PROTECTIVE MUCOSAL IMMUNITY ELICITED BY INTRANASAL DNA VACCINATION EXPRESSING HA1 OF EQUINE-2 INFLUENZA VIRUS

By

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NOMENCLATURE

APC	Antigen Presenting Cells
ATCC	American type tissue culture collection
BSA	Bovine serum albumin
CD	Cluster differentiation
CDC	Center for Disease Control
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CpG	Cytidine-phosphate guanosine dinucleotides
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
ddH_2O	Distilled & deionized water
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetracetic acid
EH	Equine hemagglutinin
ELISA	Enzyme linked immunosorbent assay
Eq	Equine
FBS	Fetal bovine serum
FDAH	Forte Dodge Animal Health

GFP	Green fluorescent protein
HA	Hemagglutinin
HHS	Department of Health and Human Services
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
ISCOM	Immune stimulating complex
Kb	Kilobases
KY	Kentucky
LB	Luria-Bertani
M	Matrix protein (of influenza virus)
MALT	Mucosa associated lymphoid tissue
MDCK	Madin-Darby Canine Kidney
MHC	Major Histocompatability complex
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NALT	Nasal associated lymphoid tissue
NP	Nucleoprotein
NS	Nonstructural protein
OIE	Office Des Internationale Epizootics
ORF	Open reading frame
PA	Polymerase A

PBS	Phosphate-buffered saline
pCMV	Cytomegalovirus promoter
PFAM	Protein Family database
pNPP	Para-nitrophenyl phosphate
RBC	Red blood cell
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SV40	Simian virus 40
TAPs	Transporter associated with antigen processing
TGF-β	Transforming growth factor-β
Th	Helper T cells
UV	Ultra-violet

CHAPTER 1

INTRODUCTION

1.1 Rationale of the Study:

Equine influenza is one of the major diseases of the horse (Mumford, 1992; Lunn et al, 1999) and causes significant economic problems for the equine industry (Lai et al., 2001). It is also one of the most common problems requiring veterinary attention (Traub-Dargatz et al., 1991). It is an acute and contagious disease caused by two distinct subtypes: subtype 1 (H7N7) and subtype 2 (H3N8) of influenza A viruses, while subtype 2 viruses are the major cause of respiratory disease in horses (Daly, Newton and Mumford, 2004).

Equine influenza includes symptoms like fever, dyspnoea, anorexia, nasal discharge, coughing and weakness and it can lead to severe complications in horses (Chambers et al., 1995a, b; CDC Flu Home, 2005). Influenza A viruses are found in many animals, including ducks, chickens, pigs and horses, while Influenza B viruses are mainly found among humans (CDC, Flu Home, 2005).

Equine-1 influenza virus (H7N7) has not been recorded in circulation for more than twenty years (Webster, 1993). Though conventional vaccines are available for equine-2 influenza virus (H3N8), it is still common in circulation (Daly, Newton and Mumford, 2004).

It has been found that current vaccines do not provide complete protection against equine influenza. Currently available vaccines are inactivated, live attenuated or vectored equine influenza vaccines (Daly, Newton and Mumford; 2004). It has been found that inactivated vaccines consisting of whole virus subtypes 1 and 2, or their subunits, do not provide complete protection for a longer period. The continuous emergence of new variants of viruses in the circulation and antigenic mismatching between viruses present in circulation and vaccines, are major reasons behind current vaccine failures (Palese and Garcia-Sastre, 2002; Daly, Newton and Mumford, 2004).

Therefore, a safe and protective DNA vaccine is useful that can provide complete protection and can generate immunity for a longer duration. A DNA vaccine that can encode the hemagglutinin protein (HA) of equine influenza virus can have an important role in providing protection against equine influenza. This study aims to compare the effectiveness of DNA vaccines pTOPOKY98 and pVAXKY98 that encode HA1 from equine-2 virus, A/Eq/Ky/98 in eliciting protective immunity in mice. It also aims to compare the effectiveness of two vaccines using liposome and metastim as adjuvants.

1.2 Objectives of the study:

a). Specific objective:

The specific objective of the study was to evaluate the effectiveness of designed DNA vaccines pTOPOKY98#6 & pVAXKY98/11-9 in eliciting protective immunity in mice.

b). General objectives:

The general objectives of the study were as follows;

- To compare the effectiveness of designed DNA vaccines pTOPOKY98#6 and pVAXKY98/11-9 expressing HA1 gene from equine-2 influenza virus.
- To verify the expression of HA1 in Vero cells and Madin-Darby Canine Kidney (MDCK) cells.
- To determine efficacy of intranasal DNA vaccination in mice.
- To determine the level of protection by a weight loss model and by elevated IgG and IgA levels in mice serum after vaccinations.
- To compare effectiveness of two vaccines using liposome & metastim.

1.3 Research Hypothesis:

A DNA vaccine consists of a plasmid that encodes one or more genes of interest that can generate protective immunity against infection in a host. It is expected that after delivering designed DNA vaccines, HA1 protein will be produced in the antigen presenting cells and will be expressed in its native form in the host. The HA1, a segment of hemagglutinin protein, should be able to generate the protective immunity. This antigen presentation pathway is very much similar to that of a natural infection; therefore, a good immune response is expected towards this protein.

Gurunathan et al. reported that when plasmid DNA is introduced into host cells, it is capable of the high level translation of its antigen and an immune response is elicited in response to antigen which is mediated by the cellular and humoral immunity of the immune system (Gurunathan et al., 2000). As reported by Abdelnoor, antigen presenting cells (APCs) play an important role in the induction of immunity by presenting vaccine peptides on MHC class I molecules and MHC class II molecules after antigen capture (Abdelnoor, 2001).

Overall, humoral immunity along with mucosal immunity will be generated and an anamnestic response will also be generated after delivering a booster vaccination.

1.4 Methodology used in the study:

In this study, two DNA vaccines pTOPOKY98#6 and pVAXKY98/11-9 were used for vaccination trial in mice. The cloning of vectors, design and characterization were done by Lai et al (unpublished data). The HA1 gene from equine-2 influenza virus, A/Eq/Kentucky/98, was cloned according to the manufacturer's instruction (Invitrogen, CA) into these vectors and the both DNA preparations were amplified in *Escherichia coli* cells.

For confirmation of correct DNA insert, the tissue transfection was performed using Vero cells and Madin-Darby canine kidney (MDCK) cells, in order to verify the expression of HA1 in cell cultures. For that, lipofection and electroporation methods were used to transfect cells with the DNA construct. Following the tissue transfection and incubation, cytosolic and nuclear fractions of cells were used and ELISA and PCR were performed for confirmation of HA expression in cells.

A vaccination trial was designed using Balb/c mice to determine the effectiveness of the constructed DNA vaccines using a weight loss model and serum antibody levels. Mice were randomly selected and distributed into seven groups consisting of five mice in each group. Four groups of mice were vaccinated delivering designed DNA vaccines into the nasal cavity. A booster vaccination was given on day 21, after three weeks following the primary dose. A virus challenge was performed with homologous equine-2 influenza, A/Eq/KY/98 on day 42, after three weeks of booster vaccination.

The body weight of mice was recorded before and after vaccination and also after virus challenge. The HA specific IgG and IgA antibodies in serum were collected after the first vaccination and booster vaccination and also after virus challenge. A PROC GLM (ANOVA) method using SAS program (SAS Institute, 2004) was performed to determine significant differences in weight loss between the vaccinated and the unvaccinated groups and also to find out the significant differences in serum antibody levels before and after vaccinations.

1.5 Significance of the study:

DNA vaccination is a relatively new technology which has several advantages which include simple technology, genetic manipulation and chemical as well as biological stability (Whalen, 1996). Abdelnoor reported that plasmid DNA is thermostable and easy to freeze dry and at the same time it does not replicate in the host (Abdelnoor, 2001).

DNA vaccines are found to be useful against infections, especially against viral diseases. After immunization, the antigens are produced in their native conformation for the generation of effective antibody responses. The protective immunity is generated by cellular and humoral responses (Donnelly, 1997; Abdelnoor, 2001). Therefore, DNA vaccines are not only safer than traditional vaccines, but also capable of providing increased protection (Johnson et al, 2000). Therefore, DNA vaccines will also be capable of providing protection against equine influenza.

CHAPTER 2

LITERATURE REVIEW

2.1 Equine Influenza virus:

The equine influenza viruses are type A influenza viruses (Daly and Mumford, 2001). There are two major subtypes of equine influenza virus, equine-1 influenza (H7N7) and equine-2 influenza (H3N8) viruses which are mainly found causing disease in horses (Lai et al., 2001; Daly, Newton and Mumford, 2004). Equine influenza virus is still a major infectious agent causing diseases in horses despite the regular administration of inactivated and live attenuated virus vaccines (Mumford et al., 1998; Daly, Newton and Mumford, 2004).

2.1.1 Structure of Influenza virus:

Influenza virus is a single stranded RNA virus. It is a pleomorphic virus, mostly found as spherical or ovoid structures. The size of virus ranges from 80-120 nm in diameter. The outer surface of lipid envelope consists of two types of glycoproteins, the hemagglutinin (HA), and neuraminidase (NA) (Flint et al., 2004). In the core, there is nucleoprotein which comprises eight segments of negative- stranded RNA. The matrix protein (M) is found in the inner side of the envelope.

The 'RNP' is found in a helical shape associated with three polymerase polypeptides. PA, PB1 and PB2 are RNA-dependent polymerases present in virus while NS1 and NS2 are non-structural proteins associated with replication (Flint et al., 2004).

2.1.2 Structure of hemagglutinin:

Hemagglutinin (HA) is the glycoprotein found on the virus surface (Flint et al., 2004). The protein consists of two segments HA1 and HA2 joined by disulfide linkages and each monomer has a stalk that extends from the membrane and a globular head outside the membrane. This stalk is composed of an alpha-helix. The disulphide linkage between two segments dissociates during virus and host cell fusion (de Lima et al., 1995). The globular head consists of HA1 residues and the sialic acid binding pocket. There are four recognized antigenic sites: A, B, C, and D found around the receptor-binding pocket.

2.1.3 Structure of neuraminidase:

Neuraminidase (NA) is an important viral surface glycoprotein which plays a role in viral release after replication (Flint et al., 2004). It is responsible for removing sialic acid from newly synthesized HAs and NAs which are sialylated by cellular enzymes (Liu et al., 1995). In the absence of NA, virus release is inhibited and virions are found attached to the cell surface forming clumps on cell surface (Liu et al., 1995). von Itzstein et al. reported that NA could be considered a suitable target for antiviral drugs because it

possesses an active site whose amino acid sequence is conserved among all types and subtypes of influenza virus (von Itzstein et al., 1993).

2.2. Classification of Influenza viruses:

Influenza viruses are members of the Orthomyxoviridiae family and consist of two genera: influenza A and B viruses; and one additional influenza C virus. Influenza viruses are distinguished from one another on the basis of proteins M and RNP. Influenza A viruses are classified into subtypes based on the surface viral proteins: HA and NA. There are fifteen different HA and nine different NA subtypes of influenza viruses (Flint et al., 2004; CDC, Flu Home, 2005).

2.3 Immunological phenomena in influenza virus:

2.3.1 Antigenic drift:

Antigenic drift is the immunological phenomenon found in influenza virus due to spontaneous mutations in the surface proteins; HA and NA (Flint et al., 2004; CDC Flu Home, 2005). These mutations may cause slight changes in the amino acid sequence of the HA protein and will cause changes in antigenicity. Therefore, a drifted subtype of virus becomes selected in a population of partially immune hosts. Antigenic drift increases from year to year and is responsible for annual vaccine failures (Cann, 1993).

2.3.2 Antigenic shift:

Antigenic shift is another important immunological phenomenon seen in influenza virus that results from genetic reassortment of the RNA segments (Flint et al, 2004; CDC, Flu Home, 2005). It takes place during replication, when two different viruses infect the same cell and when one virus takes HA genes from another virus. This phenomenon results in a major change in the antigenic structure of the virus (Gething et al., 1980) and generates mutant viruses of different subtypes. These new subtypes of virus emerge and can cause a pandemic.

