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Immunogenicity of DNA Plasmid Expressing HA1 of (A/equine/Egypt/vrlcu/2008 (H3N8)) Equine-1 *Influenza virus* in Wistar Rat Model

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ABSTRACT

The unpredictable fast evolution of H3N8 equine *Influenza virus* and its recent transmission to dogs have raised the importance of a rapid vaccine production strategy. Currently available EIV vaccines are problematic due to their production cost, time and transportation restrictions. DNA Plasmids expressing immunogenic gene (s) are good alternative as it has the advantages of modified living vaccines without the risks of infection and/or reversion to virulence as well as ease of production and handling. In this study, DNA plasmid expressing HA1 (PcDNArHA1) of an H3 Egyptian equine influenza isolate was prepared and its immunogenicity in female wistar rats was evaluated. HA1 protein of PcDNArHA1 was expressed in Vero cell line and detected in the cytoplasm by indirect immunofluorescence. Two doses of ultrapurified PcDNArHA1, 100 µg each were then inoculated 15 day apart in the quadriceps muscle of female wistar rats test group while placebo group inoculated only PBS. Rats were euthanized and the serum collect.

Key words: H3N8 equine-1, *Influenza virus*, DNA plasmid, immunogenicity, wister rats, HA1 protein

INTRODUCTION

H3N8 is the cause of almost all recent Equine Influenza outbreaks (Daly *et al.*, 2011). Being the cause of most important respiratory disease in horses made it a critical point to be considered (Murcia and Wood, 2011). Attention was raised after the unexpected jump to dogs leading to existence of Canine Influenza (CI) (Crawford *et al.*, 2005; Payungporn *et al.*, 2008). H3N8 EIV is an *influenza A* virus containing eight single stranded negatively oriented RNA genome segments that encode for at least eleven known proteins, 3 of which are exposed to outside of the viral envelope, namely Hemagglutinin (HA), Neuraminidase (NA) and Matrix (M2) ion channel protein (Wright *et al.*, 2007).

Despite the fact that Pathogenesis and/or virulence of any *influenza A* virus is a multigenic process where HA, NA, PB2, PB1, PA and NS1 are at least involved (Bogs *et al.*, 2010), a great fraction of host immune response is directed toward HA. HA is the major influenza surface protein representing the main target of host defences which is composed of a homotrimer from the monomer encoded by segment 4 mRNA of *influenza A* virus, containing at least seven epitopes (Ozaki *et al.*, 2001). Structurally, HA1 contains the receptor binding site and the five known antigenic sites (A, B, C, D and E) (Barbic *et al.*, 2009), forming the globular head, it is the target of most neutralizing antiviral antibodies.

Efforts have been made along a period of fifty years to control equine influenza infection but, it continues to evolve and affect vaccinated as well as unvaccinated equine populations

(Newton *et al.*, 2006; Barquero *et al.*, 2007; Cowled *et al.*, 2009). This obvious failure could be attributed mainly to fast evolution of equine *Influenza virus* as well as, the inefficiency of currently used vaccines.

DNA plasmids expressing immunogenic protein (S) still to provide a better vaccine solution, as the protein is synthesized by De-novo protein synthesis pathway and thus resembles infection but, without disease danger unlike living vaccines and is also able to stimulate greater fraction of cell mediated immunity unlike protein and peptide vaccines, despite other advantages regarding production, storage, update and cost (Mor, 1998). In the present study, we were able to clone HA1 of A/Equine/Egypt/VRLCU/2008(H3N8) in the PcDNA™ 3.1 Directional TOPO® mammalian expression plasmid. We expressed the recombinant HA1 (rHA1) *in vitro* (VERO cell transfection) and *in vivo* (Wistar rat inoculation) and detected antibody response in inoculated rats by means of hemagglutination inhibition and neutralization assays. Vectored HA1 appeared to be a promising solution however a challenge against virulent H3N8 virus stills a must.

MATERIAL AND METHODS

Construction of recombinant DNA plasmids: The mammalian expression vector was constructed by cloning of HA1 gene of A/Equine/Egypt/VRLCU/2008(H3N8) into PcDNA™3.1 Directional TOPO® expression plasmid (INVITROGEN®, UK). One thousand fifty four bp from HA gene were amplified after total RNA extraction using BioZol total RNA Extraction reagent (BIOFLUX, Japan). Primers used were HA3DF (5' CACC ATG AMG ACA ACC ATT ATT TTG ATA CTAC 3') a modified form of the primer authored by Bryant *et al.* (2009) and our own authored reverse primer H31R (5'-CCG CTA TTG CTC CAA AGA TTC C-3'). The amplicon was mixed with the open vector and then transformed into TOP10 chemically competent *E. coli* (INVITROGEN®, UK) and propagated for 12 h.

