXSY_I-neo3 allows insertion of the target gene into the cloning sites in a way that proteins are expressed either intracellularly or secreted in to the culture medium.

Amplification of HA gene and cloning in to the pLEXSY_I-neo3 vector

In order to facilitate secretory expression, the transmembrane and cytoplasmic tail was deleted from the HA gene. Then the customized HA gene was amplified from p JET2.1- HA following slightly modified protocol based on that of Hoffmann et al. 2001[32], using a forward and reverse primers consisting of *Xba*I and *Kpn*I restriction sites at their 5' ends respectively. The primer sequences were: H9N2 *Xba*I_FP_HA 5'- CCGTCTAGACATGGAAATAATATCACTGATAACTATAC-3' and H9N2 *Kpn*I_RP_HA 5'-TACCGGGTACCTTTGTAAGTACCCTCAGATTCCAG-3'. The restriction sites for *Xba*I and *Kpn*I are underlined. The PCR product and thepLEXSY_I-neo3 vector were digested with *Xba*I and *Kpn*I (New England Biology, Biolabs) and purified from 1% agarose gel (GeneJET Gel Extraction kit, Thermo Scientific, Darmstadt, Germany). After determination of the concentration of purified DNA, the prepared digested DNA and plasmid was ligated using T4-DNA ligase (New England Biology, Biolabs). The ligation mixture was transformed into the DH10B competent cells and grown overnight at 30°C on LB-amp plates supplemented with 100 µg/ml ampicillin.

The expression vector contained the natural signal sequence of HA gene and C-terminal His_6 stretch. The signal sequence of the vector (derived from *Leishmania Mexicana*) was also maintained. Positive clones were identified by PCR using HA specific primers and also with the primer pairs included with the LEXinduce3 kit (Jena Bioscience, Jena, Germany). Finally the recombinant plasmids of positive clones were isolated using QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) and sequenced by a commercial facility using forward P1442 and reverse A264 primers (Jena Bioscience, Jena, Germany).

Cultivation and transfection of the LEXSY host strain T7-TR

In order to transfect the linearized expression cassette into the *L. tarentolae* T7-TR strain, the expression plasmid containing HA gene was digested with *Swa*I (FastDigest, Thermo Scientific, Germany). In the meantime, *L. tarentolae* were cultivated at a 1:10 dilution in Brain Heart Infusion (BHI) media containing penicillin and streptomycin, hemin, LEXSY NTC and LEXSY Hygro at 26°C. When the cultures reachedthe OD_{600} value of 1.4, motile cells @ $6x10^7$cells/ml were subjected to transfection by electroporation at 1000V and 160µsec. After transfection cells were transferred to fresh LEXSY BHI medium in a ventilated tissue culture flask and incubated overnightat 26°C as static suspension culture. Approximately 20 hrs after electroporation, selection LEXSY antibiotics were added. After selection, confirmation of genomic integration of the expression cassette containing HA sequences was performed by diagnostic PCR as recommended by the manufacturer.

Growth of *L. tarentolae* containing recombinant HA and induction of HA expression

Recombinant parasites were cultivated in 50 ml BHI medium supplemented with hemin and antibiotics at 26°C in the dark. In order to induce the production of the recombinant HA protein, 10µg/ml of tetracycline was added at the time of inoculation of

parasite and high cell densities (OD_{600}: 2.5). For optimization of induction of the T7 promoter driven transcription, different concentration (10, 20 and 30 µg/ml) of tetracycline was added into the supplemented medium at the time of inoculation of the parasites. Culture were harvested 72 h after induction and centrifuged at 4000x g for 30 min. Supernatants were filtered (0.22 µm) and analyzed for the evaluation of the recombinant HA by SDS-PAGE and western blot.For large scale expression of HA protein 1L leishmania culture was cultivated in 2L Erlenmeyer flaskand agitated at 100 rpm.