2.3.3 Original antigenic sin:

This is an immunological phenomenon seen in influenza virus where the host's immune system fails to recognize new epitopes during subsequent infections caused by different strains of virus (Parham, 2005). This phenomenon is so named because original strain infection hampers the types of antibody made in response to future infections with different strains (Parham, 2005). In this process, the immune system uses only B cells which are rapidly mobilized. This allows virus a chance to escape from the protective immunity of a host; while the host's immune system is prevented from responding to the changes in the virus. Therefore, a protective immunity to influenza is not acquired for a longer period (Parham, 2005).

2.4 Epidemiology:

Influenza is an acute respiratory infection that has affected humans and animals since ancient times. Among equine viruses, two subtypes of equine influenza viruses were found in circulation, designated as the H7N7 and the H3N8 subtypes respectively (Oxburgh and Klingeborn, 1999). Webster reported that the first equine virus was isolated in Czechoslovakia in 1956 and this virus has not been recorded during the last two decades (Webster, 1993). Likewise, the first equine-2 influenza virus was isolated in 1963 in Florida and found to be prevalent in most equine populations (Daly, Newton and

Mumford, 2004). It has been found in different parts of America, Europe and Africa except Australia and New Zealand (Oxburgh and Klingeborn, 1999).

Oxburgh and Klingeborn reported that equine-2 influenza virus (H3N8) diverged into two lineages; European and American (Oxburgh and Klingeborn, 1999). Similarly, Lai et al reported that three American and one Eurasian lineages of the H3N8 virus have been identified (Lai et al., 2001). They were also reported in Europe and the United Kingdom. Lai et al. reported that only one strain of the virus belonging to the Eurasian lineage has been isolated in the Western Hemisphere (Lai et al., 2001).

It has been found that equine-2 viruses are continuously emerging as new strains at regular time intervals (Lai et al., 2001; Daly and Mumford, 2001). The basis for this emergence is antigenic variation in HA and NA surface proteins i.e. antigenic drift and antigenic shift. As reported by Chambers, the outbreaks of equine influenza still occur irrespective of repeated vaccinations (Chambers, 1999). Furthermore, Daly, Newton and Mumford, reported that equine influenza epidemics caused by new strains of equine-2 virus have been found in Europe, the American content and Asia since 1989 outbreaks in Europe, and therefore, there are antigenic variant strains of viruses in circulation (Daly, Newton and Mumford, 2004). The available inactivated and live attenuated vaccines are targeted to provide specific antibodies for currently circulating HA proteins (Daly and Mumford, 2001), while there is continuous emergence of new strains of equine-2 influenza virus (H3N8) and this can be one of major reasons for vaccine failure.

There is an another phenomenon, known as the antigenic mismatching, which occurs between viruses that are currently circulating and viruses that are used as vaccine strains, is causing a great problem in the selection of strains for the development of vaccines (Palese and Garcia-Sastre, 2002; Daly, Newton and Mumford, 2004). The continuous emergence of new variants of viruses in the circulation is the reason behind antigenic mismatching. Therefore, the antigenic variation and evolution of new variants, and antigenic mismatching between viruses present in circulation and vaccines, are the major reasons behind current vaccine failures.

2.4.1 Transmission of equine influenza:

The equine influenza virus generally transmits through the air by droplets and particles excreted through coughing or sneezing (EquiFlunet, 2005). Aerosol transmission is the most important way of virus spread in horses. Disease spreads rapidly among the crowded population. Cold and dry weather promotes the viral survival outside the body; therefore, influenza epidemics are common during the winter season (Miller, 2000).

It has been found that the unexposed and unvaccinated animals are found to show severe symptoms; while sub-clinical infection is also one of the significant ways for the spread of the virus (Daly, Newton and Mumford, 2004). It has been found that even vaccinated animals can infect others (Mumford, 1992).

The transportation of horses is an important factor for transmission of disease over distances (Powell et al., 1995). Several factors like the contacts in close confinement (Powell et al., 1995), contaminated water, personnel and transport vehicles are found to be other major factors in disease transmission as reported by earlier studies (Guthrie, Stevens, and Bosman, 1999; Miller, 2000).

2.4.2 Symptoms and complications of equine influenza:

Equine influenza is an infection of the respiratory tract in horses and causes flu like symptoms (EquiFlunet, 2005). Generally, horses will show symptoms of disease within a period of one to five days and may recover in about two to three weeks. The disease is characterized by fever, chills, dry cough, nasal discharge, dyspnoea, and malaise (Wilson, 1993; Miller, 2000; EquiFlunet, 2005). Weakness and refusal of food and drink consumption are other symptoms during infection. Many horses may develop bacterial secondary infections and show severe complications (Miller 2000; EquiFlunet, 2005).

2.5 Immune system: role in influenza

2.5.1 Innate immunity:

Innate immunity is an important immunity in restricting harmful substances to enter the body that can cause disease. It is a non-specific immunity and works as the first line of defense. Some of important components of innate immunity against viruses are the skin, mucous, macrophage, complement, interferon and natural killer cell (Wise, Carter and Flores, 2005). It is also important for defense against infections caused by influenza virus (Tamura and Kurata, 2004).

Interferons and natural killer cells are important for protection against viral infection (Wise, Carter and Flores, 2005). It was found that macrophages and natural killer cells in the lungs are capable of acting as antigen presenting cells and in destroying virus respectively (Price et al., 2000). Similarly, complement proteins are very important components of innate immunity, and play an important role in the neutralization of virus (Bernet et al., 2003).

2.5.2 Acquired immunity:

Acquired immunity is another important immunity which comes into play after antigen stimulation. In acquired immunity, there are two types of lymphocytes, B lymphocytes and T lymphocytes, which play an important role in generating immunity. It has been found that B lymphocytes are capable of producing antibodies while T

lymphocytes are capable of recognizing antigens associated with cells and destroying them (Wise, Carter and Flores, 2005).

2.5.2.1 Cell mediated immunity:

Cell mediated immunity plays a very important role in influenza**.** Zinkernagel and Doherty reported that cytotoxic T lymphocytes (CTLs) lyse virus infected cells in a major histocompatibility complex-restricted manner (Zinkernagel and Doherty**,** 1979). Similarly, Braciale et al. reported that the HA protein of influenza A virus is a target antigen for CTLs (Braciale et al, 1984). Bennik et al. reported specific CTL responses in mice and humans to proteins like NP, PB1, PB2, and PA, M, and NS1 (Bennik et al., 1982). Likewise, Townsend et al. showed that the NP of influenza A viruses are also important antigens for CTLs in mice and humans (Townsend et al., 1984). In addition, NS1 and the HA2 subunits of influenza A virus are also capable of inducing a protective CTL response in mice (Kuwano et al., 1990).

2.5.2.2 Humoral immunity:

Humoral immunity is one of the very important forms of immunity for removing virus from the host. It, not only removes virus from the body, but also helps to generate specific memory responses. Antibodies are the main components of humoral immunity and it has been found that antigen presenting cells stimulate plasma cells to produce antibodies. Generally, IgM is produced in the primary response, while other isotypes such as IgG and IgA are produced later on maturation (Baumgarth et al., 1999). IgA is a major

component of mucosal surfaces, while IgG is found in serum and in the lower respiratory tract (Gonchoroff et al., 1982) and considered to be major protective antibody.

2.5.3 Mucosal immunity:

Mucosal immunity is vital in influenza because influenza viruses are respiratory pathogens. As reported by Ban et al., mucosal immunity is a major component of the immunologic response in the nasal passages (Ban et al., 1997). Similarly, Iwasaki and Kelsall reported that the mucosal microenvironment is usually found shifted toward the induction of T helper cells producing Th2 and Th3 cytokines (Iwasaki and Kelsall, 1999). It has been also shown that efficient B cell isotype switching to IgA depends on both T cells and the presence of cytokines such as IL-4, IL-5, IL-10 and TGF-β (Iwasaki and Kelsall, 1999).

In mucosal surfaces, specialized lymphoid tissues, known as the Mucosa-associated lymphoid tissues (MALT) and Nasal associated lymphoid tissues (NALT), are found which play important roles in influenza. It has been found that T cells stimulate B cells to produce IgA and IgE (Davis, 2001). Several studies have shown that IgA has an important role in mucosal immunity (Kaetzel et al., 1991; Davis, 2001).

2.6 DNA vaccination:

DNA vaccines are plasmid vectors that contain one or more genes of interest inserted under the control of a eukaryotic promoter that allows high level expression in mammalian cells (Abdelnoor, 2001). It is a relatively recent development in vaccine methodology.

Considered as one of the major breakthrough works in DNA vaccination, Wolff et al found that the direct intramuscular inoculation of plasmid DNA encoding several different reporter genes could induce protein expression within muscle cells (Wolff et al, 1990). Many other reports were published after the breakthrough study of Wolff et al. The findings of Tang et al. study (Tang et al., 1992) further supported the observation of Wolff et al.. Tang et al demonstrated that mice injected with plasmid DNA encoding hGH (human growth hormone) could elicit antigen-specific antibody responses (Tang et al, 1992). Likewise, Ulmer et al reported in *Science* that DNA vaccines could protect mice from influenza infection (Ulmer et al., 1993) and it is considered one of the major breakthroughs in DNA vaccine history. Similarly, Robinson et al reported that DNA vaccine could protect chickens from influenza (Robinson et al., 1993) and also contributed to DNA vaccine studies.

There are several reports of investigations in many animals such as mice, ferrets, primates and chickens that reported the effectiveness of DNA vaccination against influenza infection (Montgomery et al.; Ulmer et al., 1993, Fynan et al., 1993, Iwasaki et

al., 1997, Wong et al., 2001; Epstein et al., 2002). However, there are not many reports in horses as compared to other animals.

Fynan et al. reported that plasmid DNA expressing HA protein using different routes like parenteral, mucosal and gene gun inoculation methods could initiate protective immunity in mice (Fynan et al., 1993). They found 95% protection in mice using the gene gun method. They also found that the DNA quantity required was less in mucosal routes like trachea or nares and was successful in inducing protection. In the study of Wong et al, viral HA was delivered intranasally with liposome and was found to induce protection (Wong et al., 2001). In this study, both serum IgG and IgA were observed in response to vaccination.

Similarly, Bot et al. reported that a plasmid bearing a HA gene from a virulent strain A/WSN/33 of influenza virus, was successful in generating protective immunity in newborn and adult mice (Bot et al., 1997). They found that a strongly biased Th1 response was induced in adult mice whereas mixed Th1/Th2 responses occurred in neonates. They reported that a significant increase in survival was observed in these mice. Likewise, Johnson et al. reported that direct intramuscular injection of plasmid DNA induced Th1 responses and a high level of protection against live influenza virus challenge (Johnson et al., 2000).

Some studies have been conducted in horses using DNA vaccines (Lunn et al., 1999, Soboll et al., 2003). Lunn et al reported that delivery of HA encoding plasmid at

skin and mucosal sites in ponies was found to stimulate IgG responses and a poor IgA response (Lunn et al,. 1999). Similarly, Soboll et al reported that the DNA encoding HA and its co-administration with cholera toxin induced primary and mucosal IgA responses in ponies (Soboll et al., 2003). They also found local production of IgGb in nasal secretions and a significantly better virological protection in ponies was observed.

2.6.1 Immunology of DNA vaccination

It has been found that DNA vaccine induces both humoral and cellular responses (McDonnell and Askari, 1999; Abdelnoor, 2001). It is not very clear exactly how plasmid DNA is processed and presented to the immune system. Gurunathan et al. reported that there are at least three mechanisms by which the antigen is processed and presented to elicit an immune response. Those three mechanisms include direct priming by somatic cells like keratinocytes, myocytes, direct transfection of professional APCs like dendritic cells and cross-priming between somatic cells and APCs (Gurunathan et al., 2000).

