ABSTRACT

Title of Dissertation: AVIAN PARAMYXOVIRUS-VECTORED VACCINES AGAINST INFECTIOUS BRONCHITIS VIRUS AND HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS

Edris Shirvani, Doctor of Philosophy, 2019

Dissertation directed by: Dr. Siba K. Samal
Professor of Virology
Department of Veterinary Medicine
College of Agriculture and Natural Resources

Highly pathogenic avian influenza (HPAI), infectious bronchitis (IB), and Newcastle disease (ND) are highly contagious and economically important diseases in poultry. Vaccination is the major strategy which is implemented to combat highly pathogenic avian influenza virus (HPAIV), infectious bronchitis virus (IBV), and Newcastle disease virus (NDV), worldwide. However, among these viruses, some NDV strains are naturally avirulent and have been used as highly safe vaccines for more than 60 years. Live attenuated IBV vaccines that are produced by passaging virulent strains in eggs have safety concerns and are genetically unstable. Inactivated

IBV and HPAIV vaccines also are less efficacious and affordable. Therefore, development of alternative vaccines against IBV and HPAIV is highly needed. In this multistep study, we have employed NDV vector and other novel avian paramyxovirus (APMV) vectors to develop improved IBV and HPAIV vaccines.

Firstly, we conducted a study to investigate the contributions of the S1, S2, and S proteins of IBV in protection against virulent IBV, and to develop a safe and efficacious recombinant NDV-vectored IBV vaccine. We generated recombinant (rNDV) strain LaSota viruses expressing S1, S2 or S protein of IBV using reverse genetics. We evaluated the protective efficacies of rNDVs against virulent IBV and NDV challenges. Our results showed that the S protein, which contains the S1 and S2 neutralizing epitopes in correct confirmation is the best protective antigen of IBV. These results suggest that the rNDV expressing the S protein of IBV is a safe and effective bivalent vaccine candidate for both IBV and NDV.

Secondly, besides rNDV strain LaSota vector, we employed a novel chimeric rNDV/avian paramyxovirus serotype-2 (rNDV/APMV-2) vector that replicates less efficiently and a modified NDV strain LaSota (rLaSota-527) vector that replicates more efficiently to develop a likely improved viral vectored vaccine against IBV. We generated rNDV/APMV-2 or rLaSota-527 virus expressing the best protective protein

of IBV (S protein), which was found in the first study. The protective efficacies of rNDV/APMV-2 or rLaSota-527 virus expressing the S protein was evaluated against IBV in chickens. Our results showed that immunization of chickens with either chimeric rNDV/APMV-2 expressing the S protein, which is a better candidate for *in ovo* vaccination, or rLaSota virus expressing the S protein provided protection against IBV. Most importantly, compared to prime-boost vaccination or vaccination with rLaSota-527 virus expressing the S protein, single immunization of chickens with rLaSota virus expressing the S protein induced better immune responses against IBV.

Thirdly, we conducted a study to evaluate the contributions of HA1 and HA2 subunits of HPAIV HA protein in the induction of neutralizing antibodies and protection in chickens, using rNDV strain LaSota vector. Our results showed that the HA1 and HA2 subunits when expressed separately, neither provided protection nor induced neutralizing antibodies. To be effective the HA protein must be incorporated into a vaccine as an intact protein. These results also highlight the importance of using chickens in HPAIV vaccine studies as they are susceptible natural hosts.

Finally, we employed APMV-3 strain Netherlands as a vaccine vector, for its high efficiency replication in multiorgans of host, to generate an improved vaccine against HPAIV. Our results showed that immunization of chickens with either rAPMV-3 expressing the HA protein (rAPMV-3/HA) or rNDV expressing the HA protein

(rNDV/HA) provided complete protection against HPAIV challenge. However, the immunization of chickens with rAPMV-3/HA induced higher levels of neutralizing antibodies than that induced by rNDV/HA. These results suggest that mass-vaccination with a rAPMV-3/HA might provide better protection against H5N1 HPAIV in field conditions.

In conclusion, the individual subunits of the S protein of IBV or the HA protein of HPAIV when expressed separately, neither provided protection nor induced neutralizing antibodies. To provide protective efficacy, the intact HA or S protein must be incorporated into vaccine. The rNDV expressing the S protein is a safe and efficacious bivalent vaccine against IBV and NDV. Other than rNDV strain LaSota, rNDV/APMV-2 and rAPMV-3 are promising vaccine vectors for development vaccines against IBV and HPAIV, respectively.

# AVIAN PARAMYXOVIRUS-VECTORED VACCINES AGAINST INFECTIOUS BRONCHITIS VIRUS AND HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS

By

Edris Shirvani

Dissertation submitted to the Faculty of the Graduate School of the

University of Maryland, College Park, in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

2019

Advisory Committee:

Professor Siba K. Samal, Chair

Professor Jeffrey DeStefano

Professor Xiaoping Zhu

Associate Professor Yanjin Zhang

Associate Professor George Belov

© Copyright by

Edris Shirvani

2019

# Dedication

I dedicate my dissertation to my family for providing me with their support and encouragement. I also dedicate this work and give special thanks to my advisor, Professor Siba K Samal, to believe on me and for his guidance, support and patience.

# Acknowledgements

I would like to express my deepest appreciation to my advisor, Professor Siba K Samal who has been a tremendous mentor for me. I am extremely grateful to him for providing me with great ideas, valuable advices, helpful discussions, generous support, patience and encouragement throughout all stages of my Ph.D. I would also like to extend my sincere thanks to my committee members: Professor Jeffrey DeStefano, Professor Xiaoping Zhu, Dr Yanjin Zhang and Dr George Belov, not only for their time and patience, but also for their insightful suggestions and thoughtful comments.

I gratefully acknowledge the assistance of all my previous and present lab members. I would like to recognize all their guidance, technical support and useful discussions. Special thanks to the assistance of Dr Anandan Paldurai, Dr Vinoth K Manoharan and Mr Berin P Varghese to perform the animal studies in biosafety level-3 plus facilities.

I also thank all faculties, staffs, postdoctoral fellows and graduate students of the department of Veterinary Medicine and my friends at the University of Maryland for their assistance and support.

I would like to express my deepest gratitude to my wonderful family for all their support and encouragement.

I thank the University of Maryland and the Elanco Animal Health for providing the financial resources for this research.

# Table of Contents

Dedication ii

Acknowledgements iii

Table of Contents iv

List of Tables xi

List of Figures xii

List of Abbreviations xvii

Chapter 1: Intruduction

Background ..... 1

Research objectives ..... 8

Chapter 2: A review on IBV, HPAIV, NDV

A brief review on IBV ..... 9

Classification ..... 9

Genome structure ..... 9

Virion structure ..... 10

Viral proteins ..... 11

IBV life cycle ..... 13

Assembly and release of virion ..... 14

Pathogenesis ..... 16

Immunity against IBV ..... 17

Vaccine ..... 18

A brief review on HPAIV ..... 20

Classification ..... 20

Genome structure ..... 21
Virion structure ..... 21
The haemagglutinin protein ..... 22
Life cycle ..... 23
Vaccine ..... 24
A brief review on NDV ..... 25
Classification ..... 25
The genome structure ..... 26
Virion structure ..... 27
Viral proteins ..... 27
Life cycle ..... 30
Pathogenesis and virulence ..... 32
NDV as a vector ..... 33
Chapter 3: A recombinant NDV expressing the S protein of IBV protects chickens against IBV and NDV.
Abstract ..... 36
Introduction ..... 37
Materials and Methods ..... 39
cells and viruses ..... 39
Generation of rNDVs expressing S1,S2 or S protein of IBV ..... 43
Evaluation of the expression of the S1,S2 and s protein of IBV ..... 43
Growth characteristics of rNDV constructs ..... 44

The protective efficacy of rNDVs expressing S1, S2 or S protein of IBV in chickens against virulent IBV challenge ..... 44
The protective efficacy of rNDV expressing S protein of IBV against virulent NDV challenge ..... 48
Serological analaysis ..... 49
Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) ..... 50
Statistical analysis ..... 51
Results ..... 51
Generation of rNDVs expressing S1,S2 or S protein of IBV ..... 51
Evaluation of the expression of S1,S2 and s protein of IBV ..... 53
Growth characteristics of rNDV constructs ..... 58
The protective efficacy of rNDVs expressing S1, S2 or S protein of IBV in chickens against virulent IBV challenge ..... 59
The protective efficacy of rNDV expressing S protein of IBV against virulent NDV challenge ..... 67
Antibodies produced against IBV and NDV ..... 68
Discussion ..... 69
Chapter 4: Comparative protective efficacy of novel APMV-vectored vaccines against virulent IBV in chickens
Abstract ..... 74
Introduction ..... 75
Materials and Methods ..... 78
cells and viruses ..... 78

Generation and growth characteristics of rNDV/APMV-2 or rLaSota-527 expressing S protein of IBV ..... 79
Expression of S protein by rNDV-APMV-2/IBV-S and rLaSota-527/IBV-S .... 80
The protective efficacy of rNDV-APMV-2/IBV-S or rLaSota expressing S protein against IBV challenge in SPF chickens ..... 80
The protective efficacy of rNDV-APMV-2/IBV-S or rLaSota expressing S protein against IBV challenge in broilers ..... 81
The protective efficacy of rLaSota-527 or rLaSota expressing S protein against IBV challenge in SPF chickens ..... 82
Statistical analysis ..... 83
Results ..... 83
Generation and growth characterization of rNDV/APMV-2 or rLaSota-527 expressing S protein of IBV ..... 83
Expression of S protein of IBV by rNDV-APMV-2/IBV-S and rLaSota-527/IBV-S ..... 85
The protective efficacy of rNDV-APMV-2/IBV-S or rLaSota expressing S protein against IBV challenge in SPF chickens ..... 86
The protective efficacy of rNDV-APMV-2/IBV-S or rLaSota expressing S protein against IBV challenge in broilers ..... 88
The protective efficacy of rLaSota-527 or rLaSota expressing S protein against IBV challenge in SPF chickens ..... 91
Discussion ..... 92

Chapter 5: Contributions of HA1 and HA2 subunits of HPAIV in induction of neutralizing antibodies and protection in chickens

Abstract ..... .98

Introduction ..... .99

Materials and Methods ..... 102

cells and viruses ..... 102

Generation of rNDVs expressing HA1, HA2, or HA protein of HPAIV H5N1 ..... 103

Expression of HA1, HA2, and HA proteins of HPAIV H5N1 ..... 105

Growth characteristics of rNDVs expressing HA1, HA2, or HA protein of HPAIV H5N1 ..... 106

The protective efficacy of rNDVs expressing HA1, HA2, or HA protein against H5N1 HPAIV ..... ..106

Serological assays ..... ..107

Statistical analysis ..... 108

Results ..... 108

Generation rNDVs expressing HA1, HA2, or HA protein HPAIV H5N1 ..... 108

Expression of the HA1, HA2 and HA proteins of HPAIV H5N1 ..... 111

Growth characteristics of rNDVs expressing HA1, HA2, or HA protein of HPAIV H5N1 ..... 113

The protective efficacy of rNDVs expressing HA1, HA2 or HA protein in chickens against HPAIV H5N1 challenge ..... 114

Antibody response against AIV ..... 115

Discussion……..117

Chapter 6: A recombinant APMV-3 expressing HA protein protects chickens against H5N1 HPAIV challenge

Abstract ..... 122

Introduction ..... 123

Materials and Methods ..... 126

cells and viruses ..... 126

Construction of rAPMV-3 expressing HA protein of H5N1 HPAIV ..... 127

Expression of HA protein by rAPMV-3 or rNDV expressing HA protein of H5N1 HPAIV ..... 128

Growth characterictics of rAPMV-3 or rNDV expressing HA protein of H5N1 HPAIV ..... 129

The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV challenge in SPF chickens ..... 129

The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV challenge in broilers ..... 130

The protective efficacy of rAPMV-3 or rNDV expressing HA protein against virulent NDV challenge in SPF chickens ..... 131

Serological analysis ..... 131

Statistical analysis ..... 133

Results ..... 134

Generation of rAPMV-3 expressing HA protein of H5N1 HPAIV and expression of HA protein by rAPMV-3 or rNDV ..... 134