A check orientation PCR was conducted to check the presence as well as the orientation of HA1 using the same forward primer (HA3DF) in conjunction with BGH universal reverse primer.

Cell culture transfection: It is very demanding step to check the expression of any cloned gene prior to animal experiment. Vero cell line, passage 112 were maintained in an antimicrobial free Eagle's MEM (SIGMA, USA) containing 10% FCS (SIGMA, USA). One day before transfection, cells were cultured into 6 well plate. At the day of transfection, 50 uL of the isolated plasmid were diluted in 1450 µL MEM and so for the transfection reagent, Lipofectmine 2000 (INVITROGEN®, UK). Tubes were mixed, incubated and the mixture was added to cells and incubated for 6 h at 37°C then the transfection mix was replaced by enriched-antimicrobial free Eagle's MEM (SIGMA, USA) containing 20% FCS (SIGMA, USA).

Cells were incubated for 48 h then assayed for transgene expression using indirect fluorescent antibody technique.

Indirect fluorescent antibody technique (IFAT): Growth medium were discarded, cells were fixed by ice-cold methanol for 3 min, methanol discarded and cells were left for air dry, washed once with PBS containing 1% BSA (SIGMA, USA). Anti-H3 equine influenza serum (Kindly donated by Dr. Mahmoud Kabbany, SERVAC, Abbasia, Egypt) was incubated with cells for 1, cell washed 3 times and then anti-Equine FITC conjugate (SIGMA, USA) was diluted according to manufacturer recommendation and incubated for 1 with cells, cells washed for 3 times, mounted and examined for expression using Olympus CX41 fluorescent microscope.

Immunization of wistar rats with the vectored HA1: Starter culture was prepared from the positive colony glycerol stock by adding 100 μL preserved colony culture to 10 mL LB broth containing 50 $\mu\text{g mL}^{-1}$ ampicillin with vigorous shaking for 8 h. One milliliter of the starter culture was added to 500 mL LB broth containing 50 $\mu\text{g mL}^{-1}$ ampicillin with vigorous shaking for 14 h. Plasmid was extracted from the cell pellet using Qiagen Plasmid Maxi Kit (QIAGEN®, Germany). Purity and concentration of the plasmid were determined using Qubit® 2.0 Fluorometer (INVITROGEN®, UK). The solution was adjusted 2 $\mu\text{g } \mu\text{L}^{-1}$ and mixed with 2X PBS (Calcium-Magnesium free) (SIGMA, USA) and stored in -20°C for use. A total 25 female Wistar rats were kept in 2 cages, inoculated group $n = 15$, control group $n = 10$, both groups were fed and watered as recommended. Inoculated group received 100 μg recombinant plasmid inoculums twice, in the anterior tibial muscle with 2 weeks interval between inoculums. Control group inoculated only PBS. First euthanasia applied 2 weeks after 2nd inoculum and continued weekly for other 2 weeks; serum was separated and stored at -20°C for testing.

Hemagglutination inhibition assay (HI): HI was performed on sera collected from inoculated as well as control Wistar rats. The test performed against 4HAU/50 μL virus according to the instructions of OIE Manual (OIE, 2009). Test results were read on plate and recorded.

Neutralisation assay: Both Alpha and Beta procedures were performed using the previously collected sera in the allantoic sac of SPF embryonated chicken eggs (SPF-ECE) following the instructions of OIE manual (OIE, 2009). Beta assay was performed against 50EID₅₀/50 μL . Test results were read using rapid slide hemagglutination for the presence/absence of active virus.

RESULTS

Results of RT-PCR, transformation and check orientation PCR

Results of transfection and indirect fluorescence

Results of HI and neutralization assays: The Highest HI titre obtained was 32 HIU/50 μL serum, three weeks after 2nd inoculum. One log increase was observed between the results of the 2nd week to those of the 3rd week. Control group failed to show any HI titre.

Neutralization index obtained from Alpha procedure neutralization test was 0.75 and the antibody titre regarding Beta procedure was 2NU/50 μL serum.

DISCUSSION

To date, immunization continues to be the best solution for controlling and/or preventing viral diseases. Continuous evolution of Equine H3N8 constitutes a great obstacle in the face of efficient vaccine development (Paillot *et al.*, 2006). Current vaccine production strategies involved in the control of Equine influenza infection depends on inactivated whole virus vaccine production which has several drawbacks, mainly being directed to the production of humeral immune response (Lewis, 2006).