Evaluation of the HA expression

Initially, about 20 ml sterile-filtered supernatant were concentrated with tricholoacetic acid (TCA) as recommended by manufacturer (Jena BioScience, Jena, Germany). Concentrated protein (15µl) was analyzed for the presence of the expected protein by 10% SDS-PAGE. The proteins were transferred into nitrocellulose membranes (Roti-PVDF, Roth, Nürnberg, Germany). The blotting was performed in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) at 160 mA for 1 h followed by 3 times washing with Phosphate Buffered Saline Tween-20 (PBST).The non-specific binding sites on the membrane were blocked by incubation with PBST containing 10% non-fat dry milk for 1 h at room temperature. For His-tag analysis, the blocked membrane was incubated overnight with 1:100 mouse anti-His-tag antibodies (Dianova, Hamburg, Germany) at 4°C. After washing with 1× PBST, the membrane was incubated with HRP polyclonal rabbit anti-mouse Immunoglobulin (Dako, Hamburg, Germany) at a concentration of 1:1000 at room temperature for 2 h. The membrane was washed with PBST and developed with PBST containing 5 mg of 3, 3′diaminobenzidin tetra-hydrochloride (DAB) with 0.03% hydrogen peroxide (H_2O_2). Expression of HA protein was also evaluated by Western blot analysis using Influenza A H9N2 HA specific rabbit antibodies pAb (antibodies-online Inc. Atlanta, USA). Expressed HA proteinwas separated by SDS-PAGE and transferred onto nitrocellulose membrane and then incubated with 1:100 influenza A H9N2 HA specific rabbit antibodies pAb at4°C. After washing with PBST the membrane was incubated with HRP polyclonal swine anti-rabbit Immunoglobulin (Dako, Hamburg, Germany) in a concentration of 1:1000 at room temperature for 2 h. The membrane was washed with PBST and color was developed with DAB solution.

Purification and deglycosylation of *L. tarentolae* expressed HA protein

Histidine-tagged recombinant HA proteins were purified by Immobilized Metal Ion Affinity Chromatography (IMAC) using Ni-NTA agarose (Qiagen, Hilden, Germany). Briefly, cell culture medium containing the expressed HA protein was concentrated 5-fold using a Vivacell 100/10K concentration system at a cut-off of 10K (Sartorius Stedim Biotech GmbH, Goettingen, Germany). After washing the Ni-NTA agarose (Qiagen, Hilden, Germany) with wash buffer (pH 8) containing 50 mM NaH_2PO_4, 300 mM NaCl, 0.05% Tween 20 and 20 mM Imidazole, added to the concentrated cell culture medium and mixed properly with Ni-NTA agarose and incubated 1-2 hon the overhead shaker at 4°C. The mixture was centrifuged for 5 min at 1000x g and then the recombinant HA was eluted at pH 8 using elution buffer with 500 mM imidazole. Purified 10µg HA proteins were deglycosylated using Endo H (New England Biology, Biolabs) according to the manufacturer instructions and the digested products were subjected to SDS-PAGE and Western blot analysis. The protein concentration was measured by using the *NanoDrop* (Peqlab, Erlangen).

RESULTS AND DISCUSSION

Avian influenza viruses are able to induce huge economical losses to poultry farmers around the world and, with its zoonotic potential, also have a significant effect on public health. Thus, vaccination is a proven and effective way to combat influenza. Present day influenza vaccines primarily depend on egg-based vaccines which is a lengthy process and heavily dependent on a safe egg supply [33]. This interferes with a fast response to a pandemic crisis [34]. Alternatives are badly needed to supplement the conventional egg based technology for developing effective and reliable vaccines against influenza. Furthermore, as the antigenic nature of a pandemic virus is unpredictable, the rapid production of vaccines by a method or system which can be adapted quickly to circulating strains of the virus is needed. Nowadays many strategies have been used for production of influenza vaccines including baculovirus expressing either a subunit or VLPs of influenza virus [35] or subunit vaccine expressed in yeast [27] which has already been described to elicit antigen specific immune response in animals. However, there are some limitations associated with the antigens expressed in baculovirus expression system such as antigen yield and unavailability of effective delivery methods. On the other hand, *L. tarentolae as a eukaryotic expression system has the potential to produce large quantities of recombinant proteins associated with increased cell densities. Therefore, this study was aimed to develop L. tarentolae expressed recombinant HA as a vaccine candidate against AIV specially the H9N2 subtype.*

In order to express the HA protein in the inducible *L. tarentolae* expression system, the HA gene of influenza virus H9N2 was amplified from pJET 2.1-HA, digested with *Xba*I and *Kpn*I and cloned into a similarly digested pLEXSY-I-neo3 vector. The clones were confirmed by restriction digestion with *Xba*I and *Kpn*I which displayed a product of 1569 bp corresponding to the HA (without the transmembrane region and cytoplasmic tail) and a product of 6.9 kb corresponding to the pLEXSY-I-neo3 vector (Figure 1A). Subsequently the cloned HA gene was sequenced completely. The pLEXSY-I-neo3-HA was transfected into *L. tarentolae*. The integration of the HA expression cassette into the *odc* locus was confirmed by PCR (Figure 1B) using primers provided in the LEXSinduce3 kit (Jena BioScience). Amplification of the HA gene from leishmania cultures was confirmed using the H9 gene specific primers described above (Figure 1C). DNA sequence analysis revealed 100% homology to that of the cloned HA. Twenty hours after the transfection the selective LEXSY antibiotics provided in the kit were added. The recombinant *L. tarentolae* growth subsequently made the cultures turbid. In contrast, the negative control (cells transfected without DNA) remained clear.