Growth characterictics of rAPMV-3 or rNDV expressing HA protein of H5N1 HPAIV ..... 136

The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV challenge in SPF chickens ..... 138

The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV challenge in broilers ..... 141

The protective efficacy of rAPMV-3 or rNDV expressing HA protein against virulent NDV challenge in SPF chickens……..144

Discussion ..... 145

Chapter 7: Conclusion

Conclusion ..... 151

Future directions and perspectives ..... 156

Bibliography ..... 163

# List of Tables

**Table 1.** The groups of broilers immunized in single or prime-boost regimens…. 83

**Table 2.** The groups of broilers immunized in single or prime-boost regimens….133

# List of Figures

**Figure 1.** The schematic diagram showing IBV genome structure (A), and virion structure (B)……...……..10

**Figure 2.** The schematic diagram illustrating the spike protein of IBV structure. The signal sequence, S1 NTD and CTD, fusion peptide, HR1, CH, HR2, TM&CT positions are showed……12

**Figure 3.** The IBV life cycle. (The figure was obtained from *Jawetz, Melnik & Adelberg's Medical Microbiology, 27th ed* and modified)……...15

**Figure 4.** The influenza A virus genome and virion structure (A). The schematic diagram showing HA protein structure (B)…….....22

**Figure 5.** The influenza A virus life cycle. (The figure was obtained from *Mark VI, Nature Review,2007*)……23

**Figure 6.** The NDV virion structure……....27

**Figure 7.** The NDV life cycle. (The figure was obtained from *Jawetz, Melnik & Adelberg's Medical Microbiology, 26th ed* and modified)……31

**Figure 8.** The schematic diagram showing the NDV genome and the reverse genetics to recover recombinant NDV from NDV antigenomic cDNA……..35

**Figure 9.** Schematic diagram of recombinant NDV constructs containing IBV genes……52

**Figure 10.** Western blot analysis of rNDVs expressing S or S2 protein of IBV……...55

**Figure 11.** Western blot analysis of rNDV expressing S1 protein of IBV…… 57

**Figure 12.** Multicycle growth kinetics of the rNDV constructs in DF-1 cells……59

**Figure 13.** The protective efficacy of rNDV constructs in one-day-old immunized SPF chickens against virulent IBV challenge (IBV protection experiment 1) at 21 days post-immunization……... 61

**Figure 14.** The protective efficacy of rNDV expressing codon optimized S protein of IBV against IBV challenge in immunized SPF chickens at 4-week-old age……... 62

**Figure 15.** The protective efficacy of rNDV expressing codon optimized S protein of IBV and neutralizing antibody response against high dose of IBV challenge in immunized SPF chickens at 4- week-old age……... 64

**Figure 16.** The protective efficacy of rNDV expressing codon optimized S protein of IBV against IBV challenge in immunized SPF chicks at 1-day-old age, by the intraocular challenge route…….. 67

**Figure 17.** The protective efficacy of rNDVs against virulent NDV challenge and antibody responses against NDV in SPF chickens immunized at 1-day-old…… 69

**Figure 18.** The schematic diagram for the construct and plaque morphology of rNDV-APMV-2 or rLaSota expressing the S protein of IBV…….. 84

**Figure 19.** The expression of S protein of IBV by rNDV-APMV-2/IBV-S or rLaSota-527/IBV-S……... 86

**Figure 20.** The protective efficacy or rNDV-APMV-2/IBV-S or rLaSota/IBV-S against IBV challenge in SPF chickens……87

**Figure 21.** The protective efficacy or rNDV-APMV-2/IBV-S or rLaSota/IBV-S against IBV challenge in broilers……. 89

**Figure 22.** The antibodies induced against rNDV-APMV-2 and rLaSota in broilers. ……..90

**Figure 23.** The protective efficacy of rLaSota-527/IBV-S or rLaSota/IBV-S against IBV challenge in SPF chickens…… 92

**Figure 24.** Construction of recombinant NDVs containing H5N1 HPAIV HA1, HA2, or HA gene……. 109

**Figure 25.** The incorporation of HA1, HA2 and HA proteins of HPAIV into NDV particles and the expression of HA1, HA2 and HA proteins by rNDVs……. 110

**Figure 26.** Growth kinetics of rNDVs expressing HA1, HA2 or HA protein in DF-1 cells……... 113

**Figure 27.** Survival rate of immunized chickens with rNDVs expressing HA1, HA2, or HA protein of HPAIV against H5N1 HPAIV challenge…….114

**Figure 28.** Antibody response against AIV. SPF chicks were immunized at day one of age and sera were collected at day 21 after immunization…….. 116

**Figure 29.** Antibody response against NDV…… 117

**Figure 30.** The schematic diagram for the construct of recombinant avian paramyxovirus serotype 3 (rAPMV-3) containing HA gene of HPAIV……. 134

**Figure 31.** The expression of HA protein by rAPMV-3 or rNDV and incorporation of HA protein into rAPMV-3 or rNDV particles…… 136

**Figure 32.** Multicycle growth kinetics and plaque morphology of rNDV or rAPMV-3 expressing HA protein in DF1 cells……137

**Figure 33.** The induction of neutralizing antibodies and protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV in SPF chickens (experiment 1)……139

**Figure 34.** The induction of neutralizing antibodies and protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV in SPF chickens (experiment 2)……141

**Figure 35.** The protective efficacy of rAPMV-3 or rNDV expressing HA against H5N1 HPAIV in broilers……..……142

**Figure 36.** The antibody responses against APMV-3 and NDV by rAPMV-3 or rNDV expressing HA in broilers…….. 143

**Figure 37.** The antibody responses and the protective efficacy rAPMV-3 or rNDV expressing HA against NDV strain GB Texas in immunized SPF chickens……...145

# List of Abbreviations

| | |
|---|---|
| HPAIV | highly pathogenic avian influenza virus |
| IBV | infectious bronchitis virus |
| NDV | Newcastle disease virus |
| rNDV | recombinant NDV |
| APMV-2 | avian paramyxovirus serotype 2 |
| APMV-3 | avian paramyxovirus serotype 3 |
| OIE | World Organization for Animal Health |
| Kb | kilobase |
| UTR | untranslated region |
| S | spike protein |
| IBV-M | IBV-membrane protein |
| E | envelope protein |
| N | nucleocapsid protein |
| F | fusion protein |
| HN | haemagglutinin neuraminidase protein |
| HA | haemmaglutinin protein |
| NA | neuraminidase protein |
| LPAIV | low pathogenic avian influenza virus |
| SPF | specific pathogen free |
| FPCS | fusion protein cleavage site |
| NTD | N-terminal domain |
| CTD | C-terminal domain |

| | |
|---|---|
| HR | heptad repeat |
| CH | central helix |
| TM | transmembrane domain |
| CT | cytoplasmic tail |
| RBD | receptor binding site |
| ER | endoplasmic reticulum |
| ERGIC | ER-Golgi intermediate compartment |
| PB | polymerase basic gene |
| PA | polymerase acid gene |
| NP | neucleoprotein |
| NS | nonstructural protein |
| HI | haemagglutination inhibition assay |
| RNP | riboneucleoprotein |
| NDV-M | NDV-matrix protein |
| L | large polymerase protein |
| GS | gene start |
| GE | gene end |
| IGS | intergenic sequences |
| cDNA | complementary DNA |
| DF1 | chicken fibroblast cell |
| HEp-2 | human epidermoid carcinoma cell |
| ATCC | American Type Culture Collection |
| DMEM | Dulbecco's minimal essential medium |

FBS fetal bovine serum

$EID_{50}$ 50% embryo infectious dose

$TCID_{50}$ 50% tissue culture infectious dose

IACUC Institutional Animal Care and Use Committee

$CLD_{50}$ 50% chicken lethal dose

RT reverse transcription

qPCR quantitative polymerase chain reaction

kDa kilodalton

ON oculonasal

IN intranasal

IO intraocular

BC Beaudette C

MDA maternal derived antibody

ADE antibody dependent enhancement

HVT turkey herpesvirus

FPV fowl pox virus

ILTV infectious laryngotracheitis

MDV Marek's disease virus

MDCK Madin-Darby canine kidney cell

IFA immunofluorescence assay

PFU plaque forming unit

TPCK N-tosyl-1-phenylalanine chloromethyl ketone

IBDV infectious bursal disease virus

# Chapter 1

# Introduction

## *Background*

Highly pathogenic avian influenza (HPAI), infectious bronchitis (IB), and Newcastle disease (ND) are highly contagious and economically important diseases in poultry. They cause significant economic losses worldwide and among the poultry diseases they rank the highest in potential consequences (1,2,3,4). According to the classification of diseases notifiable to the World Organization for Animal Health (OIE), HPAI and ND are in the list A "transmissible diseases that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal products", and IB is in the list B "transmissible diseases with socio-economic importance within countries and with significant effects on the international trade of animals and animal products" (5). HPAI, ND and IB are highly infectious respiratory diseases (1, 2, 3).

IB is usually characterized by ocular and nasal discharges, gasping, coughing, and sneezing. It also affects renal and reproductive tracts. Respiratory distress, poor carcass weight, substandard egg quality and decreased egg production resulting from IB cause huge economic losses for the poultry industry worldwide (6).

Infectious bronchitis virus (IBV) is a member of the family *Coronaviridae* (7). The genome of IBV is a non-segmented, positive-sense RNA virus with about 27.6

kilobase (kb) in length. The genome encodes five basic and four accessory genes in the order 5'-leader-replicase-untraslated region (UTR)-S (spike)-3a,3b-M (membrane)-E (envelope)-5a,5b-N (nuclocapsid)-UTR-polyA-3'. IBV is an enveloped virus and the virions contain S, M, E and N proteins (8,9). The RNA genome of IBV is bound to the N protein and forms the helical nuclocapsid. However, unlike the non-segmented single-stranded negative-sense RNA viruses, the N protein of coronaviruses does not protect the genome from nuclease activity (8). The random template switching during coronaviruses RNA synthesis also causes high rate of recombination in the entire genome during the replication (8).

The S glycoprotein is the major antigen against which neutralizing and protective antibodies are produced. It forms a homotrimer on the virus surface. Each S monomer is synthesized as an inactive precursor protein S0 (8). The S protein is cleaved into the amino-terminal S1 and to the carboxy-terminal S2 subunits post translationally by a furin-like protease of host, which are still noncovalently linked (10). The S1 subunit is responsible for viral attachment to host cells (11,12). The S1 is highly variable among different isolates of IBV and contains major neutralizing epitopes (8,13,14). The S2 subunit is highly conserved among IBV strains and contributes to viral fusion activity and elicits some minor, but broadly reactive neutralizing antibodies (8,14). Although it was shown that S1 and S2 subunits contain major and minor neutralizing epitopes, respectively, the best protective antigen for the development of vector based IBV vaccines is not known.

Vaccination is the most efficacious approach to control IB. Currently, live attenuated and inactivated vaccines are used against IB (1). However, inactivated vaccines do not elicit mucosal and strong cell mediated immunity (15,16). The costly production and administration are other limiting factors for inactivated vaccines (17,18). On the other hand, currently used live attenuated vaccines which are developed by serial passaging of field isolates of IBV in chicken embryonated eggs also have limitations. (i) Live attenuated IBV vaccines can revert back to the virulence through the circulation in the field (17,18). (ii) They create new variant viruses through recombination and mutations (19,20). (iii) They can cause pathological signs in immunized birds, particularly, in young chicks (21,22). (iv) The protective efficacy of live attenuated vaccines is not guaranteed (23). (v) The currently used live IBV vaccines cannot be used for *in ovo* administration (21). Therefore, the development of a novel safe, efficacious and cost-effective vaccine against IBV is highly needed.

Avian influenza viruses (AIVs) are members of the genus *Alphainfluenzavirus* in the family *Orthomyxoviridae* (7). They cause a wide range of clinical disease in chickens. Alphainfluenzaviruses are classified into combinations of 18 H (H1-H18) and 11 N (N1-N11) subtypes, based on antigenic difference on the two major surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). H1-H16 and N1-N9 subtypes have been found in avian species (19). AIVs are divided into low pathogenic avian influenza viruses (LPAIVs) that cause restricted respiratory and/or intestinal disease without mortality in specific pathogen free (SPF) chickens, and highly pathogenic avian influenza viruses (HPAIVs) that cause high mortality following an

acute systemic infection in SPF chickens (2,20). Of the H subtypes, H5 and H7 subtypes have high pathogenicity in poultry and cause severe disease (2). Furthermore, H5 and H7 subtypes of HPAIV are serious threats to public health (21, 22).