2.6.2 MHC molecules and antigen processing pathways:

MHC molecules are the gene products of the major histocompatability complex (MHC) gene locus (McDonell and Askari, 1999; Kumanovic et al., 2003). These molecules play a major role in presenting processed antigens to T-lymphocytes (Abdelnoor, 2001). As reported by Abdelnoor, MHC class I molecules are expressed on the surface of practically all nucleated cells and consists of two polypeptide chains; an alpha chain and beta-2-microglobulin (Abdelnoor, 2001; Kumanovic et al., 2003).

It has been found that only antigen presenting cells express MHC class II molecules and cytokine such as gamma-interferon may induce other cell types to express them (Abdelnoor, 2001). MHC class II molecules consist of two non-covalently linked polypeptides; the alpha and beta chains (Abdelnoor, 2001, Kuwanovic et al, 2003).

Some of the examples of professional APCs which are important in antigen processing mechanism are dendritic cells, macrophages, Langerhans cells of the skin and B-lymphocytes (Abdelnoor, 2001). Abdelnoor also reported that APCs degrade protein antigens into smaller peptides and present the peptides to T-lymphocytes, and T-lymphocytes are activated (Abdelnoor, 2001).

2.6.2.1 MHC class I presentation and cell mediated immunity:

The MHC class I pathway, also known as the endogenous pathway, is important in stimulating cell-mediated immunity. McDonnell and Askari reported that DNA vaccines are capable of stimulating a cellular immune response in addition to the humoral response (McDonnell and Askari, 1999). McDonnell and Askari also reported that DNA vaccines are able to generate cellular immunity because the foreign protein which they induce production, of is processed intracellularly and presented to the immune system in the context of the MHC class I system (McDonell and Askari, 1999). Traditional vaccines are processed via the MHC Class II system and therefore are not effective for cell-mediated immunity.

Abdelnoor reported the mechanism for the MHC class I system processing and showed that the MHC class I system identifies and displays intracellular antigens. The intracellular proteins are cleaved into short peptides by proteosomes in the cytosol and these peptides bind to MHC class I molecules (Abdelnoor, 2001) at the lumen of the smooth endoplasmic reticulum with the help of TAP (transporters associated with antigen processing) molecules. The MHC-antigen complex reaches the cell surface through the Golgi apparatus and is expressed on the cell surface (Abdelnoor, 2001).

Abdelnoor further reported that on the cell surface, the MHC-antigen complex is recognized by MHC class I restricted CD8+ cytotoxic T-cells. As a result, these CTLs are activated and amplified. In this manner, a cell-mediated immune response is generated

and CTLs are capable of identifying foreign proteins within all nucleated cells. In this way, cells infected with a virus may be detected and destroyed (Abdelnoor, 2001).

2.6.2.2 MHC class II presentation and humoral immunity:

The MHC Class II system which is also known as the exogenous pathway is the antibody pathway. This system processes exogenous antigens and presents them to stimulate helper T cells, which then stimulate B lymphocytes and antibody production (McDonell and Askari, 1999). MHC molecules are present on macrophages, neutrophils, some lymphocytes, dendritic cells and other antigen presenting cells (McDonell and Askari, 1999, Kuwanovic et al., 2003). But only specified antigen presenting cells express MHC class II molecules (Abdelnoor, 2001). These cells take up foreign antigen by phagocytosis or endocytosis (Kuwanovic et al, 2003).

2.6.3 Dendritic cells in antigen presentation:

Dendritic cells (DCs) are the most competent professional antigen presenting cells and important cells in antigen presentation to MHC molecules after DNA vaccination (Iwasaki and Kensall, 1999). The exact mechanisms responsible for the generation of MHC class I-restricted immune responses by DNA vaccination are still poorly understood (Corr et al., 1996), however, as reported by Gurunathan et al, a direct

transfection of professional APC or antigen transfer between transfected non-professional APC and professional APC are the most possible mechanisms for CTL induction upon DNA vaccination (Gurunathan et al, 2000).

Gurunathan et al. reported that direct transfection of professional APC facilitates the use of the conventional Tap-dependent MHC class I presentation (Gurunathan et al, 2000). As reported by Bot et al., among the professional APCs, the DCs play a critical role in the priming of MHC class I and II-restricted T cells (Bot et al., 2000). It is not clear to what extent DC that migrate from the area of DNA inoculation are responsible for the induction of MHC class I immunity but one possible explanation would be that the plasmid is picked up and expressed by the intraepithelial dendritic cells resident at the mucosal epithelium.

2.6.4 Requirements of DNA vaccinations:

The most important thing about a vaccine is that it should be able to provide protective immunity against infection. Some of the important requirements of DNA vaccine are cloning sites, enhancer promoters, selectable markers, polyadenylation sequences and a bacterial origin of replication (Gurunathan et al., 2000). The plasmid DNA vaccine should contain cloning sites where the gene of interest can be inserted. Likewise, it should contain selectable markers like antibiotic resistance genes to enable selection and also contain a bacterial origin of replication which is required for replication in bacteria. As reported by Robinson et al., a viral promoter for enhanced

expression in mammalian cells is useful (Robinson et al, 1993) and for that purpose, viral promoters from cytomegalovirus and SV40 virus are normally used. Polyadenylation sequences derived from SV40 virus or from bovine growth hormones are incorporated to stabilize mRNA transcripts (Gurunathan et al., 2000).

2.6.5 Routes of DNA vaccinations:

The reported common routes for DNA vaccinations are intra-muscular, subcutaneous, intra-peritoneal, intra-dermal, subcutaneous, intravenous, oral, rectal, intra-tracheal, vaginal and intra-nasal routes (Simmonds et al, 1997; Abdelnoor, 2001). Out of these routes, the most common routes of administration in use are intra-muscular or intra-dermal using a hypodermic needle or using a gene gun.

Intranasal routes have been found to be useful in inducing mucosal immunity. Glueck reported that intranasal delivery of DNA influenza vaccines has been found to be able to induce protective mucosal immunity (Glueck, 2001). The intranasal administration of DNA vaccines is easier in comparison to other methods. The nasal mucosa is easily accessible and highly vascular, and has a large surface for absorption. Davis reported that this route was capable of eliciting both systemic and mucosal responses and distant mucosal cells can also be involved in the response due to dissemination of effector cells (Davis, 2001).

2.6.6 Delivery vehicles of DNA vaccines:

Liposomes are the most commonly used vehicle for vaccines (Mannino and Gould-Fogerite, 1988; Gao and Huang, 1995). It has also been found that after being phagocytosed by antigen presenting cells or macrophages, liposomal antigen readily escapes from endosomes into the cytoplasm. It has also been found that liposomal peptide antigen can enter either the Golgi apparatus or the endoplasmic reticulum and thereby interact with MHC class II or class I molecules. (Audibert and Lise, 1993).

MetaStim has been commonly used in equine vaccines for many years by Forte Dodge Animal Health (FDAH, 2005). According to the Forte Dodge Animal Health (FDAH, 2005): "*MetaStim has proven itself over the years to provide excellent stimulation of the immune system and does not stimulate local inflammation*". This adjuvant system has been found to increase both short term and long term immune responses (Divers et al., 2000).

2.6.7 Advantages of DNA vaccinations:

As reported by Bot et al., plasmid DNA induces both cell mediated and humoral immunity (Bot et al, 1997). It is found to be non-infectious and does not replicate in the host (Abdelnoor, 2001). It is thermostable, easy to freeze dry and reconstitute, and can be manufactured in large quantities (Spier, 1996).

Likewise, according to Abdelnoor, plasmid DNA does not contain heterologous protein components to which the host may respond (Abdelnoor, 2001) and has the capacity to induce in vivo expression of antigens conserving the native confirmation of epitopes (Abdelnoor, 2001). Conservation of the native confirmation of epitopes is important for the induction of specific antibodies and cellular responses (Abdelnoor, 2001). Plasmid DNA may include more than one immunogene and will have the advantage of potentially decreasing the number of vaccinations required in children (Schodel et al, 1994).

CHAPTER 3

MATERIALS AND METHODS

3.1 Growth and amplification of virus:

A standard embryonated chicken egg inoculation method as described by Mahy and Kangro was used for virus cultivation (Mahy and Kangro, 1996). Equine-2 influenza virus, A/Eq/KY/98 was grown in 10-12 day old embryonated chicken eggs at 37^0C for 72 hrs. and extracted from an allantoic fluid. The allantoic fluid was harvested as described by Mahy and Kangro (Mahy and Kangro, 1996). After clarification by centrifugation at 1000g for 15 minutes, a hemagglutination assay was used to determine the virus titer.

3.2 Hemagglutination assay:

A standard method of hemagglutination assay was followed as described by Barrett and Inglis (Barrett and Inglis, 1985). After clarification of virus, the hemagglutinin assay was determined using serial dilutions of sample and mixing it with 1% chicken red blood cells in a hemagglutinin assay plate. The plate was observed for hemagglutination after 30 minutes incubation at room temperature and virus titer was determined.

3.3 Selection of DNA vaccines:

Two DNA vaccines pTOPOKY98#6 and pVAXKY98/11-9 were used for immunization in Balb/c mice. The DNA vaccines were designed and characterized by Lai et al (unpublished data). Two mammalian expression vectors pcDNA3.1/V5/His (pTOPOV5/His) and pVAX1 (Invitrogen, CA) were used for construction of DNA vaccines.

As reported in its manual: "*pcDNA3.1/V5/His is a 5.5 kb plasmid vector and has multiple TOPO cloning sites. It consists of pCMV and T7 promoters, V5 epitope and C-terminal polyhistidine tag, BGH reverse priming site, BGH polyadenylation signal, SV40 early promoter & origin, neomycin resistance gene and ampicillin resistance gene*" (Invitrogen, pcDNA3.1-TOPO TA expression Kit, 2004).

Similarly, according to its manual: *"pVAX1 is a 3.0 kb plasmid vector and specially designed for development of DNA vaccines. It is a derivative of pcDNA3.1/V5/His with reduced size and replacement of ampicillin resistance gene by kanamycin resistance gene"* (Invitrogen, pVAX1, catalog no. V260-20, version B)

3.3.1 Construction of DNA vaccine pTOPOKY98#6:

The DNA vaccine pTOPOKY98#6 was prepared by Lai et al. The HA1 gene of equine-2 influenza virus was cloned into the vector pTOPOV5/His by two different ways.

One clone was prepared without a 3' stop codon, and its open reading frame (ORF) was in frame with the C terminal His tag**.** The second clone was prepared by using 3' stop codon.

In this study, the clone prepared without a 3' stop codon was used as a DNA vaccine, designated as pTOPOKY98#6. One shot competent *E. coli* cells supplied by the manufacturer (Invitrogen, CA) were transformed with the pTOPOKY98#6. Clones were selected for the presence of ampicillin resistance. These clones were constructed and characterized by Lai et al.

3.3.2 Construction of DNA vaccine pVAXKY98/11-9:

The DNA vaccine pVAXKY98/11-9 was prepared by Lai et al. The HA1 gene in pTOPOKY98/11-9 was cut using restriction enzyme *Hind*III and *Bst*X1. The gene was ligated into eukaryotic expression vector pVAX1 according to the manufacturer's instruction (Invitrogen, CA). The clone was designated as pVAXKY98/11-9. These clones were constructed and characterized by Lai et al (unpublished data). As described previously, these clones were further characterized in vitro, for use as a DNA vaccine for equine influenza virus.

3.4 Extraction of plasmid DNA:

For extraction, DNA constructs were amplified in *Escherichia coli*. After amplification, DNA was extracted using a MaxiPrep plasmid DNA isolation method and subjected to restriction digestion and PCR for verification of the presence of the insert.