Hannant *et al.* (1988) and Bryant *et al.* (2009) stated that better protection against re-infection was produced via natural EI infection without high level of circulating antibodies. Plasmids expressing immunogenic genes, known as Gene or DNA vaccine present a better strategy for immunization by mimicking infection without disease induction and so stimulating better antibody and cell mediated responses (Mor, 1998).

Regarding influenza, heamagglutinin is the main target for host defences and so plasmids expressing HA will induce virus neutralizing antibodies and is considered a good vaccine candidate

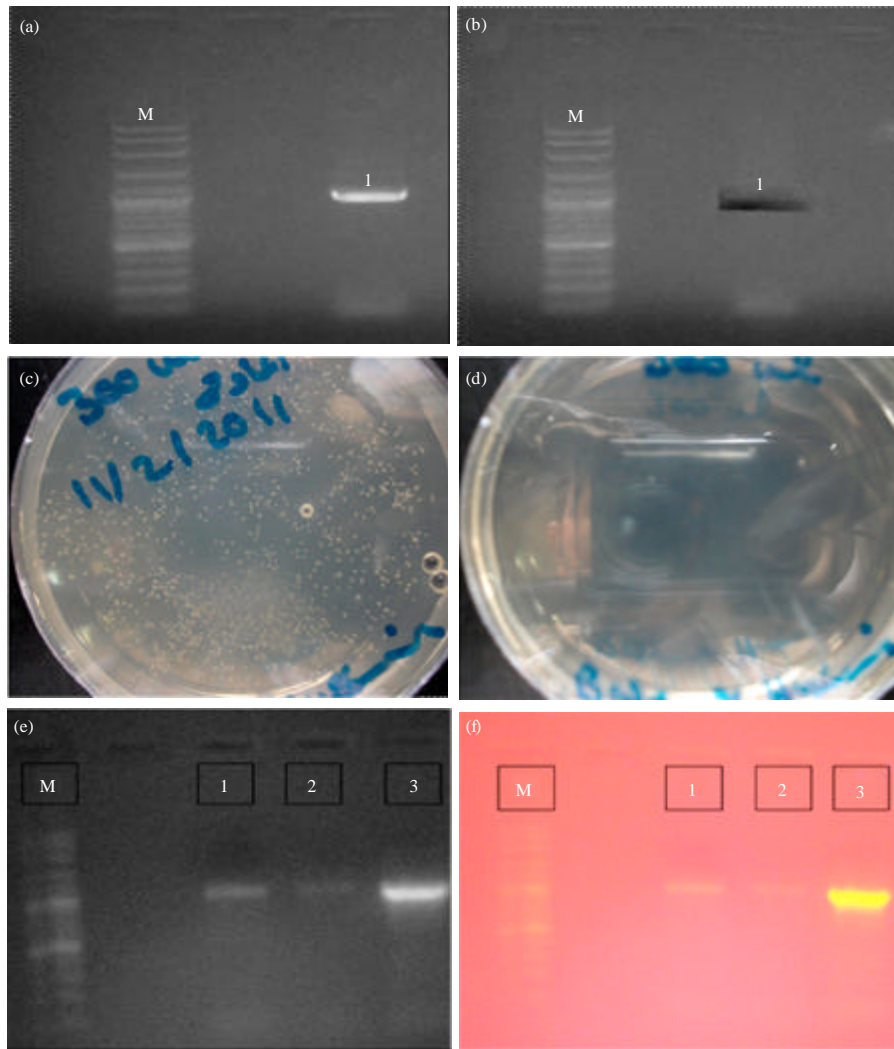


Fig 1(a-f): (a) Represents the amplification of HA1 against molecular weight molecular, M before cutting the band, (b) Represents the cut band from the gel, (c) Represent positive colonies after transformation of top 10 cells, (d) Control plate without colonies, (e) Represents check orientation PCR results, 1 represents colony H31D3, 2 represents H31D5 and 3 represents H31D18 which was selected for completion of the work and (f) Represents the same Fig (E) in colour

(Brown *et al.*, 1998). As these plasmids will not induce anti-nucleoprotein antibodies, they will allow for successful DIVA strategy, essential for global trade of equines (Ji *et al.*, 2011).