Vaccination with inactivated virus vaccines and/or viral vectored HA-based vaccines is one of the major strategies that is currently implemented against HPAIV in chickens in many countries (23). Although the vast majority of AIV vaccines (95.5%) currently used in the field are inactivated vaccines, they are not the best choice to combat HPAIV infection in poultry. Because not only the effectiveness of these vaccines is suboptimal, but also the processes of production and administration of inactivated vaccines are expensive, time consuming and labor intensive. Furthermore, vaccination programs with inactivated vaccines cause difficulty to differentiate vaccinated birds from infected ones that can result in trade restrictions (24,25). On the contrary, viral-vectored HA protein vaccines are a desirable alternative choice for inactivated vaccines (26-32). However, the advantages and disadvantages of each virus should be considered when it is chosen as a vector for the development of a HA-based vaccine against HPAIV (33).

The HA is an important multifunctional protein of influenza virus. The HA protein mediates binding of the virus to host cell receptor. It is the major antigen against which neutralizing antibodies are induced during infection. The HA protein is a homotrimer at the virus surface. Each HA monomer is synthesized as an inactive precursor protein HA0 (19). The cleavage of HA0 protein into HA1 and HA2 subunits by a cellular protease is required for viral infectivity (34,35). Following cleavage, the

HA1 and HA2 subunits are held together by disulfide bonds. The HA1 binds to sialic acid receptors on cell surface and initiate virus entry by endocytosis. Due to acidic pH-induced conformational change of HA2 in endosome, the fusion of viral and endosomal membranes occurs (19). The HA1 subunit, which forms the globular head, is highly variable among subtypes and contains major neutralizing epitopes. The HA2 subunit, which forms most of the stalk domain, is considerably conserved among subtypes and contains minor but cross-reactive neutralizing epitopes (19,36,37). Viral vectored vaccines containing the intact HA protein of H5N1 HPAIV have been investigated in chickens in several studies (33,38,39). The individual contributions of HA1 and HA2 subunits of HPAIV HA protein in induction of neutralizing antibodies and protection has not been investigated in chickens. However, they were investigated in mice and ferrets (40-43), though they are not natural host model for influenza viruses (44,45). In contrary, chickens are highly susceptible to H5N1 HPAIV infection and play a critical role in the spread of the virus (2,20). Hence, chickens can be an excellent model to determine the contributions of HA1 and HA2 subunits of H5N1 HPAIV in induction of neutralizing antibodies and protection.

Avian paramyxoviruses (APMVs) belong to the family *Paramyxoviridae.* To date, twenty classified serotypes (APMV-1 to APMV-20) and one unofficially classified serotype (APMV-21) of paramyxoviruses have been reported (7). APMV-1 belongs to the genus *Orthoavulavirus* (7). APMV-1 viruses are classified into velogenic (highly virulent), mesogenic (moderately virulent) and lentogenic (low virulent) strains based on their pathogenicity in chickens. Velogenic and mesogenic

strains that cause Newcastle disease (ND) are only called Newcastle disease virus (NDV) (46,47). To avoid complexity, in this chapter we will mention all APMV-1 strains as NDV. The molecular nature of NDV genome and the biological characteristics of NDV make it an attractive viral vector to develop vaccines against IBV and HPAIV. (i) Avirulent NDV strains such as LaSota and B1, with restricted replication in respiratory tract, have been safe live vaccines against virulent NDV over 60 years (3). (ii) NDV replicates through respiratory tract and elicits strong mucosal, humoral and cell-mediated immune responses against the virus (iii) The RNA genome of NDV is tightly encapsidated with nuclocapsid protein. The genomic and antigenomic RNA are not found free during the RNA replication in cell; therefore, recombination in NDV genome is rare. (iv) NDV is genetically stable and the mutation rate in its genome is very low. (v) The NDV replication occurs in the cytoplasm; therefore, there is no interaction between NDV and host cell genomes (47,48). (vi) The matrix protein of paramyxoviruses including the matrix protein of NDV forms flexible shape; hence, NDV can packages the membrane-integrated glycoproteins of other viruses such as the S protein of IBV and the HA protein of HPAIV in its envelope (48). (vii) NDV replicates efficiently in egg and cell culture, which is a merit for the development of vaccines in industrial scales. (viii) A wide range of APMV serotypes make it possible to employ other APMV serotype vectors and novel chimeric APMV vectors for the development of improved vaccines against avian pathogens.

Novel chimeric APMV vectors and improved NDV vectors also can be used for the development of improved vaccines against avian pathogens. A novel chimeric

NDV/APMV-2 vector in which the ectodomains of F and HN proteins of NDV were replaced with ectodomains of corresponding proteins of APMV-2 were developed previously (49). Due to the differences between cleavage sites residues of APMV-2 and APMV-1 strain LaSota F proteins, compared to recombinant LaSota (rLaSota), recombinant NDV/APMV-2 (rNDV/APMV-2) possessed less fusion activity and less efficient replication *in vitro*. It was also shown that compared to rLaSota, rNDV/APMV-2 was less pathogenic for chicken eggs embryos, due to the differences between F and HN proteins of the two viruses (47,48). Hence these features make rNDV/APMV-2 as a potential viral vector for *in ovo* vaccination. Another improved rAPMV-1 strain LaSota in which a Y527A mutation in F protein cytoplasmic tail (rLaSota-527) resulted in increased fusion activity, replication and immunogenicity was developed (50). The novel rNDV/APMV-2 and rLaSota-527 vectors can be employed to develop a likely improved viral vectored IBV vaccine.

Among APMV serotypes which have been identified to date, APMV-3 strain Netherlands, because of its multibasic fusion protein cleavage site (FPCS) residues, replicates more efficiently in wider range of chicken and turkey organs (51,52). Despite possessing multibasic FPCS, APMV-3 is safe in chicken and it is not a Select Agent. Therefore, APMV-3, which has high efficiency replication in a greater range of host organs, can be employed as a vaccine vector against HPAIV.

*Research objectives*

1. To develop the NDV vectored IBV vaccine, and to investigate the contributions of S1, S2 and S proteins of IBV in protection against virulent IBV in chickens.
2. To develop the novel chimeric APMV vectored IBV vaccines in chickens.
3. To investigate the contributions of HA1 and HA2 subunits of HPAIV in induction of neutralizing antibodies and protection in chickens.
4. To develop the APMV-3 vectored HPAIV vaccine in chickens.

# Chapter 2

# A review on IBV, HPAIV and NDV

## *A brief review on IBV*

### Classification

IBV belongs to the genus *Gammacoronavirus* in the family *Coronaviridae* (7). Like other viruses in *Nidovirales* order, coronaviruses possess distinctive characteristics including; (i) having the largest RNA genome among viruses (ii) two third of genome encoding a very large replicase gene located at 5'end (iii) expressing a nonstructural replicase-transcriptase polyprotein as a result of ribosomal frameshifting (iv) expression of downstream genes by transcription of 3'-nested sub-genomic mRNAs. The last feature means "nest" in Latin and is used in the name nido of the order *Nidovirales* (8). The family *Coronaviridae* is classified into two subfamilies, *Orthocoronavirinae* and *Letovirinae*. The subfamily *Orthocoronavirinae* (also called coronaviruses), based on serology and phylogenic clustering is divided into four genera; alpha, beta, delta and gammacoronaviruses (7). All gammacoronaviruses (also called avian coronaviruses) except one isolate were isolated from avian species (8).

### Genome structure

IBV has a large nonsegmented, single-stranded, positive-sense RNA genome with 27.6 kilobase (kb) in length. The genome has a eukaryotic 5' terminal cap and a

3’ polyadenylate (poly A) tail that allow the genome to play its role as mRNA. The genome contains five basic and four accessory genes. The genes are in the constant order 5’- leader-replicase-UTR-S-3a,3b-E-M-5a,5b-N-UTR-polyA-3’(8). The two-third of the genome is occupied by replicase, encoding a polyprotein, replicase-transcriptase. The S, M, E and N genes are expressed from sub-genomic mRNAs. The replicase products are essential for replication. On the contrary, the products of accessory genes (3a, 3b, 5a and 5b) are not essential for the replication of IBV in cell culture. However, they may play roles in virus pathogenesis (Fig.2.1A)(8, 53,54,55).

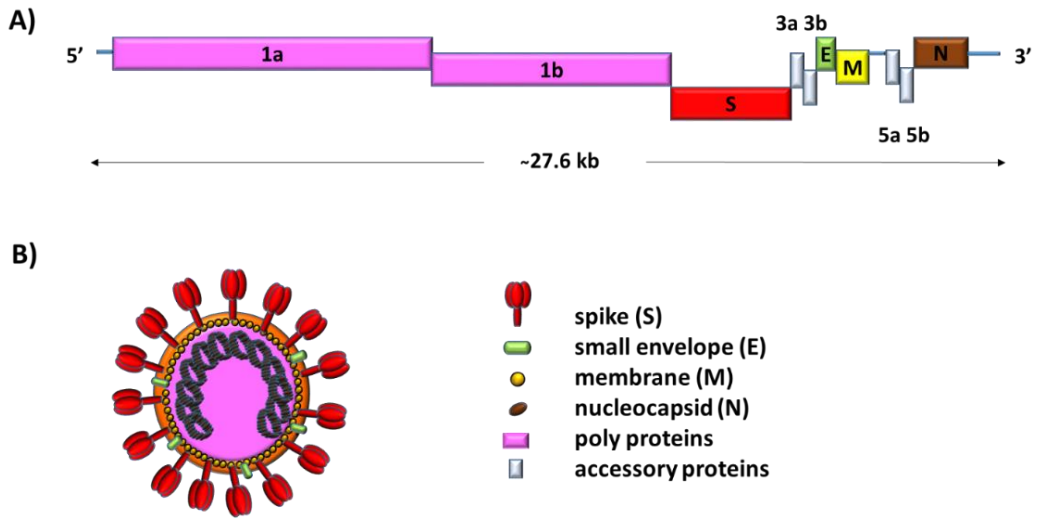


**Figure 1.** The schematic diagram showing IBV genome structure (A), and virion structure (B).

**Virion structure**

IBV is enveloped and roughly spherical in shape with a diameter of approximately 120 nm. The spike protein projects from virion surface. The name of coronavirus is derived from the corona-like shape of virion. Like other coronaviruses,

IBV also has a nuclocapsid with helical symmetry, which is highly uncommon for positive-stranded RNA viruses. Other positive-stranded viruses have icosahedral capsids. IBV particles contain S, M, E and N proteins (Fig.2.2B) (8).

**Viral proteins**

**The Spike glycoprotein**. The S protein is a heavily N-glycosylated trimer molecule on the virion surface. It is a class I membrane-integrated viral fusion protein. The S protein is cleaved into two subunits: 530 amino acid N-terminus S1 and 630 amino acid C-terminus S2 by a furin-like host cell protease. The two subunits are still bound by disulfide linkage. However, the exact position of disulfide bond in S protein has not been mapped yet. The cleavage of S protein is required for IBV infectivity (8,10,56). The cryo-EM structure of IBV strain Mass-41 spike protein in the pre-fusion state illustrates that there is a cleavable 23-amino acid signal peptide in the N-terminal of S protein which plays a crucial role in the formation of glycosylated S protein via trafficking to ER and in membrane insertion of S protein. The S1 subunit consists of N-terminal domain (NTD) and C-terminal domain (CTD). The fusion peptide (FP) is located at the N-terminus of S2 protein. The S2 protein also contains heptad repeats 1 and 2 (HR1 and HR2) and central helix (CH). The transmembrane (TM) and cytoplasmic tail (CT) domains are located at the C-terminus of S2 subunit (Fig.2.2) (57). The S1 subunit is responsible for viral attachment to the cell receptors on epithelial cell (11,12). It contains major neutralizing epitopes, and is highly variable among IBV strains (8,13,14). The S2 subunit contributes to viral fusion activity via its

fusion peptide. It contains some minor epitopes, and is highly conserved among IBV isolates (8,14).