3.5 DNA band with Restriction digestion:

The different combinations of restriction enzymes such as *Hind*III and *Pst*I, *Hind*III and *Xho*I and *Hind*III and *Xba*I were used for restriction digestion. The preparations were designed as follows;

Table 3-1

Chemicals	Quantity
Plasmid DNA	10.0 µl
Reaction buffer	2.0 µl
HindIII	0.5 µl
*Pst*I (or *Xho*I or *Xba*I)	0.5 µl
Distilled water	7.0 µl

Table 3-1: Chemicals for restriction digestion

Ten µl of DNA was taken in an eppendorf tube and 0.5 µl of *Hind*III, 0.5 µl of *Pst*I (or *Xho*I or *Xba*I), 2.0 µl of reaction buffer and 7.0 µl of distilled water were added

to it to make a total reaction volume of 20.0 µl. The mixture was incubated for 2 hours on water bath at 37^0C.

The agarose gel electrophoresis was used for confirmation of insert in digested DNA. For that, 5 µl of digested DNA was mixed with 1 µl of blue tracking dye and loaded on a 1.5% agarose gel. The gel was run for 20 minutes at 100 V in TBE buffer. The gel was stained with ethidium bromide (1.0 µg/ml) for 10 minutes. The gel was visualized and the presence of DNA fragments was confirmed.

3.6 Confirmation of insert by PCR:

The lyophilized 2X Master Mix buffer (Promega, Wisconsin) was prepared by Lai et al. (Lai et al., 2001) to a volume of 17.0 µl with nuclease free water. Master mix buffer composition is given in Table 3-2.

Table 3-2

Chemicals	Quantity
Taq DNA polymerase	50 units/ml (pH 8.5)
dNTPs (dATP, dGTP, dCTP, dTTP)	400 mM
Tris HCl	50.0 mM (pH 9.0)
$MgCl_2$	3.0 mM
Nuclease free water	To a volume of 17.0 µl

Table 3-1: Chemicals used for preparation of PCR 2X Master mix buffer

PCR was performed using 17.0 µl of 2X Master mix buffer and using 1.0 µl of extracted plasmid DNA. The chemical preparation for PCR was performed as shown in Table 3-2.

Table 3-3

Chemicals	Quantity
Plasmid DNA	1.0 µl
Forward primer (T7 specific)	1.0 µl
Reverse primer	1.0 µl
Master Mix buffer	17.0 µl

Table 3-3: preparations for PCR

The different combinations of 1.0 µl of forward primers (forward primer specific to T7, EH_3 451^+) and 1.0 µl of reverse primers (EH_3 450^-, EH_3 1061^- and EH_3 832^-) were added to the tube to make a total volume of 20 µl. The PCR conditions provided were; denaturation at 95^0C for 30 seconds, annealing at 42^0C for 30 seconds, extension at 72^0C for one minute for a total of 30 cycles using a PCR thermocycler (Perkin, California). The presence of the DNA fragment was confirmed by agarose gel electrophoresis.

3.7 Transfection of HA in cell cultures:

In transfection experiments, two cell cultures, Vero cells and MDCK cells were used for expression of HA in tissue transfection and in-vitro protein expression. For cell culture, Dulbecco's modified Eagle's medium (DMEM) was used and antibiotics such as ampicillin, streptomycin and amphotericin (Cellgro, Virginia) were added to the medium. The standard cell culture method as described by George et al. (George et al., 1996) with some modifications was followed.

3.7.1 Transfection by lipofection:

Lipofection was used to transfect Vero cells and MDCK cells with designed DNA plasmids utilizing lipofectin**.** About eighty percent confluent cell cultures were used for transfection. Lipofection was performed for transfection of HA genes as follows;

a). Preparation of cell culture:

Cell culture plates were washed with 1x PBS and 1 ml of Trypsin-EDTA was added. Cells were incubated in a CO_2 incubator for 10 minutes. About 0.5 ml of cells were passed into 35 mm petri-dishes and 5 ml of DMEM with 5% fetal bovine serum (FBS) was added. The plates were incubated overnight in a CO_2 incubator. The Following day, cells were resuspended to 3-5 x 10^5 cells/ml.

b). Preparation of DNA-liposome mixture:

Two hundred µl serum free DMEM was placed in three separate tubes and 2.0 µl of pTOPOKY98, pVAXKY98 or pGFP DNA were added to tubes separately. Twenty-four µl of lipofectin was added to 600 µl of serum free DMEM in a separate tube and mixed well. Then, 200 µl of this solution was added to each of the DNA tubes. DNA-liposomes mixtures were prepared and incubated at room temperature for 30 minutes.

c). Transfection of DNA into cell cultures:

The DNA-liposome mixtures were transferred onto a monolayer of Vero cells and MDCK cells in a 35 mm Petri-dish separately after washing with 1x PBS. Six hundred µl of serum-free DMEM medium was added to each petri-dish separately. The plates were incubated for 7 hours in a CO_2 incubator and 1.0 ml DMEM containing 10% FBS was added to them. The plates were further incubated overnight in a CO_2 incubator.

d). Extraction of cells:

After overnight incubation, the resulting monolayer of cells was scraped and detached with the help of a sterilized rubber policeman. Cells were washed three times in PBS, and re-suspended in hypotonic saline solution (0.9%) for 10 minutes. The cells were centrifuged at 1000g for 1.0 minute and the supernatant was collected as the cytosol

fraction. The pellet was resuspended in sterile distilled and deionized water (ddH_2O), and centrifuged at 1000g for 1.0 minute and the supernatant was collected as the nuclear fraction. ELISA and PCR were performed for HA expression protein using both cytosolic and nuclear fractions to verify the presence of the DNA as a measure of transfection.

3.7.2 Transfection by electroporation:

Electroporation is the transfection of cells with plasmid utilizing electric current. Electroporation methods were adopted from methods as described by Chu et al with modifications (Chu et al., 1987). Electroporation was performed as follow:

a). Preparation of cell culture:

Cell cultures were prepared following the same way as with lipofection above. About eighty percent confluent Vero cells and MDCK cells were harvested by washing the cell sheet with PBS, and incubating at 37^0C for ten minutes after addition of 0.5 ml of Trypsin-EDTA. The cells were collected into a sterile 5 ml tube and washed with PBS three times by centrifugation at 500g for 1.0 minute. They were re-suspended to 2-5 x 10^6 cells/ml in DMEM without serum.

b). Preparation of DNA for eletroporation:

The cell suspension in DMEM was distributed in 400 μl volumes in three eppendorf tubes. Twenty μl of purified plasmid DNA, pTOPOKY98, pVAXKY98 and pGFP were added to the tubes and allowed to stand for 10 minutes at room temperature.

C). Electroporation:

A BTX electroporator (BTX-ECM 39, Genetronic) was used for transfection of the DNA preparation. The DNA mixture was added into electroporation cuvettes and pulsed in electroporator providing the following conditions: 260 V, 1050 μF, and 29 microseconds. After electroporation, the cells were incubated in the CO_2 incubator for 48 hours.

3.8 Mice vaccination study:

3.8.1 Design and schedule:

Mice vaccination and virus challenge study were performed in the Animal Laboratory Facilities, Center for Veterinary Health Sciences, Oklahoma State University, March-May, 2004. Seven groups of five, 4-8 weeks old female Balb/c mice were selected at random and separated into the following groups:

Table 3-4

Group	Treatment	Commonly used name
Group 1	negative control group (PBS only)	unvaccinated group
Group 2	positive control group (UV-inactivated KY98)	Inactivated group
Group 3	vaccinated group (pTOPOKY98#6, Liposome)	pTOPO (L) group
Group 4	vaccinated group (pTOPOKY98#6, MetaStim)	pTOPO (M) group
Group 5	vaccinated group (pVAXKY98/11-9, Liposomes)	pVAX (L) group
Group 6	vaccinated group (pVAXKY98/11-9, MetaStim)	pVAX (M) group
Group 7	non-related plasmid DNA group (pGFP, liposome)	pGFP group

Table 3-4 Table showing different groups of mice, treatment and common name

Table 3-5

Schedule	Experiments
Day 0	Body weight measurement, Serum collection and vaccination
Day 21	Body weight measurement, Serum collection and booster vaccination
Day 42	Body weight measurement, Serum collection and Virus challenge
Day 49 (from day 42 to 49)	Body weight measurement daily for 7 days, serum collection at 7 days post infection
Day 52	Serum collection and Euthanasia

Table 3-5: Table showing schedule of the vaccination and virus challenge

3.8.2 Mice vaccination:

The following vaccination protocols were used for delivering DNA vaccines in mice:

a). Vaccine dosage:

A vaccine dosage of 25µl dose/mouse containing 0.01 µg DNA per gram body weight was designed. Before vaccination, the DNA preparation diluted in DMEM to 20 µg/ml and lipofectamine in DMEM (20µg/ml) were mixed and allowed to stand at room temperature for 20 minutes. Twenty five µl of respective preparation of DNA was inoculated per nostril intranasally to each mouse of vaccinated groups.

b). Intranasal inoculation:

For mice groups 3 to 6, vaccination was performed following intranasal inoculation with the designed primary dose of the respective preparation of DNA vaccines on day 0. A booster dose was given on day 21 following the same route and dose.

For the unvaccinated group, 25 µl of PBS was administered per nostril intranasally. For the inactivated or positive control group, the uv-inactivated A/Eq/Ky/98 virus was administered intranasally. The inactivated virus preparation was prepared from

an allantoic fluid culture of A/Eq/Ky/98 virus with a HA titer of 1:16. The virus was inactivated by exposure to ultra-violet radiation by placing 1.0 ml of the virus suspension in a petri-dish, without a lid, at a distance of 15 inches from a 30 W UV lamp, for 15 minutes. This method was adapted from the methods of Ramamoorthy (Ramamoorthy, 2001).

c). Virus challenge:

On day 42, all groups of mice were challenged with homologous A/Eq/Ky/98 virus with a HA titer of 16 HA units by intranasal inoculation of 25 μl per nostril. The mice body weights were recorded on day 0, 21, 42 before challenge and everyday after challenge for 10 days. All mice were bled on the 7^{th} day post challenge for serum collection and euthanized on the 10^{th} day using isofluorane as anesthesia, followed by cervical dislocation.

d). Serum collection:

A retro-orbital bleeding method was used to collect blood from mice. The procedures were carried using Isofluorane (Fluorane,1-chloro-2, 2, 2-trifluoroethyldifloromethyl ether) anesthesia. In a closed glass jar, a cotton pad soaked with 0.5 ml of 2 % isofluorane was placed and mice were held until loss of pedal and ocular reflexes occured. For that an average time of one and a half minutes was allowed.

The blood samples were collected during the time period when mice had the effect of anesthesia.

3.9 ELISA for viral specific IgG and IgA titers:

ELISA methods were adopted from Deck et al (Deck et al., 1997) with the following modifications performed in the following steps:

a). Coating of ELISA plates:

ELISA plates (Nalge Nunc, NY) were coated with a suspension of purified influenza virus. The virus was diluted in 50mM $NaHCO_3$ solution to 0.6 HA units/ml, and 100 µl of this virus suspension was added to each well of the ELISA plate. The plates were left at room temperature for 24 hours for the antigen to be coated onto the plate.

b). Blocking buffer:

Before using blocking buffer, the plates were washed three times with 1x PBS. A blocking buffer (PBS containing 2% BSA and 0.5% skim milk) was prepared and 100 µl of blocking buffer was added to wells of the ELISA plates. Plates were incubated at room temperature for 1 hour.

c). Addition of primary antibody:

The different dilutions of mouse serum (1:25, 1:50, 1:100 and 1:200) were prepared in PBS with 2% BSA and 100 µl of diluted serum was added in triplicate to plates. The plates were washed three times again with 1x PBS before adding primary antibody. The plates were incubated at room temperature for 1 hour.

d). Addition of secondary antibody:

The 1:2000 dilutions of anti-mouse IgG and IgA conjugate (Chemicon, CA) were prepared and 100 µl of each preparation was added to the plates in the respective wells after washing the plates three times with 1x PBS. The plates were incubated at room temperature for 1 hour.

e). Addition of substrate:

1 mg/ml of pNPP (p-nitrophenyl phosphate, Sigma, MO)) was prepared in glycine buffer and used as a substrate. After incubation, the plates were washed with 1x PBS three times and 100 µl of substrate was added. The plates were incubated for two and a half hours at room temperature.

f). Serum titers:

After incubation, O. D. readings were taken at 405 nm using the ELISA plate reader (Biotek Instruments). Mice serum titer observed on day 0 was used as a normal sera titer. Titers were expressed as the mean of triplicates after subtracting the blank (control) and normal sera titer.