H3 HA1 contains the receptor binding domain and the five known antigenic sites (Barbic *et al.*, 2009). We cloned HA1 in PcDNA™ 3.1 Directional TOPO® mammalian expression plasmid and proved the correct orientation Fig. 1, to ensure Maximal identity with native viral sequence we used a proofreading polymerase, Platinum® Pfx DNA Polymerase (INVITROGEN) to create a DNA

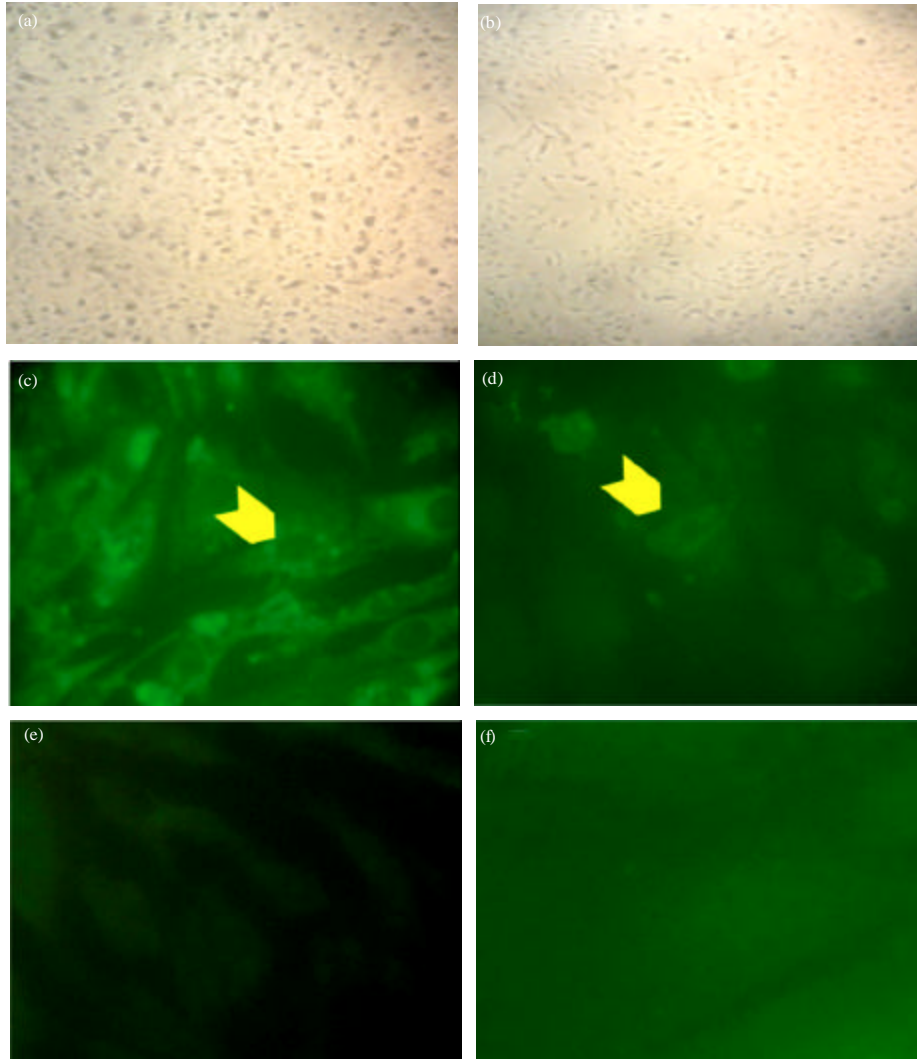


Fig. 2(a-f): (a) Represents transfected Vero cells with the recombinant plasmid and lipofectamine 2000™, (b) Represents non-transfected cell control, (c) and (d) Represent positive fluorescence reaction; note the cytoplasmic fluorescence and empty nuclei, (e) and (f) Represent control non-transfected negative FA cells

template from the H3 cDNA. The transgene was successfully expressed *in vitro* Fig. 2, characterized by indirect immuno-fluorescence using anti-H3N8 serum and anti-Equine FITC conjugate.

The success of several previous studies in using rats from different types as *Influenza virus* animal models (Daniels *et al.*, 2003) and to evaluate vaccination response (Alarcon *et al.*, 2007), encouraged us to use the institutionally available Wistar rat (Clause, 1998). Preliminary results for evaluation of the immunogenicity of the construct using HI and neutralization assay were promising.

In vivo expressed, recombinant HA1 (rHA1) induced a good level of anti-H3 antibodies marking the construct as a possible, cost effective and easily updated Equine flu candidate vaccine. Being synthesized intracellularly ensures the stimulation of cellular immune response.

The study opens the door for studying the feasibility, optimum application regime and delivery system for the construct, also gives an insight about the possible use of Wistar rat as a model for studying benign influenza.

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