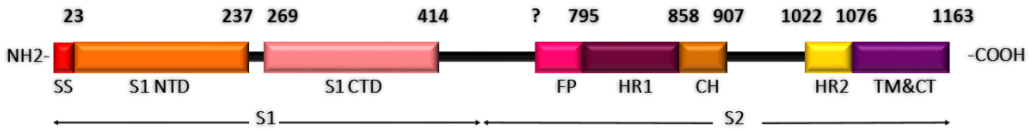


**Figure 2.** The schematic diagram illustrating the structure of spike protein of IBV. The amino acid positions of the signal sequence, S1 NTD and CTD, fusion peptide, HR1, CH, HR2, TM&CT positions are shown.

**The membrane protein**. The largest amount structural protein in IBV virion is M protein. It is a small protein with 230 aa in size. The M protein is likely found in dimers in virion. It likely forms the virion shape. The M protein is found with two different conformations; (i) the compact form that enhances membrane curvatures and is thought to give virion shape, and (ii) the elongated form that interacts with the nucleocapsid(8, 56).

**The envelope protein.** The E protein is a small protein of approximately 100 aa in length and has small portion in virion. It is an integrated membrane protein. However, it does not have cleavable signal sequence and is a non-glycosylated protein. It may assist the M protein in assembly and release of virus and play a role in ion channel activity and apoptosis (8, 59). However, these roles are not well understood.

**The nucleocapsid protein.** The N protein is around 420 aa in length and composed of a N-terminal domain (NTD) and a C-terminal domain (CTD). The N protein is the only

protein that binds to the RNA genome and forms the helical nuclocapsid. However, unlike the nucleocapsid protein (N) of paramyxoviruses, the N protein of coronaviruses cannot protects the genome from nuclease (8). The N protein also binds to the M protein and replicase complex (60).

**Life cycle**

The attachment of the S protein to its cognate surface host cell receptor is the initiation of IBV infection (11). The S1 subunit binds to host cells through its receptor binding domains (RBD). Although the receptors for gammacoronaviruses are not identified, α 2-3 sialic acid receptors on the respiratory, renal and reproductive tracts are thought to be the common receptors for most IBVs (61,62). In addition to α 2-3 sialic acid receptors, it has been also shown that attenuated IBV strain Baudette binds to heparin sulfate receptors (63). The S1 subunit RBDs and host cell receptors binding followed by proteolytic cleavage of S protein at the furin consensus motif, RRFRR↓S (in most IBV strains), and exposure to low pH environment, cause the exposure of fusion peptide of S2 subunit. The fusion peptide merges the virion and host cell membranes resulting in the delivery of nucleocapsid to the cytosol (9, 64).

In cytoplasm, the expression of the pp1a and pp1ab polyproteins is the perquisite for expression of structural proteins and RNA replication. The pp1a and pp1ab polyproteins are translated from two ORFs of replicase gene, rep1a and rep1b, respectively. These rep1a and rep1b are produced through frameshifting mechanism (65). Polyproteins pp1a and pp1ab are cleaved to nsp2-nsp16 through activity of the

papain-like proteases, encoded by nsp3 and a serine type protease encoded by nsp5 (66,67). The repicase-transcriptase complex (RTC) is formed after nsps production and provides prerequisites for RNA replication and transcription of subgenomic mRNAs. The nsps also have other activities, such as RNA-dependent RNA polymerase, RNA helicase and RNA 5'triphosphatase that are essential for IBV infection. Some of these nsps antagonize innate immunity roles, and the functions of some nsps are unknown (8). RNA synthesis occurred after RTC assembly. The negative-sense antigenome RNA is utilized as a template for synthesis of positive-sense genomic RNA and sub-genomic RNAs. The sub-genomic RNAs serve as mRNAs to produce structural and accessory proteins. Each sub-genomic RNA contains 5'leader, body RNA and 3'poly A. The leader fuses to body RNAs. The fusion of leader and body RNAs can be transcribed by template switching of RdRp (8,67). This feature of coronaviruses RNA synthesis, random template switching, also causes high rate of recombination occurs in entire genome during the replication. All sub-genomic RNAs together with genomic RNA form a 3'co-terminal nested set (8, 68) (Fig.3.3).

**Assembly and release of virions**

It is thought that the M and E proteins play key roles in assembling the envelope compartments and to forming the virion shape. However, the details in assembling the IBV envelope compartments and trafficking the membrane proteins inside and to the surface of host cells are not well understood. Following the translation of S, M and E proteins in the cytoplasm, they are inserted to the endoplasmic reticulum (ER), then to ER-Golgi intermediate compartment (ERGIC) and become matured through secretory

pathway. The virion buds into the ERGIC by nuclocapsid. The virions are transferred to the cell membrane by smooth double-walled vesicles and are released by exocytosis (8,70). The S protein either is packaged into the virions or is expressed on the cell surface and initiate the cell-vesicle and cell-cell membrane fusion (Fig.3)(8). It was shown that similar to the infection of Vero cells by IBV, the exogenous expression of the intact S protein of IBV alone in Vero cells leaded to develop cell-cell syncytium that the inhibitory drugs decreased the ability of syncytia formation for both IBV and IBV S protein in cells (71).

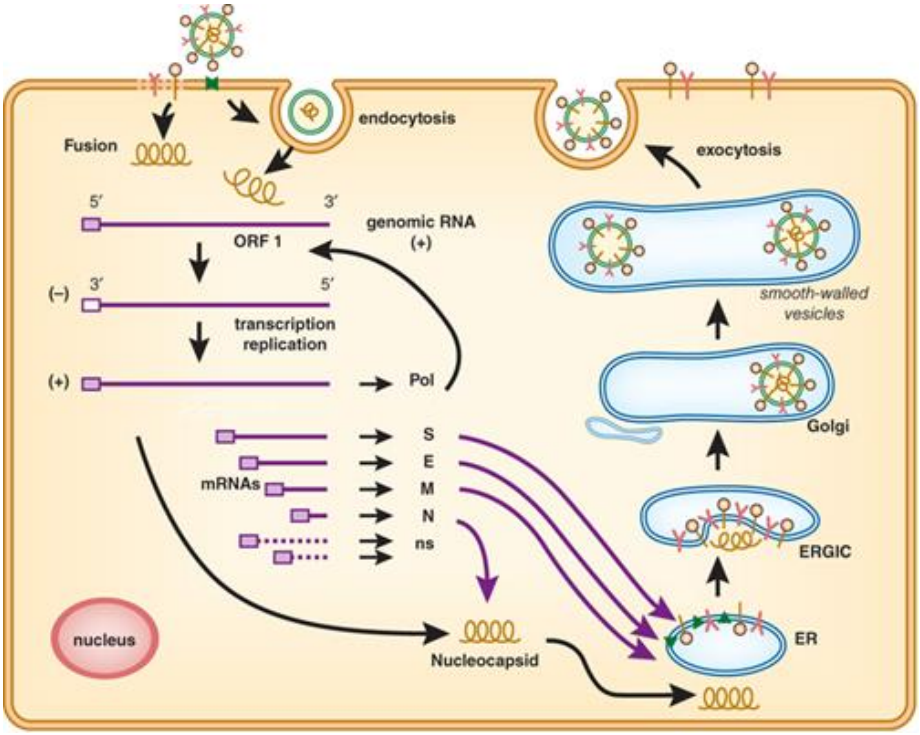


**Figure 3.** The IBV life cycle. The diagram shows the virus entry, RNA transcription and replication, protein synthesis, virion assembly and release (The figure was obtained

from *Jawetz, Melnik & Adelberg's Medical Microbiology, 27th ed*, and it was modified to show the IBV life cycle).

**Pathogenesis**

IBV infects predominantly respiratory tract. It also affects renal and reproductive tracts of poultry (9). Domestic poultry (Gallus Gallus) and pheasant are susceptible hosts for IBV. IBV-like coronaviruses have been isolated from other avian species; turkey, geese, duck, peafowl, pigeon, quail, teal, and parrot (72-77). IBV infects chickens of all ages and breed types. However compared to adult chickens, the outcomes of natural infection or challenge with IBV in young chicks are more severe and chicks show the clinical sigs for a longer period of time. It is thought that S1 protein mainly binds to α 2-3 sialic acid receptors on the respiratory, renal and reproductive tracts. IBV strain Baudette S1 protein binds to both heparin sulfate receptors and sialic acid receptors. The incubation period for IBV is 18 to 36 hours. However, dose and the route of infection affect the incubation time. Following the primary infection of Harderian gland, trachea, lung and air sacs epithelial cells, virus targets epithelia of renal and urogenital tracts and causes systemic infection. The infection may cause variable clinical sings. Respiratory clinical signs in chickens infected with virulent IBV includes lacrimation, frothy conjunctivitis, nasal discharges, sneezing, gasping, coughing, tracheal rales and dyspnea. The infected birds appear dull with ruffle feather and edematous face. They lose weight and are not alert and may not move. Nephropathogenic strains of IBV cause depression, wet droppings and excessive water

intake in broilers. IBV infection in urogenital system leads to irreversible decrease of egg production in quantity and quality (6,77).

**Immunity against IBV infection**

Adaptive immunity is required to protect chickens from IBV infection (78). The S glycoprotein of protein contains major neutralizing epitopes. Hence the S protein is the major antigen which protective adaptive immunity is developed against (13,14). Humoral immunity, mucosal immunity, and cell-mediated immunity are important in protection against IBV infection. Humoral anti-IBV IgG antibodies are detectable from day 4 after infection. They rise to high titer at day 21 after infection and they maintain at high titer for several weeks. The presence of IgA and IgG antibodies on mucosal surfaces also are important in protection against IBV. IgG antibodies in lacrimal fluid are detected at high level at day 7 post-infection and are detectable until day 23 after infection. CD8 + T cell-mediated immunity also play a role in protection against IBV infection. The mucosal cell-mediated immunity against IBV is detected at day 3 post-infection and increased until 14 days post-infection. Altogether, duration of humoral immunity induced after IBV infection is longer than cell-mediated and mucosal immunities induced post-IBV infection (78).

The innate immunity including physical barriers such as mucus and tracheal cilia is the first line of defense against IBV. Although the innate immunity against IBV is not well characterized, studies in recent years shows that initially after immunization or infection of chickens with IBV, the virus RNA is recognized by toll-like receptor

(TLR)-3 and TLR-7. The requirement of MyD88 adaptor is resulted in transcription of interferons (IFNs) and pro-inflammatory cytokines. The secretion of type I IFN activates macrophages and natural killer (NK) cells in trachea and lung, leading to induction of an immediate response to virus replication. Activated NK cells, dendritic cells and lymphocyte T cells also secrete the IFN gamma which enhances the presentation of neutralizing epitopes by the antigenic presenting cells. The presentation of antigens by macrophages and dendritic cells causes the expression of major histocompatibility complex-1 (MHC-1) molecule and subsequently development of the adaptive immunity (18, 78).

**Vaccines**

Vaccination is the major strategy to control IBV around the world (1). Several strains and variants of IBV are circulating in different regions of the world. IBV serotype Massachusetts (Mass) vaccine was the first IBV vaccine, has been used across the world for several years. For years it was the only IBV vaccine used in the field. Later other vaccines were produced against new important serotypes of IBV circulating in local regions. In USA, vaccines derived from serotypes Mass, Arkansas (Ark), Delaware, Georgia 98(GA98), Florida and JMK, in Europe and other parts of the world, vaccines derived from serotypes Mass, 793/B, QX and Q1 are used frequently. OIE recommends that the serotypes circulating in a region should be taken into consideration for the choice of vaccine. In order to provide heterologous protection, two vaccination strategies have been used. The first one is multivalent vaccines from several serotypes. The other strategy is using two major prototypes in vaccines. Mass

and 793/B IBV serotypes have been the most widely used in the second strategy. In USA, Mass and Ark types have been used as prototypes of IBV vaccines for several years (18, 79).