3.10 Statistical analysis:

Statistical analysis was carried out using the SAS program and the PROC GLM procedure for ANOVA (Analysis of Variance) (SAS Institute, 2004). The graph construction was carried out using the Sigma-plot program (SigmaPlot, 2004). The standard error of the mean was obtained and represented graphically. A Dunett's t test was performed on the challenge experiment and ELISA data and levels of significance were determined using a 95% confidence interval ($\alpha = 0.05$).

CHAPTER 4

RESULTS

4.1 Confirmation of insert in DNA vaccine constructs:

Two DNA vaccine vectors pTOPOKY98#6 and pVAXKY98/11-9 used in the vaccination experiment were characterized by PCR and restriction digestion experiments. They were validated for the HA gene insert, and its orientation. By performing the PCR and restriction digestion, a correct size of insert and correct orientation was observed in the pTOPOKY98#6 and pVAXKY98/11-9 vectors as shown in figure 4-1 and 4-2.

4.1.1. Confirmation by restriction digestion:

The orientations of insert are given in Fig 4-1 and 4-2, photographs of agarose gels with restriction digestion samples of vectors pTOPOKY98 and pVAXKY98. The 1.0 kb band in the sample lanes 1 and 2, digested with two *Hind*III and *Pst*I and *Hind*III and *Xho*I restriction enzymes, indicates that the 1 kb HA1 fragment was correctly inserted into the vector.

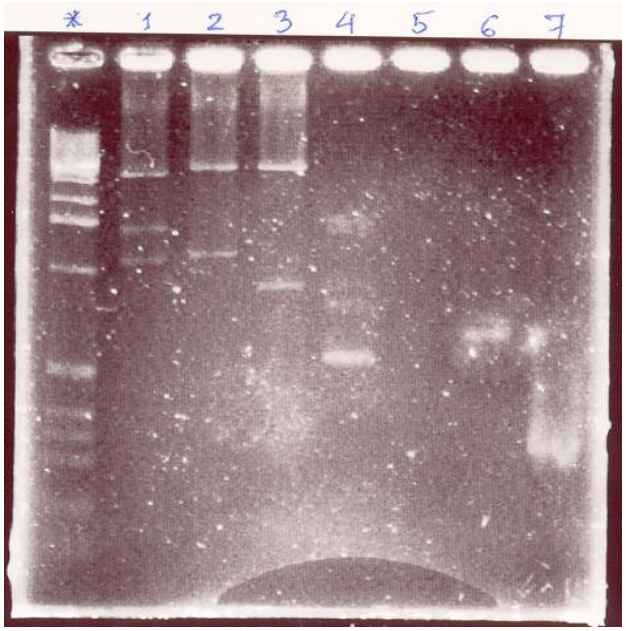

Fig 4-1: Restriction digestion and PCR of pTOPOKY98#6: Lane *: 1 Kb DNA ladder; Lane 1: *Hind*III + *Pst*I; Lane 2: *Hind*III + *Xho*I; Lane 3: *Hind*III + *Xba*I; Lane 4: forward primer specific to T7 promoter + EH_3 450^-; Lane 5: T7 plus EH_3 1061^-; Lane 6: EH_3 451^+ + EH_3 1061^- and Lane 7: EH_3 451^+ + EH_3 832^-

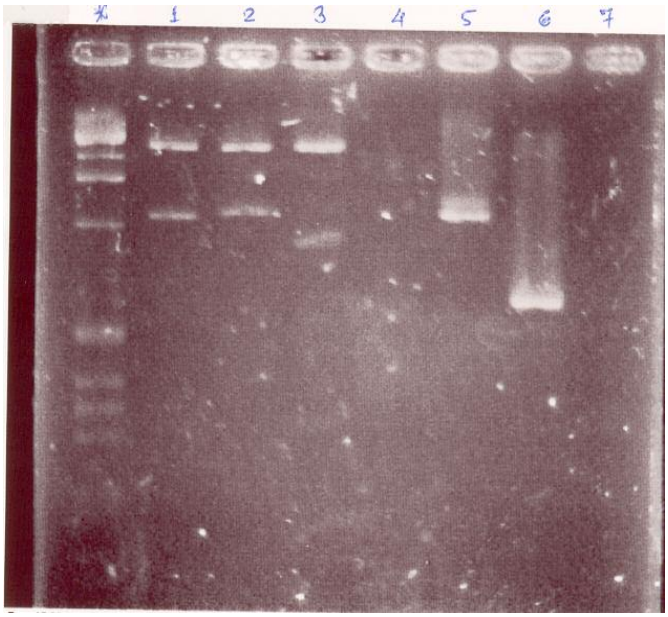

Fig 4-2: Restriction digestion and PCR of pVAXKY98/11-9: Lane *: 1 Kb DNA ladder; Lane 1: *Hind*III + *Pst*I; Lane 2: *Hind*III + *Xho*I; Lane 3: *Hind*III + *Xba*I; Lane 4: forward primer specific to T7 promoter + EH_3 450^-; Lane 5: T7 plus EH_3 1061^-; Lane 6: EH_3 451^+ + EH_3 1061^- and Lane 7: EH_3 451^+ + EH_3 832^-

4.1.2 Confirmation by PCR:

The PCR method was used for verification of the orientation of the insert in DNA construct. The orientation was verified using a forward primer specific to the 5' T7 promoter and a reverse primer specific to an internal site 415 bps down stream of the HA gene. The expected product was found as seen lanes 6 and 7 as shown in Fig. 4-1.

4.1.3 PCR for transfected HA:

The PCR was used for cytosol and nuclear fractions from cell cultures transfected with pTOPOKY98#6 and pVAXKY98/11-9. For this experiment, a forward primer EH_3 29^+ and a reverse primer EH_3 1061^- were used. The expected 1kb bands were not observed, however HA expression proteins were detected by ELISA (data not shown). The plasmid pGFP was used as a negative DNA control (figure not shown).

4.2 Mice vaccination study:

A weight loss model was designed in mice to evaluate the effectiveness of vaccination. The body weight of each mouse was taken daily after virus challenge and the results were expressed as the percentage change in body weight after challenge. The body weight of each mouse on the day of virus challenge was considered as day 0 body weight. The graphs are made plotting mean weight loss in each group with the standard error of the mean (SEM) against the days after challenge as shown in Fig. 4-3.

a). Disease symptoms:

After virus challenge, mice were observed for clinical symptoms for next ten days to determine the severity of disease in each group. The prominent "fluffy coat" appearance and inactivity were observed in the unvaccinated group mice for 3 days after virus challenge and they took longer time for recovery. A lesser severity of disease was observed in the vaccinated group mice in comparison to the unvaccinated mice and found that they recovered more quickly. Likewise, similar symptoms were observed in the pGFP group as found in the unvaccinated mice group but recorded a lesser weight loss than that of the unvaccinated group. The symptoms were found for a longer period than the vaccinated groups and almost similar to the unvaccinated group.

b). Weight loss result:

A maximum of 8.4% mean body-weight loss on day 3 was recorded in the unvaccinated group mice and they did not recover the initial body-weight for more than 7 days. Maxima of 1.2% and 0.82% body weight losses were observed in pTOPOKY98 and pVAXKY98 vaccinated group mice which were significantly different from the unvaccinated group mice.

Likewise, a maximum of 6.5% weight loss on day 3 in the pGFP group mice was recorded that is still less than that of the unvaccinated group. As symptoms were found for a longer period than the vaccinated groups and almost similar to the unvaccinated group; it is believed that this type of observation is due to the nonspecific protection elicited by the DNA vaccine.

c). Statistical significance of weight loss model:

The body weight loss in the vaccinated groups was found to be significantly different from that unvaccinated group ($P<0.001$). The pGFP group showed no significant difference with a P value of 0.23. There were no significant differences observed between the vaccinated groups pTOPOKY98 and pVAXKY98 ($p=0.67$); likewise no significant differences were observed between liposome and metastim treatment groups ($p=0.30$). The output of statistical analysis using SAS program is given in Appendix-A.

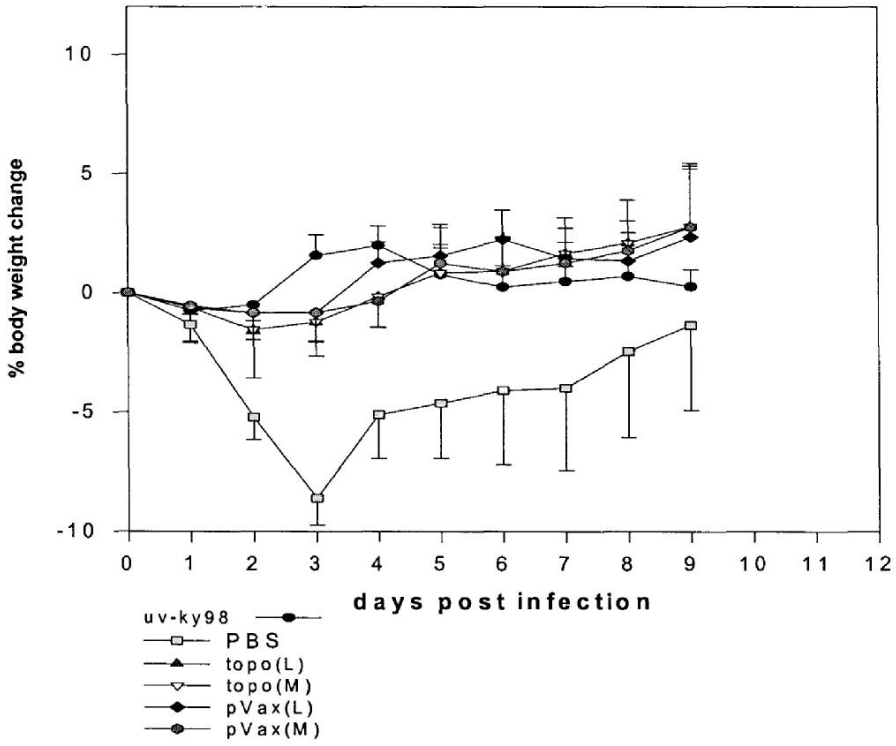

Fig 4-3: Figure showing weight loss in mice after virus challenge. (uv-ky98: inactivated group; PBS: unvaccinated group, topo (L) (pTOPO L); topo (M) (pTOPO M); pVax (L); pVax (M); vaccinated groups.)

4.3.1 Viral specific IgG (mean O.D.) value:

The mice sera were collected at day 0, 21, 42 and 49 (day 7 post-infection) and these samples were assayed for virus-specific IgG mean O.D. value. An increased optimal density (O.D.) at 405nm was observed in sera after vaccinations and expressed as the mean of triplicates after subtracting the blank (control) and normal sera titer. The different dilutions 1:25, 1:50, 1:100 and 1:200 of the sera were used to assay the virus-specific IgG titers and found that 1:25 dilution was found to provide an optimal O.D. value. The mean IgG titers are given in Table 4-1 and plotted in Fig 4-5, 4-6, 4-7 and 4-8.

a). Observation of IgG (mean O. D.) value on day 21:

A mean O.D. increase of 0.43 ($p<0.01$) units above normal serum was observed in the inactivated group. In pTOPO (L) and pTOPO (M) groups, an increase of 0.36 ($p<0.01$) and 0.43 ($p<0.01$) O. D. units were observed respectively whereas in pVAX (L) and pVAX (M) groups, an increase of 0.40 ($p<0.01$), and 0.49 ($p<0.01$) O.D. units, were recorded respectively. An increase of 0.20 ($p = 0.70$) was observed in the pGFP group. On day 21, the vaccinated groups and the inactivated group showed significant increase in titers when compared to the unvaccinated group.

b). Observation of IgG (mean O.D.) value on day 42:

An increased of 0.72 ($p<0.01$) O.D. units was recorded in the inactivated group. In pTOPO (L) and pTOPO (M) groups, an increase of 0.52 O.D. units ($p<0.01$), and 0.88

O.D. units ($p<0.01$) were recorded respectively. Whereas, for the pVAX (L) and pVAX (M) groups, an increase of 0.59 ($p<0.01$) and 0.72 ($p<0.01$) O. D. units were recorded respectively. No significant increase for the pGFP group ($p=0.69$) was recorded. The output of statistical analysis is given in Appendix-B.