The recombinant HA from influenza virus A/chicken/Bangladesh/VP01/2006 (H9N2) (BVP01) was evaluated by SDS-PAGE which is shown in (Figure 2A). Western-blot analysis of

the protein using anti-His-tag mouse monoclonal antibodies and the influenza A H9N2 HA specific antibody pAb was conducted. Expressed protein was visualized with these antibodies at the expected protein size as shown in (Figure 2B and C). A major band of~ 68 kDa corresponding to the expected size of the HA molecule without the transmembrane region and cytoplasmic tail was obtained. The induction of the protein expression was performed at different time points. Western-Blot analysis revealed that the highest protein expression was achieved when the cultures were induced with tetracycline immediately at the time point of parasite inoculation (data not shown) suggesting that the HA protein was tolerated by the *L. tarentolae*. To further optimize the induction of protein production different concentration (10, 20 and 30 µg/ml) of tetracycline were added at the time of parasite inoculation. More protein expression was obtained when the T7 driven transcription was induced with 20 and 30 µg/ml of tetracycline (Figure 2B and 2C), although there are some variation in the OD_{600} values of leishmania culture measured at different time points. This suggested that increased concentrations of tetracycline up to 30µg/ml have no harmful effect on the leishmanial growth. The recombinant *L. tarentolae* expressed HA protein was successfully purified by Immobilized Metal Ion Affinity Chromatography (IMAC) using Ni-NTA agarose. As the pLEXSY neo3 vector contains C-terminal His-tag, the expressed protein is easily purified from culture supernatants. In this study production of recombinant HA proteins was initially performed in tissue culture flasks. *It has been described that Leishmania* display good growth in biofermenters [36]. To adapt to these conditions, we performed large scale protein expression with *1L leishmanial cultures in 2L Erlenmeyer flask sunder slight agitation*. After purification of leishmanial cultures, a yield of 2-5 mg of recombinant HA per liter of initial culture was reproducibly obtained. These levels are also comparable with other eukaryotic and prokaryotic expression systems [26,37]. The production duration observed in this study suggest that the entire process of recombinant HA proteins expression in *L. tarentolae* could be performed within three months, a minimum time frame compatible for better responding to sudden disease outbreaks or pandemics.It has been reported that *L. tarentolae* has mammalian like glycosylation pattern (Jena BioScienc). Deglycosylation of the recombinant HA proteins using Endo H (NEB, New England Biology) gave rise to a~ 6 kDa smaller product, which verifies that the HA protein was glycosylated (Figure 2D). The H9 protein contains seven potential glycosylation sites which play an important roles on immunogenicity, virion export, receptor binding, and appropriate folding of the protein [38,39]. Although in this study immunogenicity of the *L. tarentolae* expressed protein was not analyzed, it has been reported that soluble HA monomers expressed in *L. tarentolae* are immunogenic in mice [38].