Live-attenuated and inactivated IBV vaccines are currently used to control IB. However, the production and application of inactivated IBV vaccines have some limitations. Inactivated IBV vaccines do not elicit strong immune responses in chickens (15,16). The production and administration of inactivated vaccines are also time consuming and costly (17,18). On the other hand, safety, attenuation and immunization outcomes of current live-attenuated vaccines, which are developed by serial passaging of field isolates of IBV in chicken embryonated eggs, are not guaranteed, particularly with regards to the age at immunization, vaccine dose, type of bird and route of vaccination (17,18,80).Because live attenuated vaccine strains are not genetically stable and have a tendency to revert back to virulence (81). Mutations and recombination leading to the creation of IBV variants also happens due to the circulation of live vaccine strains in the field (18,82,83). Live vaccines have been found as one of the sources for IB outbreaks. Although the field isolates have been passaged in eggs for many times, for instance 52 times for H52 or 120 times for H120, it has been shown that these vaccines cause disease in the field. Specifically, when vaccine manufacturers give a recommendation for using a particular live vaccine in chickens of a certain range of age in field, there is no guarantee that the vaccine strain only circulate in the population of birds with the recommended range of age (18). Moreover, live IBV vaccines may cause pathological lesions or secondary bacterial infections in young

vaccinated chicks (84,85). Therefore, there is a great need to develop an alternative IBV vaccine.

Development of viral vectored vaccines to control IB is an alternative approach. Several viruses, such as, fowl pox virus, adenovirus and Newcastle disease virus (NDV) have been evaluated as vaccine vectors for IBV (86-90). Among these viral vectors NDV holds great promise, because the natural avirulent NDV strains have been used as safe vaccines for many years(3). NDV infects chickens through respiratory tract and induces effective local and systemic immune responses (46,47). A recombinant NDV vectored IBV vaccine will serve as a bivalent vaccine against IBV and NDV; hence the complexity and dilemma of using both IBV and NDV vaccines will be resolved.

*A brief review on HPAIV*

**Classification**

Avian influenza (AI) is an infection of birds caused by any viruses of the family *Orthomyxoviridae*. Genus *Alphaorthomyxovirus* is divided into distinct subtypes based on antigenic difference of the two major surface glycoproteins, haemagglutinin (HA) and neuroaminidase (NA). To date, there are 18 (H1-H18) subtypes and 9 subtypes of (N1-N11). H17N10 and H18N11 subtypes are recognized only in bats(7,19). Avian influenza viruses are classified into high pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses. HPAI viruses that produce acute clinical disease and cause more than 75% mortality in birds to date have been associated with

H5 and H7 subtypes. However most H5 and H7 isolates are low pathogenic for avian species (H5/H7 LPAI) and naturally cause local respiratory and gastrointestinal tracts infections with minimal clinical signs. It is thought that mutations occur in HA protein through circulation of H5/H7 LPAI viruses in domestic poultry. As a result of accumulation of mutations, H5/H7 LPAI can evolve to become highly pathogenic (2,20). Furthermore, in recent years, H5, H7, H9 and H10 subtypes of avian influenza viruses caused human infections with H5 and H7 causing serious disease in human. Therefore, H5 and H7 subtypes are potential threat to public health (21,22).

**Genome structure**

Influenza viruses have a segmented negative-strand RNA genome. Alphaorthomyxoviruses possess eight RNA segments: polymerase basic 2 (PB2), polymerase basic 1(PB1), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M) and nonstructural (NS) RNA segments (Fig.4A). Segments PB2, PA, HA, NP and NA encode individual proteins, M encodes M1 and M2 proteins and NS encodes NS1 and NEP/NS2 proteins via spliced mRNAs. PB1 encodes two PB1-F2 and PB1 N40 protein from alternative open reading frames. There are segment-specific noncoding regions at both 5’ and 3’ ends of each segment. The extreme ends are conserved among all segments of alphaorthomyxoviruses(19).

**Virion structure**

Influenza viruses are enveloped and pleomorphic with diameter of 100 nm for spherical particles and of more than 300 nm for filamentous particles. The HA, NA and

M2 proteins project from envelope. The HA and NA with an approximate ratio of four to one, respectively, serves as spikes on the surface of virion. The M1 protein locates between envelope and the core of virus. The core consists RNA genome segments, PB1, PB2, PA and NP which form ribonucloprotein (RNP) complex. The NEP/NS2 also has been found in purified virions (Fig.2.4A)(19).

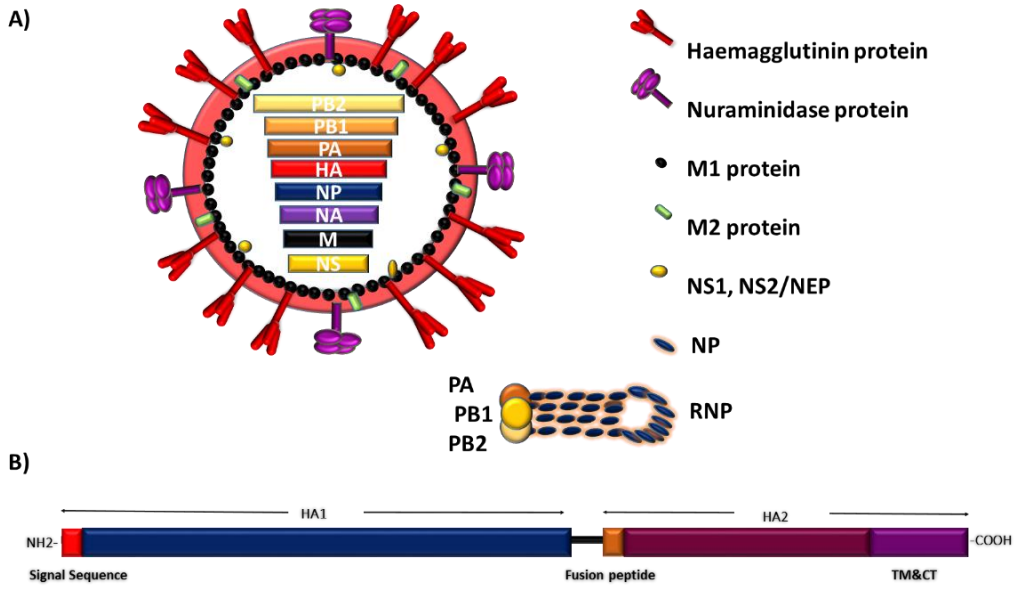


**Figure 4.** The influenza A virus genome and virion structure (A). The schematic diagram showing HA protein structure (B).

**The haemagglutinin protein**

HA is a type I viral fusion protein. It exist as homotrimeric form, consisting of three precursors (HA0), on the surface of virus (2). Each precursor of HA is cleaved by cellular proteases into HA1 and HA2 subunits, while they are still linked by disulfide bond. The cleavage of HA precursor is important for influenza virus infectivity (34,35).

The HA1 binds to sialic acid receptors on cell surface and initiates virus entry by edocytosis. Due to acidic pH-induced conformational change in HA2 in endosome, the fusion of viral and endosomal membranes occurs through fusion peptide of HA2 (2). HA1 is highly variable among subtypes and contains major neutralizing epitopes. HA2 is considerably conserved among subtypes and contains minor but cross-react neutralizing epitopes (Fig.2.4B) (36,37).

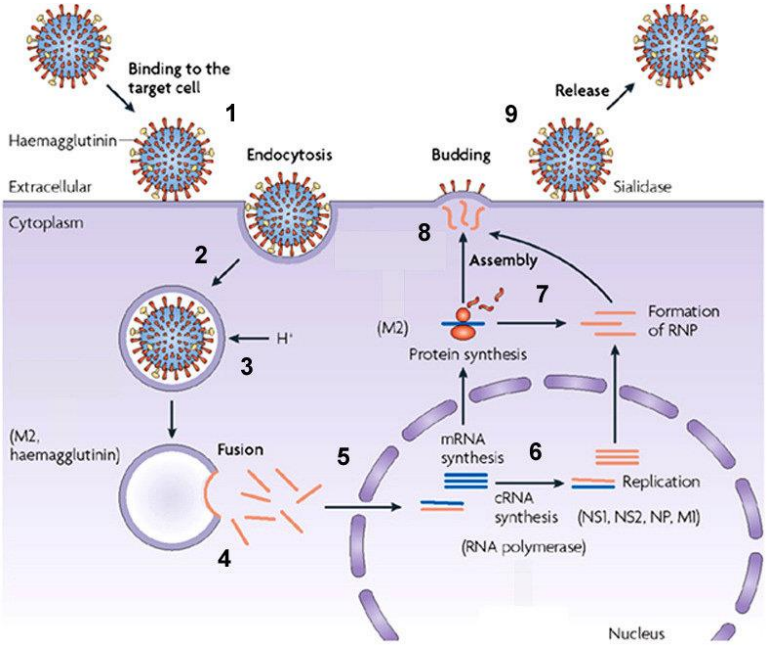


**Figure 5.** The influenza A virus life cycle. The diagram shows the virus entry, RNA transcription and replication, protein synthesis, virion assembly and release (The figure was obtained from *Mark VI, Nature Review,2007*).

**Life cycle**

At the first step of influenza viruses infection, the HA protein binds to sialic acids on the cell surface. Avian influenza viruses bind to sialic acid with an α2,3

linkage, while human influenza viruses bind to α2,6-linkead sialic acid. Virions enter the cells through endocytosis. Fusion of virion and endosomal membranes occurs within the endosome under the acidic pH. The low pH causes conformational changes in HA2 subunit, and the fusion peptide is exposed at the N-terminus of HA2 subunit (19).

The fusion peptide fuses viral and endosomal membranes, resulting in to the release of the virion contents into the cytoplasm. The viral RNPs are transported to the nucleus. In the nucleus, the mRNAs are transcribed from negative-stranded RNAs. The mRNAs become capped and polyadenylated. The viral proteins are produced through the translation of the mRNAs. For replication, a full-length, positive-sense RNA is produced from negative sense RNA. The positive-strand RNA serves as a template to produce viral RNA genome. The membrane-proteins are transported to the plasma membrane. The M1 protein plays an important role to package the virion at assembly site under the plasma membrane. The membrane proteins are packaged into virion envelop. The NA is a class II membrane-integrated protein. The NA protein removes the virus from its host cell and releases the virion through its enzymatic activities. NA activity also removes sialic acid from the viral glycoproteins; therefore, individual virus particles do not aggregate (Fig.2.5)(19).

**Vaccines**

Immunization of chickens with inactivated vaccines and/or viral vectored HA-based vaccines is one of the major policies which are currently implemented against

HPAIV in many countries (23). The greater part of currently used vaccines are inactivated vaccines. But for inactivated vaccines, there are limitations. Effectiveness of these vaccines is suboptimal. Their production and administration also are expensive, time consuming and labor intensive. Furthermore, inability to serologically differentiate infected birds from vaccinated ones is a drawback of inactivated vaccines (24,25). On the other side, viral-vectored HA protein vaccines are desirable alternatives for inactivated vaccines (26-33). However, the advantages and disadvantages of each DNA or RNA virus should be considered when they are used as a vector for the development of HA-based vaccines against HPAIV (33). Viral-vectored HPAIV vaccine candidates have been extensively studied in chickens. A NDV, a fowl pox and a turkey herpesvirus vectored HA protein of HPAIV have been licensed to use in the field, which are primarily rNDV expressing HA protein of HPAIV (2,33).

*A brief review on NDV*

**Classification**

Newcastle disease virus belongs to the family *Paramyxoviridae*. To date twenty classified avian paramyxovirus (APMV) serotypes (APMV-1-APMV-20) and one unofficially classified serotype (APMV-21) of paramyxoviruses have been identified (7). The family is divided into three genera; *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus*, based on haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (7). APMV-1 belongs to the genus *Orthoavulavirus*. Among all APMV serotypes, APMV-1 viruses rank the highest importance in poultry industry.

APMV-1 viruses are classified into velogenic, mesogenic and lentogenic strains based on their pathogenicity in chickens. Velogenic strains cause severe ND and mesogenic strains cause moderate ND. Therefore, velogenic and mesogenic strains of APMV-1 are called NDV. Lentogenic strains such as LaSota and B1 are natural avirulent strains with restricted replication in respiratory tract (3,46,47). To avoid complexity in this chapter, we will mention all APMV-1 strains as NDV.