On day 42, the vaccinated groups and the inactivated group showed significant increase in titers compared to the unvaccinated groups. Significant differences were observed between two different vaccinated groups pTOPO and pVAX on day 42 and 49 ($p<0.01$) while marginal difference was observed on day 21 ($p=0.049$). Likewise significant differences were observed between liposome and metastim treatment groups on day 42 and 49 for pTOPO ($p<0.01$) and on day 21, 42 and 49 for pVAX ($p<0.01$).

c). Observation of IgG (mean O.D.) value after virus challenge:

On day 49 (day 7 post infection), an increase of 1.4 O.D. ($p<0.01$) units was recorded in the inactivated group. In pTOPO (L) and pTOPO (M) groups, an increase of 1.0 ($p<0.01$) and 1.2 ($p<0.01$) O.D. units were recorded respectively. Similarly, in pVAX (L) and pVAX (M) groups; an increase of 1.1 ($p<0.01$) and 1.76 ($p<0.01$) O.D. units were recorded respectively. The titers of the unvaccinated group and the pGFP groups were also found to be increased by 0.66 ($p<0.01$) and 0.74 O.D. units ($P<0.01$) respectively.

Table 4-1

Virus specific IgG as measured by ELISA		
Group	Day	IgG titer (Mean O. D. +/- SEM) (Dilution: 1:25)
Gr-1 unvaccinated mice	Day 21	0.22 (+/- 0.016)
	Day 42	0.22 (+/- 0.037)
	Day 7 post infection	0.66 (+/- 0.071)
Gr-2 Inactivated group	Day 21	0.43 (+/- 0.042)
	Day 42	0.72 (+/- 0.077)
	Day 7 post infection	1.42 (+/- 0.289)
Gr-3 pTOPO (L)	Day 21	0.36 (+/- 0.044)
	Day 42	0.52 (+/- 0.014)
	Day 7 post infection	1.01 (+/- 0.110)
Gr-4 pTOPO (M)	Day 21	0.43 (+/- 0.006)
	Day 42	0.88 (+/- 0.159)
	Day 7 post infection	1.28 (+/- 0.263)
Gr-5 pVAX (L)	Day 21	0.40 (+/- 0.162)
	Day 42	0.59 (+/- 0.073)
	Day 7 post infection	1.11 (+/- 0.076)
Gr-6 pVAX (M)	Day 21	0.49 (+/- 0.017)
	Day 42	0.72 (+/- 0.078)
	Day 7 post infection	1.76 (+/- 0.331)
Gr-7 pGFP	Day 21	0.20 (+/- 0.069)
	Day 42	0.21 (+/- 0.033)
	Day 7 post infection	0.74 (+/- 0.156)

Table 4-1: Table showing viral specific IgG (mean O.D.) value for each group with SEM (standard error of the mean) after vaccinations and virus challenge. Mean O.D. and SEM were obtained using Sigma Plot, 2004. (Refer to Table 3-4 for abbreviation).

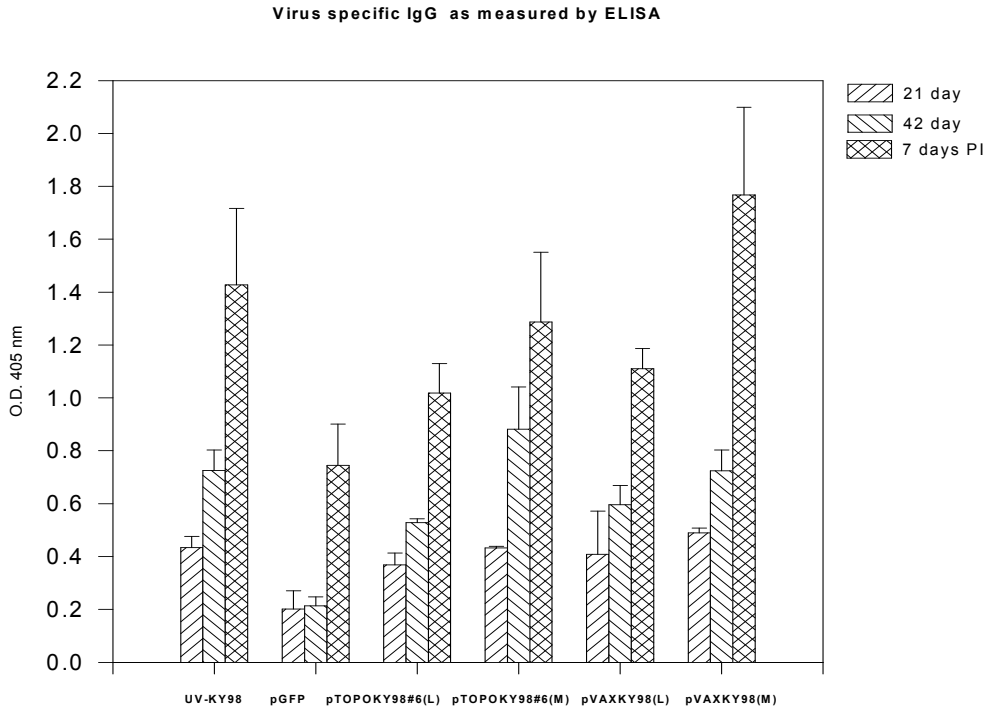

Fig 4-5: Viral specific IgG (mean O.D.) value as measured by ELISA. (uv-ky98: Inactivated group, pGFP: pGFP group, pTOPO (L); pTOPO (M); pVAX (L), pVAX (M); vaccinated groups. (Refer to Table 3-4 for abbreviation).

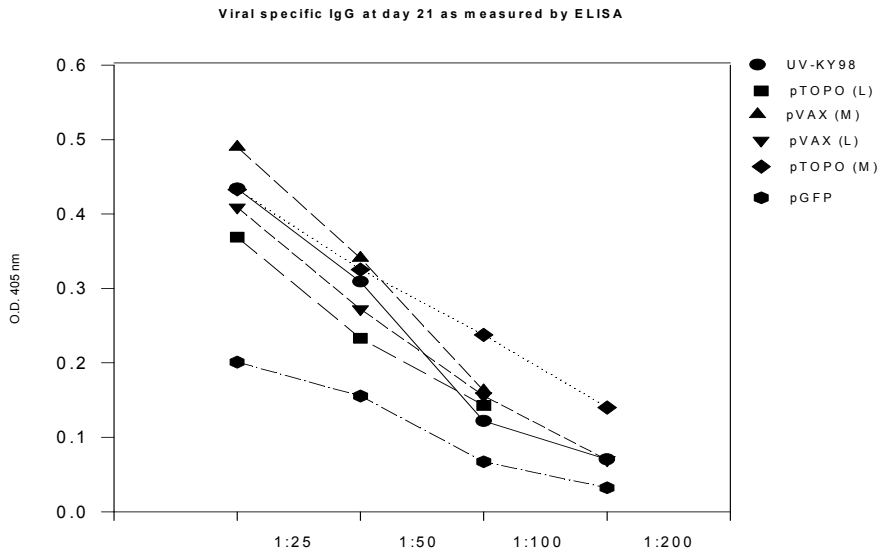

Fig 4-6: Viral specific IgG at day 21 as measured by ELISA using serum dilutions (1:25, 1:50, 1:100, 1:200)

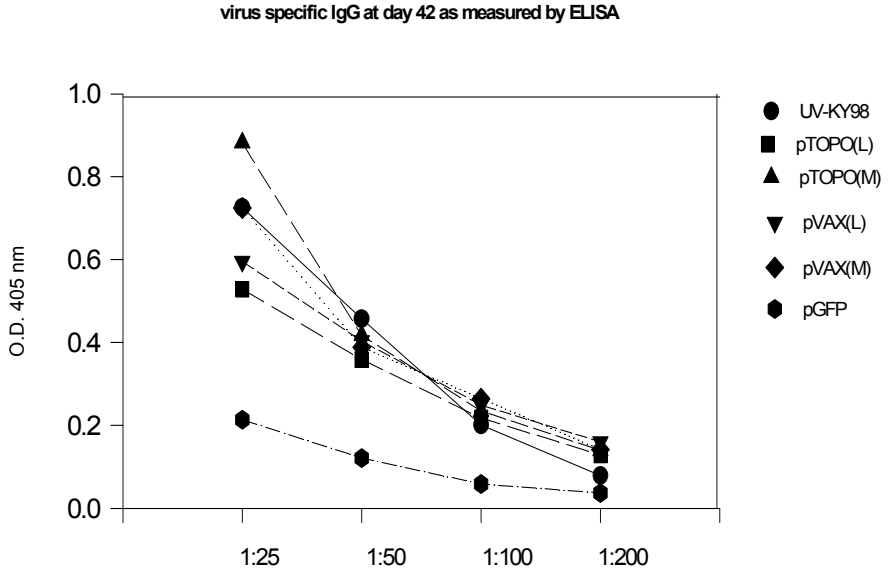

Fig 4-7: viral specific IgG at day 42 as measured by ELISA using serum dilutions (1:25, 1:50, 1:100, 1:200)

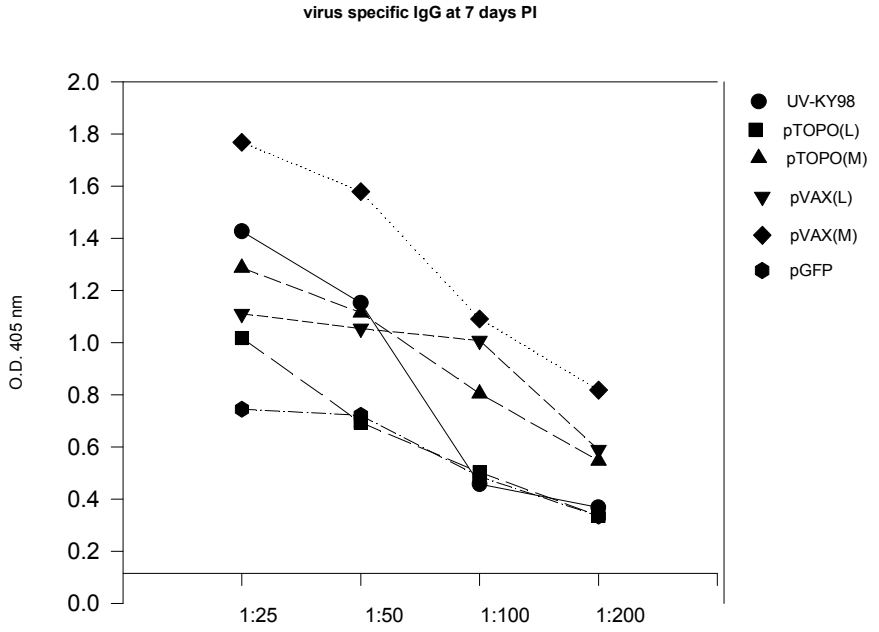

Fig 4-8: Viral specific IgG at 7 days post-challenge as measured by ELISA using serum dilutions (1:25, 1:50, 1:100, 1:200)

4.3.2 Viral specific IgA (mean O.D.) value:

The mice sera were collected on day 0, 21, 42 and 49 (day 7 post-infection) and these samples were assayed for virus-specific IgA titers. An increased optimal density (O.D.) at 405nm in sera was observed after vaccinations and is expressed as the mean of triplicates after subtracting the blank and normal sera titer. The different dilutions 1:25, 1:50, 1:100 and 1:200 of the sera were used to assay the virus-specific IgA titers. The mean IgA titers are given in Table 4-2 and plotted in Fig 4-9, 4-10 and 4-11.

a). Observation of IgA (mean O.D.) value on day 21:

A mean O.D. increase of 0.21 ($p<0.01$) units above the normal value was observed in the inactivated group. In pTOPO (L) and pTOPO (M) groups; an increase of 0.21 ($p<0.01$) and 0.22 ($p<0.01$) O. D. units were recorded respectively whereas in pVAX (L) and pVAX (M) groups; an increase of 0.23 ($p<0.01$), and 0.25 ($p<0.01$) O.D. units were recorded respectively. An increase of 0.18 ($p = 0.0023$) was recorded in the pGFP group. On day 21, the vaccinated groups and the inactivated group showed significant increases in mean O.D. values when compared to the unvaccinated groups.

b). Observation of IgA (mean O.D.) value on day 42:

An increase of 0.38 ($p<0.01$) O.D. units was recorded in the inactivated group. In pTOPO (L) and pTOPO (M) groups; an increase of 0.41 O.D. units ($p<0.01$), and 0.44

O.D. units ($p<0.01$) were recorded respectively. Whereas, in pVAX (L) and pVAX (M) groups; an increase of 0.58 ($p<0.01$) and 0.59 ($p<0.01$) O. D. units were recorded respectively. No significant increase in the pGFP group ($p=0.079$) was observed. The output of statistical analysis is given in Appendix-C.