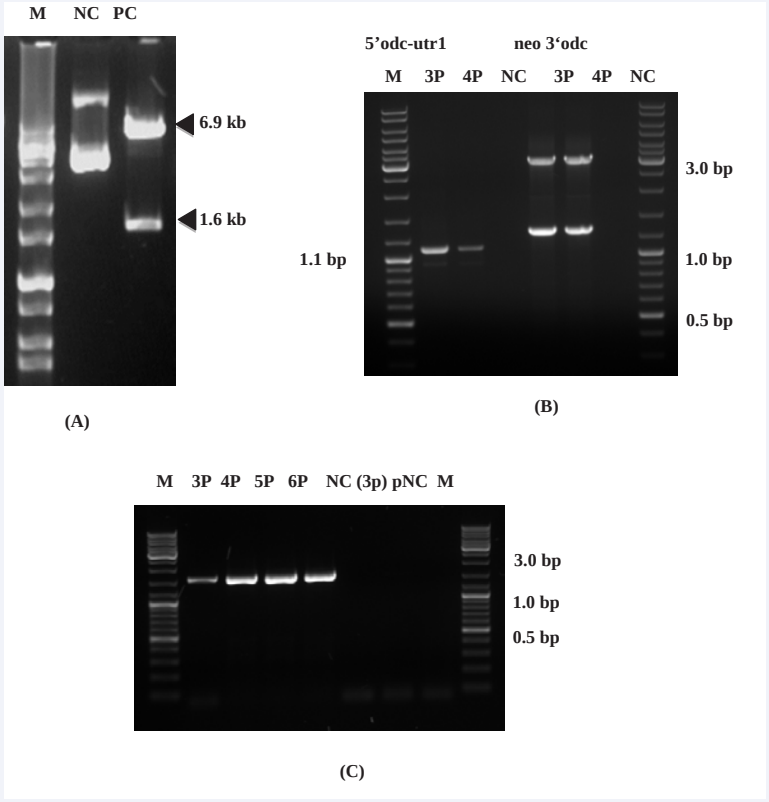

Figure 1 Cloning of HA into pLEXSY neo3 vector and determination of HA gene integration. (A) The cloning of HA into *L.tarentolae* expression vector pLEXSY-I- neo3 was confirmed by digesting the plasmid with XbaI and KpnI which released 1.6 kb HA and 6.9 kb pLEXSY vector. NC: Negative clone; PC: Positive clone. (B) Confirmation of the genomic integration of HA expression from leishmania culture 1.1 kb and 3.0 kb represented integration into 5' odc utr and neo 3' odc locus. M= Marker; 3P, 4P, = third and fourth passage respectively; NC= Negative control. (C)Confirmation of the HA gene by PCR from different passages of leishmanial culture released 1.6 kb HA; M= Marker; 3P, 4P, 5P, 6P= third, fourth, fifth and sixth passage respectively; NC (3p) = Negative control from 3rd passage; NC (5p) = Negative control from 5th passage; pNC= Negative control for PCR.

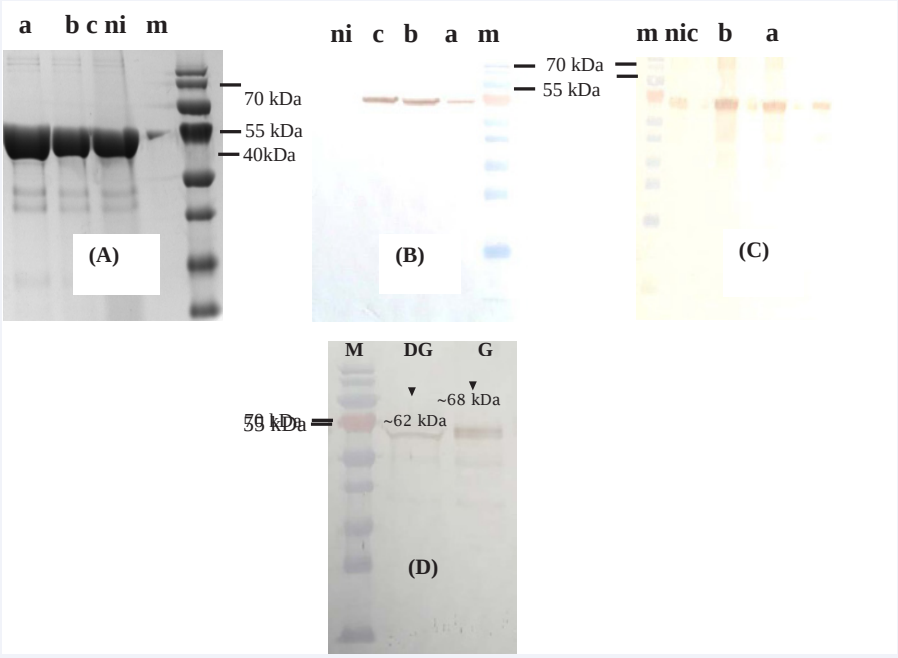

Figure 2 Evaluation of HA protein expression by SDS-PAGE (A) and Western blot using (B, C). Protein expression analyzed using His-tag Mouse mAb (B) and an anti-Influenza A H9N2 HA Antibody Rabbit pAb (C); induction performed using different concentration of Tetracycline, a=induced with 10µg/ml Tet.; b= induced with 20µg/ml Tet.; c= induced with 30µg/ml Tet.; ni= Non-induced; m= Marker; Negative control (ni) showed a thin band in Western blot, as cells were transfected with HA gene but the culture was not induced with tetracycline; (D): Deglycosylation of HA protein using Endo H , M: Marker; G: glycosylated, DG: deglycosylated.

CONCLUSION

This is the first description of a recombinant, glycosylated HA protein production from an avian influenza strain of subtype H9N2. The recombinant H9 proteins expressed in the *L. tarentolae* expression system were derived from A/chicken/Bangladesh/VP01/2006 (H9N2) (BVP01). The results of study yielding 2-5 mg of the soluble, monomeric, glycosylated HA protein per liter of initial culture show this eukaryotic parasite based expression system is a versatile tool for large scale antigen production. Further research needs to evaluate the immunogenicity of the H9 protein and the potential of the protein to induce protective immunity in vaccination/challenge trials.

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