**The genome structure**

NDV possesses a non-segmented, negative-sense, single-stranded RNA genome. The RNA genome is tightly encapsidated with N protein and forms a complex called riboneucleoprotein (RNP). This unique feature means that the free RNA is not found in cells during replication. Therefore, the recombination in NDV genome is rare (46,47,91). The genome has been found in 15,186, 15,192 or 15,198 nt in length among NDV strains (92-95). NDV genome contains a 55 nt leader and a 114 nt trailer sequences at 3' and 5' ends, respectively. The presence of leader and trailer regions in genome is required for transcription, replication and encapsidation of RNAs into virion particles. The genome encodes six genes: nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large polymerase (L). The genes are ordered 3' leader-N-P-M-F-HN-L-trailer 5'. Each ORF is flanked by the 3'-conserved transcriptional sequences known as gene-start (GS) and untranslated regions (UTR) at upstream and UTR and conserved transcriptional sequences known as gene-end (GE)-'5 at downstream. The non-coding intergenic sequences (IGS) are between each two genes. The GS and GE initiates and terminates the mRNA synthesis,

respectively. The IGSs length for N-P, P-M, M-F, F-HN and HN-L junctions are 1, 1, 1, 31 and 47nt, respectively (46,91). The roles of IGSs and UTRs in transcription and translation of NDV genes and virus replication are not well-characterized.

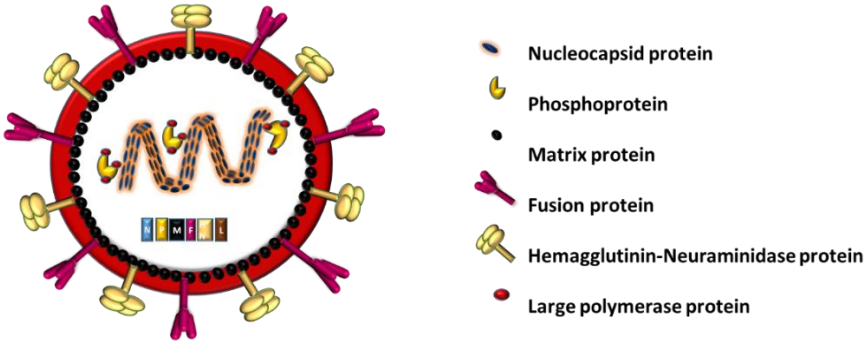


**Figure 6.** The NDV virion structure.

**Virion structure**

Virions are plasma membrane-derived enveloped in spherical and pleomorphic shape with 100nm or more in size (46). Matrix protein that lies under the envelope forms the virion shape. The F and HN proteins project like spike from envelope surface. Neucleocapsid is inside the virion. The neucleocapsid consists of RNP and L and P proteins which bind to RNP. NDV genome produces N, P/V/W, M,F, HN and L proteins; each gene encodes one protein except the P protein that encodes three products of P,V and W proteins through mRNA editing (Fig2.6)(46,48,91).

**Viral proteins**

**The nucleocapsid protein.** The N protein of NDV with 489 amino acids in length is the most abundant protein in virions. The N protein coats the negative-sense RNA

genome and positive RNA anti-genome in virion and replication site tightly. It protects genome from nuclease activity and prevent the recombination in NDV genome. The presence of N protein is crucial to maintain the proper folding of NDV nucleocapsid. In the RNP, the RNA is tightly encapsidated by N protein and as a template for the polymerase only some structural transition and local replacement of N protein, similar to the separation of two strands of DNA during transcription, might happen (96). Each N protein is associated with exactly six nucleotides of RNA, which is important for efficient transcription and replication, so called “rule of six” (97).

**The phosphoprotein.** The P protein of NDV is 395 amino acids in length. It plays a crucial role in RNA synthesis. It presents in both homooligomer and in bounded form with N or L protein. It binds to L protein to form active polymerase. Not only N-P complex prevents the binding of N protein to the cellular RNA, but also it assist N protein to bind leader and trailer regions of NDV RNA selectively(46,48,91). Insertion of one and two G residues at the P gene editing site produces V protein and W protein, respectively. It was shown that V protein may play a role in NDV virulence (98,99).

**The matrix protein.** The M protein of NDV has 364 amino acids. It lies beneath the virion envelope. It interacts with RNP, virion envelope compartments and cytoplasmic tails of F and HN proteins. M protein plays a key role in the assembly of virion and may play a role in transport of virion compartments to the assembly site (46,48,91). The M protein of paramyxoviruses including NDV forms more flexible shape than other non-segmented single-strand negative-sense RNA viruses (100).

**The fusion protein.** The F protein of NDV is a type I viral membrane protein of 553 amino acids in length(46). It exists as a homotrimer protein at virion surface. The monomer of F protein (F0) is cleaved to N-terminus F2 (117 aa) and C-terminus F1 (436 aa) subunits by proteases, while two subunits are still linked by a disulfide linkage. A cleavable signal sequence is on the N-terminus of F protein. The cytoplasmic tail and transmembrane domains are at C-terminus of F protein. The fusion peptide is at N-terminus of F1 subunit. The fusion peptide caused viral-cell membrane fusion at natural PH of endosome. The F protein is responsible for viral-cell and cell-cell membranes fusion resulting in cell entry and infection spread, respectively. Furthermore, the cleavage site motif of F protein is the major virulence factor of NDV. The F protein also contains heptad repeat domains (HRA-D), which HRA and HRB domains composes six coiled-coils in the F trimer (46,91,101).

**The haemagglutinin-neuraminidase protein.** The HN protein of NDV is a type II viral membrane protein with a varing length of 571-616 amino acids among NDV strains. It is located as a homotetramer at the surface of virion. It contains uncleaved signal sequence, cytoplasmic tail, transmembrane and stalk domains at N-terminus and globular head domain at C-terminus. HN protein is responsible for the attachment of virus to the sialic-acid receptors on host cells. It also has neuraminidase activity (NA) resulting the release of virions from sialic-acid. (46,91) Furthermore, HN protein increase the fusion activity through the interaction with F protein (102). However, the mechanism of HN and F proteins interaction is not well characterized. The HN protein also determines the tropism and contributes to the virulence of NDV(103).

**The large polymerase protein.** The L protein is the least abundant in the virion, but the longest protein of NDV with 2204 amino acids. The L protein is functional only in the association with the P protein. The L-P complex is responsible for RNA synthesis, capping, methylation and polyadelination of newly synthetized mRNAs. The L protein recognizes the encapsidated RNA with N protein as a template for RNA synthesis(46,48,91). The L protein may contributes in NDV virulence (104).

**Life cycle**

The HN protein of NDV attaches to the sialic acid receptors and virus membrane merges to the cell membrane through fusion activity of fusion peptide of F protein with assistance of HN protein. The nuclocapsid is released to the cytoplasm (104). In cytoplasm, the RNA genome encapsidated in nuclocapsid is used as a template for transcription. The polymerase binds to the RNA leader region and the NDV genes are transcribed sequentially in a 3'- 5' order by a start-stop process. The polymerization initiates when the polymerase reach to the GS and the polyadenylation occurs when the polymerase reach to the GE and the mRNA synthesis terminates. The synthetized mRNA is capped, methylated and polyadenylated and is used for protein production. The polymerase complex remains attached to the encapsidated RNA, but it does not transcript the IGS and reinitiate the polymerization while reaching to the next GS. The reinitiated polymerization is not as efficient as prior polymerization, resulting in a gradual reduction in the amount of transcribed mRNAs, based on the distance from the 3'-leader. When sufficient amount of N protein is produced, the N protein binds to the P protein and forms the soluble N-P. The soluble N-P complex is utilized in replication

of genome. Therefore, the polymerase switches the RNA transcription to the RNA replication. For the RNA replication, the encapsidated genomic RNA is used as template. Unlike the RNA transcription, for RNA replication, mRNAs are not produced and an encapsidated positive sense full-length antigenomic RNA is produced from the negative sense leader genomic RNA. The antigenomic RNA also is encapsidated with nuclocapsid and is used as a template to produce the negative sense genomic RNA. The 3' promoter in the antigenomic RNA that directs genomic RNA synthesis is stronger than the promoter in the genomic RNA that is responsible for the synthesis of both antigenomic RNA and mRNAs(46,48).

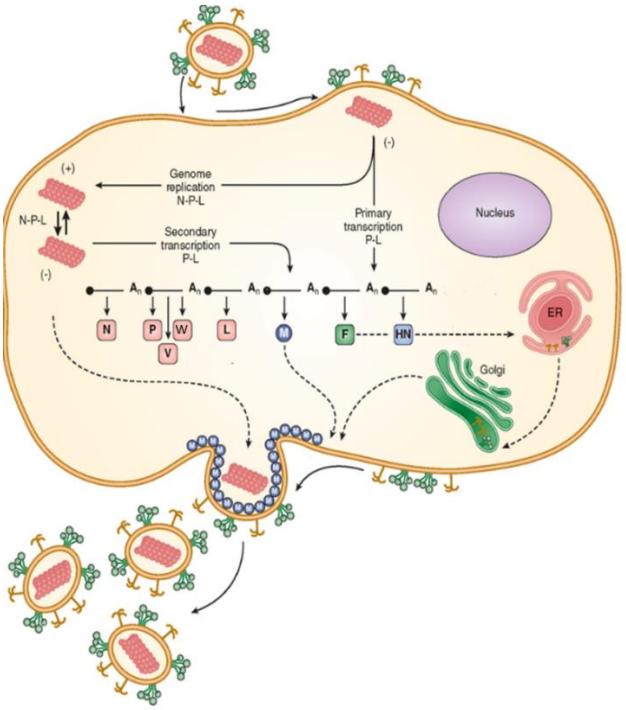


**Figure 7.** The NDV life cycle. The diagram shows the virus entry, RNA transcription and replication, protein synthesis, virion assembly and release (The figure was obtained from *Jawetz, Melnik & Adelberg's Medical Microbiology, 26th ed* and modified).

The viral proteins are produced from mRNAs in ER. The membrane glycoproteins are transported to the plasma membrane surface followed by processing in the Golgi and transporting in the secretory pathway. The M protein and nucleocapsid are transported to the assembly site close to the plasma membrane. The virion is released through budding from the plasma membrane, while the surface glycoproteins are being packaged into the virion envelop (Fig.2.7)(46,91).

**Pathogenesis and virulence**

NDV strains are classified into three pathotypes on the basis of the clinical sings in infected chickens: lentogenic (avirulent or low virulent), mesogenic (moderately virulent) and vicerotropic and neurotropic velogenic (highly virulent)(105). The major viral factor that determines the pathotypes of NDV is the F protein cleavage site (FPCS). During replication, cleavage of precursor F0 to F1 and F2 subunits is required for NDV infection. Most Lentogenic NDV strains have the sequence $^{112}$G/E-K/R-Q-G/E-R$^{116}$, contain a mono or dibasic amino acids within the FPCS and a L (leucine) at residue 117. In contrary, velogenic and mesogenic strains have the sequence $^{112}$R/K-R-Q/K/R-K/R-R$^{116}$, contain multibasic amino acids within the FPCS and a F (phenylalanine) at the residue 117 (106,107). The F0 protein of lentogenic strains can be cleaved into F1 and F2 subunits only by a trypsin-like protease that is present extracellularly in the respiratory and intestinal tracts. In contrast, F0 protein of mesogenic and velogenic NDV strains can be cleaved intracellularly by the ubiquitous furin-like protease, resulting in systemic infections. However, the different virulence between NDV strains with the same FPCS showed that other proteins may contribute

to virulence of NDV strains (108). HN protein is responsible for tropism and also contributes in virulence of NDV strains (103). Furthermore, the L and V proteins also are associated with NDV virulence (98,99,104).

**NDV as vaccine vector**

During two recent decades, live attenuated and inactivated vaccines have had undeniable impacts on the control of infectious disease in humans and animals. However, the inactivated vaccines do not provide strong immunity, and the production and administration these vaccines is not affordable and practical, particularly for veterinary application. The conventional live vaccines also may act as double-edged sword in human and animal populations of the modern world. They can cause disease, if they reverse back to the virulence. The mutations, recombination, reassortment and latency also are other unfavorable consequences of the use of these vaccines (17). Furthermore, there is a need to develop successful vaccines against many infectious pathogens, particularly, emerging infectious diseases. Among several novel approaches for the development of the next generation of vaccines, viral-vectored vaccines are the most promising. However the favorable and unfavorable consequences for the choice of each virus as a vaccine vector against a certain pathogen should be taken in consideration.