On day 42, the vaccinated groups and the inactivated group showed significant increases in mean O.D. values when compared to the unvaccinated groups. No significant differences were observed between two different vaccinated groups pTOPO and pVAX; likewise no significant differences were observed between liposome and metastim treatment groups.

c). Observation of IgA (mean O.D.) values after virus challenge:

On day 49 (day 7 post infection), an increase of 0.99 O.D. ($p<0.01$) units was observed in the inactivated group. In pTOPO (L) and pTOPO (M) groups; an increase of 0.72 ($p<0.01$) and 0.79 ($p<0.01$) O.D. units were recorded respectively. Similarly, in pVAX (L) and pVAX (M) groups; an increase of 0.79 ($p<0.01$) and 0.80 ($p<0.01$) O.D. units were recorded respectively. The mean O.D. values of the unvaccinated group and the pGFP groups were found to be increased by 0.50 ($p<0.01$) and 0.55 O.D. units ($P<0.01$).

Table 4-2

Virus specific IgA as measured by ELISA		
Group	Day	IgA titer (Mean O. D. +/- SEM) Dilution (1:25)
Gr-1 unvaccinated mice	Day 21	0.19 (+/- 0.023)
	Day 42	0.21 (+/- 0.042)
	Day 7 post infection	0.48 (+/- 0.071)
Gr-2 Inactivated group	Day 21	0.21 (+/- 0.022)
	Day 42	0.38 (+/- 0.052)
	Day 7 post infection	0.99 (+/- 0.326)
Gr-3 pTOPO (L)	Day 21	0.21 (+/- 0.033)
	Day 42	0.41 (+/- 0.012)
	Day 7 post infection	0.72 (+/- 0.182)
Gr-4 pTOPO (M)	Day 21	0.22 (+/- 0.005)
	Day 42	0.44 (+/- 0.124)
	Day 7 post infection	0.79 (+/- 0.223)
Gr-5 pVAX (L)	Day 21	0.23 (+/- 0.112)
	Day 42	0.58 (+/- 0.089)
	Day 7 post infection	0.79 (+/- 0.308)
Gr-6 pVAX (M)	Day 21	0.25 (+/- 0.028)
	Day 42	0.59 (+/- 0.062)
	Day 7 post infection	0.80 (+/- 0.221)
Gr-7 pGFP	Day 21	0.18 (+/- 0.069)
	Day 42	0.19 (+/- 0.044)
	Day 7 post infection	0.55 (+/- 0.144)

Table 4-2: Table showing viral specific IgA (mean O. D.) value for each group with SEM (standard error of the mean) after vaccinations and virus challenge. Mean O. D. and SEM were obtained using Sigma Plot, 2004. (Refer to Table 3-4 for abbreviation).

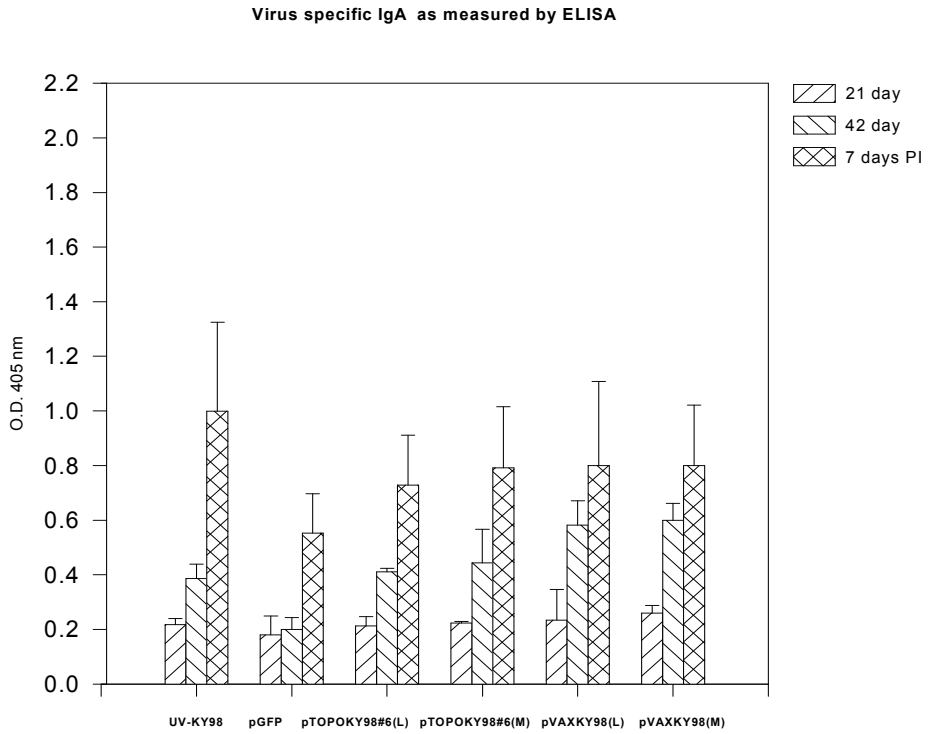

Fig 4-9: Viral specific IgA (mean O.D.) values as measured by ELISA. (uv-ky98: Inactivated group, pGFP: pGFP group, pTOPO (L); pTOPO (M); pVAX (L), pVAX (M); vaccinated groups. (Refer to Table 3-4 for abbreviation).

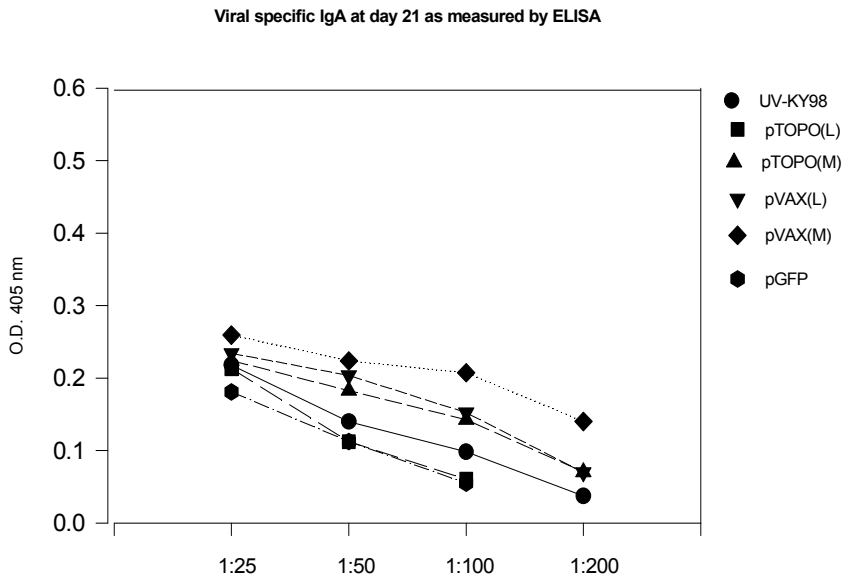

Fig 4-10: Viral specific IgA at Day 21 measured by ELISA using serum dilutions (1:25, 1:50, 1:100, 1:200)

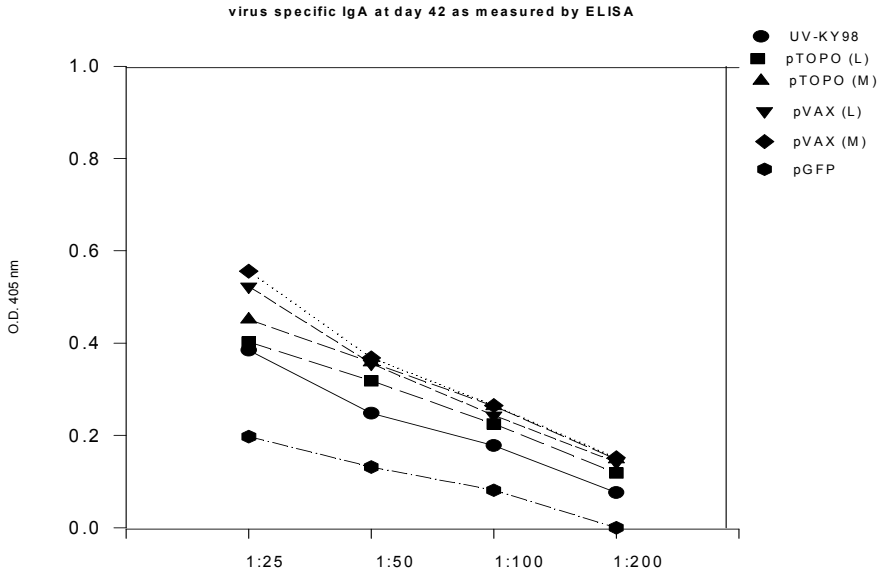

Fig 4-11: Viral specific IgA at Day 42 as measured by ELISA using serum dilutions (1:25, 1:50, 1:100, 1:200)

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Discussion:

The objective of this study was to evaluate the effectiveness of designed DNA vaccines pTOPOKY98#6 and pVAXKY98/11-9 in eliciting protective immunity in mice. In this study, the levels of serum antibody after vaccination and weight loss after virus challenge were studied, and it was found that after two intranasal vaccinations with designed DNA vaccines pTOPOKY98#6 and pVAXKY98/11-9, a significant increase of serum antibody levels was found in the vaccinated mice as shown in Fig. 4-5 and 4-9.

In this study, it was found that designed plasmids encoding the HA1 gene were effective in generating immune responses in mice. Several studies conducted by Fynan et al., Montgomery et al., Robinson et al., Bot et al., Kodihalli et al. and Johnson et al. reported that an HA expressing plasmid could induce protective immunity against influenza (Fynan et al., Montgomery et al., Robinson et al., 1993, Bot et al., 1997, Kodihalli et al., 1999; Johnson et al., 2000).

In the study of Fynan et al., it was reported that the plasmid DNA expressing HA protein using different routes of inoculation could initiate protective immunity in mice (Fynan et al., 1993). It was also reported that the DNA quantity required was less in mucosal routes like trachea or nares and was successful in inducing protection. Similarly, in the study of Wong et al, intranasal delivery of viral HA in liposome was found to provide protection, and both serum IgG and IgA were found (Wong et al., 2001).

Similarly, it was reported by Bot et al. that a plasmid bearing a HA gene was successful in generating protective immune responses in newborn and adult mice (Bot et al., 1997). In their study, mice survival was significantly increased after immunization. Likewise, it was reported by Johnson et al. that direct intramuscular injection of plasmid DNA induced Th1 responses and a high level of protection against live influenza virus challenge. It was reported by Kodihalli et al. that a DNA vaccine encoding HA provided immunity against highly virulent equine virus H5N1 infection in mice.

In this study, it was found that the expression of HA1 alone is sufficient to elicit protective immunity, which is in accordance to findings of Ramamoorthy, and Tonegawa et al (Ramamoorthy, 2001; Tonegawa et al., 2003). It was reported by Ramamoorthy that a protective immunity was observed in mice using pTOPOKY98 expressing HA1 from equine-2 influenza virus (Ramamoorthy, 2001). The increased levels of serum IgG and IgA titers were also found in the vaccinated mice. Similarly, in the study of Tonegawa et al., mice were immunized with HA1 DNA and a humoral response was observed after immunization and an increased survival rate was also found against homologous virus

challenge. However, the gene gun method was used in delivering DNA. It is expected that using an HA1 segment instead of full length HA gene in the DNA vector, will facilitate the release of HA1 protein in host cells because it will not be membrane bound in the absence of HA2. This will help to elicit better immunity and a lower dose of DNA will be sufficient to generate protective immunity (Ramamoorthy, 2001).

In this study, it was found that dose levels as low as 0.01 μg DNA/gram body-weight were sufficient to elicit protective immunity in mice. In the study of Fynan et al., it was reported that as little as 0.4 μg of DNA was sufficient to provide protection in mice (Fynan et al., 1993). The DNA dose used in this study is less than that employed in the study of Fynan et al. Similarly, it was reported by Robinson et al. that a primary dose of 0.04 μg of DNA and a booster dose of 0.004 μg of DNA were sufficient to provide protection against lethal viral challenge (Robinson et al., 1997). The primary dose used was somewhat similar to this study while the booster dose was less in the study of Robinson et al., however, gene gun was the delivery method in that study. In the study of the Tonegawa et al., it was reported that 0.006 μg dose of HA while using #1HA1 DNA and less than 0.06 μg dose of HA while using #2HA1 DNA were sufficient to induce humoral immunity and provide increased survival of mice. The DNA dose used in this study is nearly similar to that of the Tonegawa et al. study while using #2HA1; however the gene gun was the method of delivery in their study also.

It was found that intranasal immunization using these DNA vaccines elicits specific IgG and IgA antibodies to the HA1 protein. Previous studies have shown that

mucosal immunity is important in protection against influenza virus and other respiratory infections (Liew et al, 1984; Ada et al., 1986; Fynan et al., 1993; Lunn et al., 1999). It was reported by Liew et al. that IgA is responsible for protection against influenza virus infection (Liew et al., 1984). In the study of Lunn et al., it was reported that delivery of an HA encoding plasmid at skin and mucosal sites in ponies was found to stimulate IgG responses and a poor IgA response (Lunn et al,. 1999).

Many studies have shown that intranasal inoculation of a DNA vaccine for influenza virus can elicit protective immunity in mice (Fynan et al.; Wong et al, 2001, 1993, Ramamoorthy and Lai, 2001). In the study of Fynan et al., it was reported that 95% protection was found in mice after mucosal routes of immunization like trachea and nares. Likewise in the study of Wong et al., it was reported that intranasal delivery of DNA by liposomes induced protective mucosal immunity in the respiratory tract. It was reported by Ramamoorthy that intranasal inoculation of plasmid DNA induced protective immunity in mice (Ramamoorthy, 2001). It has been found that specialized lymphoid tissues known as MALT (mucosa-associated lymphoid tissues) and NALT (nasal-associated lymphoid tissue) are very much similar in all vertebrates. It is expected that these lymphoid tissues will function in the same way in horses in eliciting protective immunity against influenza infections.

In this study, it was also found that two intranasal inoculations were significantly better than one inoculation. As reported earlier by Ramamoorthy, a second booster did not result in an increase in the titers of viral specific IgG or IgA and was not necessary

(Ramamoorthy, 2001). Protective immunity was observed in mice by encapsulation of the DNA vaccine into liposome and metastim and by delivering the DNA vaccine intranasally. The protection is probably mediated by IgA and IgG as there was a significant increase of viral specific IgA and IgG in serum after the first booster vaccination.

In this study, no significant differences were observed between two different vaccinated groups pTOPOKY98 and pVAXKY98 ($p=0.67$) while using the weight loss model after virus challenge. Similarly, no significant differences were observed between liposome and metastim treatment groups ($p=0.30$) for the weight loss model. In the case of IgG levels, significant differences were observed between two different vaccinated groups pTOPO and pVAX on day 42 and 49 ($p<0.01$) while a marginal difference was observed on day 21 ($p=0.049$). IgG mean O.D. values were comparatively higher in the pVAX group than the pTOPO group on day 21, 42 and 49. Likewise significant differences were observed for the liposome and the metastim treatment groups on day 42 and 49 for the pTOPO ($p<0.01$) and on day 21, 42 and 49 for the pVAX ($p<0.01$). IgG mean O.D. values were slightly higher in the metastim group than the liposome group. While in the case of IgA levels, no significant differences were observed between two different vaccinated groups pTOPO and pVAX; likewise no significant differences were observed between liposome and metastim treatment groups. A possible explanation for the high mean O.D. value of IgG is due to anamnestic responses after booster vaccinations. The increase in mean O.D. values of IgA was not very high even after booster vaccination in comparison to IgG titer.

The continuous emergence of new variants of viruses in the circulation due to antigenic variations in HA genes and antigenic mismatching between viruses present in circulation and virus present in vaccine strains, are the major cause of current vaccine failures (Palese and Garcia-Sastre, 2002; Daly, Newton and Mumford, 2004). The conventional vaccines are found to induce specific antibodies to circulating strains of viruses, and elicit only humoral immunity, and are therefore not very effective. DNA vaccines have the potential to overcome these short-falls because they have the advantages of eliciting cell mediated responses like live vaccines and at the same time they are safer than the live vaccines. Another advantage for DNA vaccines is that multiple immunogenic genes can be engineered in the same vector. This will reduce the number of vaccinations required in horses. Similarly, it is believed that DNA vaccines can elicit immunity even in the presence of circulating antibodies and maternal antibodies will not hamper the effectiveness of DNA vaccines.

5.2. Conclusions:

Protective immunity was found in mice after intranasal vaccination with designed DNA vaccines pTOPOKY98#6 and pVAXKY98/11-9. The elevated IgG and IgA levels were recorded in sera of vaccinated groups of mice. Similarly, HA1 was found to be sufficient to elicit protective immunity against homologous challenge of equine-2 virus, A/Eq/ky/98 in mice.

It was found that the intranasal route using liposome and metastim as adjuvants was very effective. While using the weight loss model, no significant differences were observed between two different vaccinated groups pTOPOKY98 and pVAXKY98 and no significant differences were observed between liposome and metastim treatment groups. IgG mean O.D. values were higher in the pVAX group and metastim treatment group in comparison to the pTOPO group and the liposome treatment group respectively while no significant difference was found in the case of IgA mean O. D. values in these groups and treatments. More importantly, dose levels as low as 0.01 μg DNA/gram body-weight were sufficient to elicit protective immunity against a sub-lethal challenge in mice.

Therefore, DNA vaccines have a great potential to replace currently available vaccines for equine influenza virus. It is expected that these designed vaccines will prove to be effective in horses also.

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APPENDIXES

Appendix – A

The GLM Procedure for Weight Loss Model

Class	Levels	Values
group	7	group1 group2 group3 group4 group5 group6 group7

Dependent Variable: day_3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	18.84537833	3.14089639	4.75	0.0024
Error	25	16.54342167	0.66173687		
Corrected Total	31	35.38880000			

Dunnett's t Tests for day_3

This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	25
Error Mean Square	0.661737
Critical Value of Dunnett's t	2.76322

Comparisons significant at the 0.05 level are indicated by ***.

group Comparison	Difference Between Means	Simultaneous Confidence	95% Limits	
group5 - group1	2.2440	0.8224	3.6656	***
group4 - group1	1.7800	0.3584	3.2016	***
group3 - group1	1.5900	0.1684	3.0116	***
group6 - group1	1.5613	-0.0802	3.2029	***
group2 - group1	1.6805	- 0.0827	3.2018	***
group7 - group1	0.5240	-0.8976	1.9456	

Note: SAS output for Weight loss model in mice on day 3 after Virus challenge. A Proc GLM procedure was used for analysis of F value and p value and determination of significance. A Dunnett's t Test was used to compare each treatment with negative control group 1.

Appendix - B

The GLM Procedure for IgG

Class	Levels	Values
group	7	group1 group2 group3 group4 group5 group6 group7

Dependent Variable: day_42

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	1.86237988	0.31039665	85.82	<.0001
Error	25	0.09042345	0.00361694		
Corrected Total	31	1.95280333			

Dunnett's t Tests for day_42

This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	25
Error Mean Square	0.003617
Critical Value of Dunnett's t	2.76322

Comparisons significant at the 0.05 level are indicated by ***.

group Comparison	Difference Between Means	Simultaneous Confidence	95% Limits	
group4 - group1	0.65478	0.54968	0.75988	***
group2 - group1	0.49880	0.38732	0.61028	***
group6 - group1	0.49740	0.37604	0.61876	***
group5 - group1	0.36850	0.26340	0.47360	***
group3 - group1	0.30170	0.19660	0.40680	***
group7 - group1	-0.01330	-0.11840	0.09180	

Note: SAS output for IgG titer on day 42, three weeks after booster vaccination. A Proc GLM procedure was used for analysis of F value and p value and determination of significance. A Dunnett's t Test was used to compare each treatment with negative control group 1.

Appendix - C

The GLM Procedure for IgA

Class	Levels	Values
group	7	group1 group2 group3 group4 group5 group6 group7

Dependent Variable: day_42

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.59200313	0.09866719	50.69	<.0001
Error	25	0.04866660	0.00194666		
Corrected Total	31	0.64066973			

Dunnett's t Tests for day_42

This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	25
Error Mean Square	0.001947
Critical Value of Dunnett's	2.76322

Comparisons significant at the 0.05 level are indicated by ***.

group Comparison	Difference Between Means	Simultaneous Confidence	95% Limits	
group6 - group1	0.33760	0.24857	0.42663	***
group5 - group1	0.32040	0.24329	0.39751	***
group4 - group1	0.18120	0.10409	0.25831	***
group3 - group1	0.14870	0.07159	0.22581	***
group2 - group1	0.12520	0.04342	0.20698	***
group7 - group1	-0.06220	-0.13931	0.01491	

Note: SAS output for IgA titer on day 42, three weeks after booster vaccination. A Proc GLM procedure was used for analysis of F value and p value and determination of significance. A Dunnett's t Test was used to compare each treatment with negative control group 1.

VITA

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Candidate for the Degree of

Master of Science

Thesis: PROTECTIVE MUCOSAL IMMUNITY ELICITED BY INTRANASAL DNA VACCINATION EXPRESSING HA1 FOR EQUINE-2 INFLUENZA VIRUS

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Pages in Study: 79 Candidate for the Degree of Master of Science

Major Field: Microbiology, Cell and Molecular Biology

Abstract:

A vaccination trial was performed in mice by intranasal inoculation of DNA vaccines pTOPOKY98#6 and pVAXKY98/11-9 encapsulating with liposome and metastim. DNA vaccines were designed to express HA1 protein from equine-2 influenza virus, A/Eq/Ky/98. After a booster vaccination, each mouse was challenged with a sub-lethal dose (16 HAU) of homologous equine-2 influenza virus.

By using a weight loss model, it was found that the vaccinated mice showed less severe disease than the unvaccinated mice. Elevated IgG and IgA levels were recorded in the sera of the vaccinated mice. HA1 was capable of inducing protective immunity against homologous challenge. In the unvaccinated group mice, a maximum of 8.4% weight loss was observed while the observed weight losses were 1.2% and 0.82% for pTOPOKY98#6 and pVAXKY98/11-9 group mice respectively. It was found that dose levels as low as 0.01 μg DNA/gram body-weight were sufficient to elicit protective immunity.

Advisor's Approval: Dr. Kim Burnham