The nature of NDV genome and the biological characteristics of NDV make it attractive as a vaccine vector for avian and non-avian pathogens. (i) Avirulent NDV strains have been used as safe live vaccines against virulent NDV over 60 years (3). (ii)

NDV replicates through respiratory tract and elicits strong mucosal, humoral and cell-mediated immune responses against the virus. (iii) The RNA genome of NDV is tightly encapsidated with nuclocapsid and the genomic RNA and antigenomic RNA are not found free during the RNA replication in cell; therefore, the recombination in NDV genome is rare. (iv) NDV is genetically stable and the mutation rate in its genome is very low. (v) The NDV replication occurs in the cytoplasm; therefore, there is no interaction between NDV and host cell genomes (46,47,48,91). (vi) The M protein of paramyxoviruses including the M protein of NDV forms more flexible shape than other non-segmented negative sense RNA viruses; hence, NDV can package the membrane-integrated glycoproteins of other viruses such as S protein of coronaviruses and HA protein of influenza viruses in its envelope (48,100). (vii) NDV replicates efficiently in egg and cell culture, which is a merit for the development of vaccines in industrial scales. (viii) A wide range of APMV serotypes make it possible to employ other APMV serotype vectors and novel chimeric APMV vectors for the development of improved vaccines against pathogens (46,47).

To date, NDV has been used as a successful vector against several avian and non-avian pathogens (38). Reverse genetics techniques have been employed to engineer NDV vector expressing the protective antigens of other pathogens (109). NDV genome contains six genes in order 3’ -N-P-M-F-HN-L-5’. The beginning and the end of each gene contain conserved transcriptional sequences known as the GS and GE, respectively. Between the genes, there are gene junction (47, 91). Any of the gene junctions is a potential insertion site for the transcription cassette of a foreign gene.

However, it was found that the intergenic region between the P and M genes is a good site for expression of most foreign genes (110-113). The transcription cassette containing the foreign gene ORF is required to be modified to contain NDV genome elements. Therefore, the newly added ORF to the NDV genome possesses its own GS, IGS and GE and probably UTRs. The Kozak sequence also is added at the upstream of foreign gene ORF to enhance the level of the expression of inserted protein. Some extra nucleotides may be added to maintain the rule of six in entire genome of NDV. The rNDV expressing the foreign protein is recovered by employing reverse genetics through the co-transfection of the full length antigenomic cDNA of NDV with the expression plasmids containing N, P and L genes of NDV into cells under the control of a promoter/terminator system(Fig.2.8)(109).

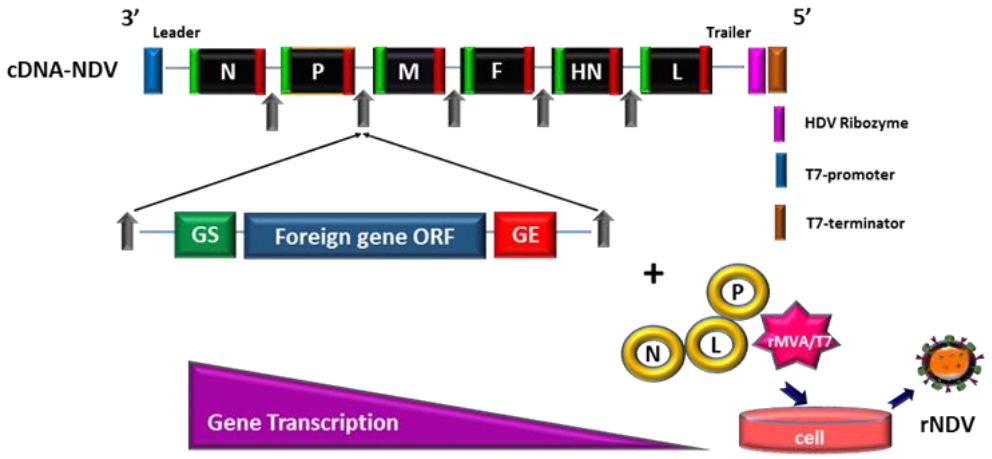


**Figure 8.** The schematic diagram showing the NDV genome and the reverse genetics to recover recombinant NDV from NDV antigenomic cDNA.

# Chapter 3

# A recombinant NDV expressing the S protein of IBV protects chickens against IBV and NDV.

*Abstract*

Infectious bronchitis virus causes a highly contagious respiratory, reproductive and urogenital tract disease in chickens worldwide, resulting in substantial economic losses for the poultry industry. Currently, live-attenuated IBV vaccines are used to control the disease. However, safety, attenuation and immunization outcomes of current vaccines are not guaranteed. Several studies indicate that attenuated IBV vaccine strains contribute to the emergence of variant viruses in the field due to mutations and recombination. Therefore, there is a need to develop a stable and safe IBV vaccine that will not create variant viruses. In this study, we generated rNDVs expressing the S1, S2 and S proteins of IBV using reverse genetics technology. Our results showed that the rNDV expressing the S protein of IBV provided better protection than the rNDV expressing S1 or S2 protein of IBV, indicating that the S protein is the best protective antigen of IBV. Immunization of 4-week-old SPF chickens with the rNDV expressing S protein elicited IBV-specific neutralizing antibodies and provided complete protection against virulent IBV and virulent NDV challenges. These results suggest that the rNDV expressing the S protein of IBV is a safe and effective bivalent vaccine candidate for both IBV and NDV.

*Introduction*

Infectious Bronchitis (IB) is an acute and highly contagious viral respiratory disease of chickens (1,4). IB causes major economic losses in commercial chickens throughout the world (1,6). It is one of the most prevalent diseases in the poultry industry. The disease is usually characterized by respiratory signs including ocular and nasal discharges, gasping, coughing and sneezing. However, the virus can also infect urogenital and reproductive tracts causing renal dysfunction and decreased egg production (6,9).

IB is caused by IBV, a member of the family Coronaviridae. The genome of IBV is a single stranded, positive-sense RNA of approximately 27.6 kb in length. It encodes five major structural proteins: S, E, M, N and fifteen non-structural proteins. The S glycoprotein is the major antigen against which neutralizing and protective antibodies are produced. The S protein is partially or completely cleaved into the amino-terminal S1 and to the carboxy-terminal S2 subunits post translationally by a furine-like protease of host (8,9). The S1 subunit is highly variable among different isolates of IBV. It is responsible for viral attachment to host cell and contains major neutralizing epitopes. The S2 subunit is highly conserved among IBV strains and contributes to viral fusion activity and elicits some minor but broadly reactive neutralizing antibodies (8,11,14,114-116).

Currently IB in commercial chickens is controlled by the use of live-attenuated and inactivated IBV vaccines. However, safety, attenuation and immunization

outcomes of current live-attenuated vaccines which, were developed by serial passaging of field isolates of IBV in chicken embryonated eggs, are not guaranteed, particularly with regards to the age at immunization, vaccine dose, type of bird and route of vaccination (80). Live vaccines have been found as one of the sources of IB outbreaks (81). Live attenuated vaccine strains also are not genetically stable and have a tendency to revert back to virulence (17,18). Furthermore, circulation of live-attenuated viruses in the environment provides a setting in which the viral population may undergo mutations and recombination leading to creation of variant viruses (82,83). Moreover, live IBV vaccines may cause pathological lesions or secondary bacterial infections in young vaccinated chicks (84,85). Inactivated IBV vaccines, which usually are administered by injection to layers and breeders are not an alternative for live-attenuated IBV vaccines. Inactivated vaccines of IBV do not elicit strong immune responses in chickens against circulating virulent IBV strains (15,16). The production and administration of inactivated vaccines are also time consuming and costly (17,18). Therefore, there is a great need to develop an alternative IBV vaccine.

Development of viral vectored vaccines to control IB is an alternative approach. One of the important advantages of vectored IBV vaccines is that it will not create variant viruses, which is a major drawback of the current live attenuated IBV vaccines. Several viruses, such as, fowl pox virus, adenovirus and NDV have been evaluated as vaccine vectors for IBV (86-90). Among these viral vectors NDV holds great promise, because it induces effective local and systemic immune responses, it is a safe vaccine vector, and it will serve as a bivalent vaccine against IBV and NDV.

NDV belongs to the genus Avulavirus in the family Paramyxoviridae. Virulent NDV strains cause a fatal neurological disease in chickens. NDV strain LaSota has been used as a safe and effective live vaccine for more than 60 years (3,46). rNDV strain LaSota has been evaluated as a vaccine vector against several avian pathogens including IBV (30,117-120). In one study, rNDV expressing S2 protein of IBV was found to induce partial protection against a virulent IBV challenge (88). In another study, it was shown that rNDV expressing S1 protein of IBV resulted in inducing protective immunity against virulent IBV challenge (89). However, the protective efficacy of the whole S protein of IBV expressed by rNDV vector has not been evaluated and it is also not known among S1, S2 and S proteins of IBV, which is the best protective antigen for the development of viral vectored IBV vaccines. In this study, we have used reverse genetics to generate rNDVs expressing S1, S2 and S proteins of IBV strain Mass-41. The protective efficacies of rNDVs expressing S1, S2 or S protein of IBV were compared in chickens against a virulent IBV strain Mass-41 challenge. Our results showed that the whole S protein is the best protective antigen of IBV. We also showed that rNDV strain LaSota expressing the S protein of IBV strain Mass-41 protects chickens against both virulent IBV and virulent NDV challenges.

*Materials and Methods*

**Cells and viruses**

Chicken embryo fibroblast (DF-1) cells and human epidermoid carcinoma (HEp-2) cells were obtained from the American Type Culture Collection (ATCC,

Manassas, VA). They were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS). The recombinant avirulent NDV strain LaSota was generated previously in our laboratory using reverse genetics (109). The rNDV and rNDVs expressing chicken codon optimized S1, S2 and S genes and non-codon optimized S gene of IBV strain Mass-41 were grown in 9-day-old embryonated SPF chicken eggs at 37°C. The virulent IBV strain Mass-41 was propagated in 10-day-old SPF embryonated chicken eggs and harvested five days after infection. The titer of virus in harvested allantoic fluid was determined by 50% embryo infectious dose ($EID_{50}$) method. Briefly, ten-fold serial dilutions of IBV strain Mass-41 was inoculated into 10-day-old embryonated SPF chicken eggs. Seven days after inoculation, infected embryos were examined for IBV specific lesions such as stunting or curling. The titer of virus was calculated using Reed and Muench method. The modified vaccinia virus strain Ankara expressing T7 RNA polymerase (MVA-T7) was propagatd in monolayer primary chicken embryo fibroblast cells.

**Generation of rNDVs containing S1, S2 or S gene of IBV**

A plasmid containing full-length antigenomic cDNA of NDV strain LaSota has been constructed previously (109). In order to develop an effective IBV vaccine the maximum neutralizing epitopes with correct conformation are needed to be displayed. Most neutralizing epitopes are located in the S protein. In this study seven transcription cassettes containing S, S1 or S2 genes of IBV were constructed to identify the best protective antigen for the development of NDV vectored IBV vaccines. The S, S1 and S2 genes were chicken codon optimized for higher level of expression in chickens. The

following transcription cassettes were designed: i) a transcription cassette containing the S gene of IBV strain Mass-41 (3489 nt) was designed to determine whether the expression of the whole S gene from NDV will lead to display the maximum neutralizing epitopes in correct conformation, ii) a transcription cassette containing the S2 subunit of S gene (1878 nt) of IBV fused with C-terminus of signal peptide sequence of S gene (69 nt) was constructed for transport of the protein from the cell iii) a transcription cassette containing the S1 subunit of S gene (1614 nt) was designed to determine the protective efficacy of S1 protein, iv) a transcription cassette containing the codon optimized S1 gene (1611 nt) fused with N-terminus of transmembrane and cytoplasmic tail of S gene (255 nt) was designed for incorporation into NDV envelop , v) a transcription cassette containing the S1 subunit of S gene without S1 protein cleavage site residues (1593 nt) fused with N-terminus of transmembrane and cytoplasmic tail of NDV F gene (171nt) was designed for incorporation of the S1 protein into envelope of NDV. vi) a transcription cassette containing the S1 subunit of S gene containing S1 protein cleavage site residues (1611 nt) fused with N-terminus of transmembrane and cytoplasmic tail of NDV F gene (171 nt) was designed to incorporate the S1 protein into NDV envelope and also to know whether adding the cleavage site residues has any effect on the fusion of two proteins, and vii) a transcription cassette containing the non-codon optimized S gene (3489 nt) was constructed to compare the level of protein expression between the codon optimized and non-codon optimized S genes.

NDV genome contains six genes: N, P, M, F, HN and L. The genes are ordered 3' -N-P-M-F-HN-L-5'. The beginning and the end of each gene contain conserved transcriptional sequences known as the GS and GE, respectively. Between the genes, there are gene junctions (46). Any of the gene junctions is a potential insertion site for the transcription cassette of a foreign gene. However, we and others have found that the intergenic region between the P and M genes is a good site for expression of most foreign genes (46,110, 112,113,122) The transcription cassettes containing IBV genes contained PmeI restriction enzyme sequence, 15 nt of UTR of NDV, NDV GE signal, one T nucleotide as intergenic sequence, NDV GS signal, extra nucleotides to maintain the rule of six (46,97) Kozak sequence at the upstream of foreign gene ORFs and PmeI restriction enzyme sequence at downstream of foreign gene ORF. The transcription cassettes of codon optimized and non-codon optimized S gene were digested from two commercially synthesized (GenScript; pUC57-IBV-Mass-41-S syn) plasmids containing codon optimized (GenScript; optimization on Gallus Gallus codons using OptimumGeneTM PSO algorithm) and non-codon optimized S gene of IBV strain Mass-41(GenBank Accession no. AY851295. 1), respectively. The transcription cassettes of codon optimized S1 and S2 genes were amplified from the commercially synthesized plasmid containing codon optimized S gene of IBV strain Mass-41 and cloned into individual shuttle vectors (pGEM®-T Easy Vector, Promega Corporation). Then the flanking DNA of transcription cassettes were digested from shuttle vectors. The transcription cassettes derived from shuttle vectors were cloned into complete individual plasmids containing cDNA of rLaSota at P and M gene junction using PmeI

site (Fig. 3.1). The correct sequences of the foreign genes were confirmed by nucleotide sequence analysis. rNDVs containing the IBV genes were recovered by reverse genetics as described previously (109). .Briefly, each full length cDNA was co-transfected with three expression plasmids containing N, P or L gene of NDV strain LaSota into MVA-T7 infected HEp-2 cells. Three days post-transfection, 200µl of supernatant of transfected cells were inoculated in 9-11 day-old SPF embryonated chicken eggs. After three days, haemagglutination test was used to detect infected allantoic fluids collected from eggs. rNDVs containing S1 gene, S1 gene fused with transmembrane and cytoplasmic tail of IBV S gene, S1 gene containing cleavage site residues of S gene of IBV fused with transmembrane and cytoplasmic tail of NDV F gene, S1 gene without cleavage site residues of S gene fused with transmembrane and cytoplasmic tail of NDV F gene, S2 gene, codon optimized S gene and non-codon optimized S gene were named rNDV/S1, rNDV/S1+IBV-S-TM&CT, rNDV/S1(cs+)+NDV-F-TM&CT, rNDV/S1(cs-)+NDV-F-TM&CT, rNDV/S2, rNDV/codon optimized-S and rNDV/non-codon optimized-S, respectively. The IBV genes were amplified from the rNDV constructs by RT-PCR.

**Expression of S1, S2 and S proteins of IBV**

Confluent monolayers of DF-1 cells were infected at a multiplicity of infection (MOI) of 0.01-0.1 with rNDV strain LaSota, rNDV/S1, rNDV/S1+IBV-S-TM&CT, rNDV/S1(cs+)+NDV-F-TM&CT, rNDV/S1(cs-)+NDV-F-TM&CT, rNDV/S2, rNDV/codon optimized-S or rNDV/non-codon optimized-S. DF-1 cells were harvested 30 hours post-infection, lysed and analyzed by Western blot. A polyclonal chicken anti-

IBV strain Mass-41 was used to detect the expression of S1, S2 and S proteins of IBV. To determine the incorporation of IBV proteins into NDV envelope, rNDV, rNDV/S2, rNDV/codon optimized-S and rNDV/non-codon optimized-S were inoculated into 9-day-old embryonated SPF chicken eggs. Three days after incubation, recombinant viral particles from infected allantoic fluids were partially purified by sucrose density gradient centrifugation and analyzed by Western blot analysis. A monoclonal anti-NDV/HN antibody also was used to detect HN protein of NDV in lysates and purified virions by one more Western blot analysis.

**Growth characteristics of rNDV constructs**

In order to determine the growth kinetics of rNDVs expressing S1, S2 or S protein of IBV, confluent monolayers of DF-1 cells in 6-well tissue culture plates were infected at a MOI of 0.1 with rNDV, rNDV/S1(cs+)+NDV-F-TM&CT, rNDV/S2 and rNDV/codon optimized-S and adsorbed for 90 minutes at 37°C. After adsorption, cells were washed with PBS, then incubated with DMEM containing 2% FBS and 10% fresh SPF chicken egg allantoic fluid at 37°C in presence of 5% CO2. Aliquots of 200 µL of supernatant from infected cells were collected and replaced with fresh DMEM including FBS at intervals of 8 hours until 64 hours post-infection. The titer of virus in the harvested samples was determined by $TCID_{50}$ method in DF-1 cells in 96-well tissue culture plates.

**The protective efficacy of rNDVs expressing S1, S2 and S protein of IBV against virulent IBV challenge**

Based on the level of expression of S1, S2 and S proteins of IBV from rNDVs, rNDV/S1(cs+)+NDV-F-TM&CT, rNDV/S2, and rNDV/codon optimized-S viruses were selected for animal study to evaluate their protective efficacy against virulent IBV challenge.

IBV protection experiment 1. In this study, the protective efficacy of rNDVs expressing S1, S2 or S protein of IBV strain Mass-41 were evaluated in 1-day-old SPF chicks. Briefly, a total of eighty 1-day-old chicks were divided into five groups of fifteen each and one group of five. Chicks of the first four groups were inoculated with $10^7$ $EID_{50}$ ($2^7$HAU) of rNDV, rNDV/S1(cs+) + NDV-F-TM&CT, rNDV/S2 and rNDV/codon optimized-S strains via oculonasal route. The fifteen chicks of group five and five chicks of group six were inoculated with PBS. Three weeks after immunization, all immunized chickens, were challenged with $10^{3.1}$ $EID_{50}$ of virulent IBV strain Mass-41. This challenge virus dose was determined by an experimental chicken infection study. The severity scores of clinical signs of IBV including, nasal discharge, ocular discharge and difficulty in breathing (0 = normal, 1= presence of mild ocular discharge, mild nasal discharge and or sneezing 2= presence of heavy ocular discharge and or heavy nasal discharge with mild tracheal rales and mouth breathing and or coughing 3= heavy ocular discharge and heavy nasal discharge with sever tracheal rales and mouth breathing, gasping, dyspnea and or severe respiratory distress) were recorded twice a day for 10 days post-challenge. In order to evaluate protective efficacy of rNDVs expressing S1, S2 and S genes of IBV in preventing shedding of virulent IBV in immunized chickens, at day five post-challenge, tracheal swab samples

were collected from fifteen birds of each group and placed in 1.5 mL serum free DMEM with 10 X antibiotics and amphotericin B. The swab samples were analyzed for quantification of viral RNA using an IBV-N gene-specific RT-qPCR.

IBV protection experiment 2. In this study, the protective efficacy of rNDV expressing codon optimized S protein of IBV was evaluated in 4-week-old SPF chickens against the OIE recommended dose of virulent IBV challenge (1). A total of fifteen 4-week-old SPF chickens were divided into four groups of five each. Five chickens of groups one were inoculated with $10^7$ $EID_{50}$ ($2^7$HAU) of rNDV/codon optimized-S via oculanasal route. Five chickens of group three were inoculated with 10 recommended doses of a commercial live attenuated Mass-type IBV vaccine via oculanasal route and chickens of group four were inoculated with PBS. Three weeks after immunization, chickens of all groups were challenged with $10^{3.1}$ $EID_{50}$ of virulent IBV strain Mass-41 by the oculonasal route. The severity scores of clinical signs of IBV, described in IBV protection experiment 1, were recorded for 10 days post-challenge. The post-challenge vial load was evaluated as described in IBV protection experiment1. The swab samples collected from five non-vaccinated, non-infected SPF chickens involved in another IBV protection study also were used as control.

IBV protection experiment 3. In this study, the protective efficacy of rNDV expressing codon optimized S protein of IBV was evaluated in 4-week-old SPF chickens against a higher dose of virulent IBV challenge. A total of thirty two 4-week-old SPF chickens were divided into four groups of eight each. Eight chickens of group one and two were inoculated with $10^7$ $EID_{50}$ ($2^7$HAU) of rNDV and rNDV/codon

optimized-S, respectively, via oculanasal route. Eight chickens of group three were inoculated with 10 recommended doses of a commercial live attenuated Mass-type IBV vaccine via oculanasal route and chickens of group four were inoculated with PBS. Three weeks after immunization, chickens of all groups were challenged with $10^{4.7}$ $EID_{50}$ of virulent IBV strain Mass-41 by the oculonasal route. The severity scores of clinical signs of IBV, described in IBV protection experiment 1, were recorded for 8 days post-challenge. At day 4 post-challenge, three chickens from each group were euthanized for tracheal ciliostasis analysis (data not shown). The post-challenge vial load was evaluated as described in IBV protection experiment1. The swab samples collected from two non-vaccinated, non-infected SPF chickens involved in another IBV protection study also were used as control for RT-qPCR assay. Each swab fluid also was tested for IBV specific lesions on chicken embryo by inoculation (0.1 ml) of one 10-day-old embryonated SPF chicken egg.

IBV protection experiment 4. In this study, the protective efficacy of rNDV expressing codon optimized S protein of IBV was evaluated in 1-day-old SPF chicks against virulent IB challenge virus infected by the intraocular route. Intraocular route was used, because this route of IBV challenge has been specified by the USDA-CFR-9 (123). A total of forty five 1-day-old SPF chickens were divided into three groups of ten each and three groups of five each. Ten chickens of group one and two were inoculated with $10^7$ $EID_{50}$ ($2^7$ HAU) of rNDV, rNDV/codon optimized-S, respectively, via oculanasal route. Ten chickens of group three were inoculated with one recommended dose of a commercial live attenuated Mass-type IBV vaccine via

oculanasal route and chickens of groups four to six were left non-vaccinated. Three weeks after immunization, chickens of all groups one to four were challenged with $10^4$ $EID_{50}$ of virulent IBV strain Mass-41 by the intraocular route, chickens of group five were challenged with $10^4$ $EID_{50}$ of virulent IBV strain Mass-41 by the oculanasal route, and chickens of group six were left non-infected. The severity scores of clinical signs of IBV, described in IBV protection experiment 1, were recorded for 10 days post-challenge. In order to evaluate the efficacy of rNDV expressing S protein of IBV in preventing shedding of virulent IBV in immunized chickens, at day 5 post-challenge, tracheal swab samples were collected from all chickens of each group and placed in 3 mL serum free DMEM with 10 X antibiotic and amphotericin B. Each fluid was tested for IBV specific lesions on chicken embryo by inoculation with 0.2 ml to each of five 10-day-old embryonated SPF chicken egg. The sample was considered positive for virus shedding, if any of the five embryos showed IBV lesions.

We performed all experiments involving virulent IBV in our USDA approved Biosafety level-2 and Biosafety level-2 plus facilities following the guidelines and approval of the Animal Care and Use Committee (IACUC), University of Maryland.

**The protective efficacy of rNDV expressing S protein of IBV against virulent NDV challenge**

The protective efficacy of rNDV expressing S protein of IBV strain Mass-41 was evaluated against a virulent NDV strain GB Texas challenge in our biosafety level 3 (BSL-3) plus facility. Briefly, a total of fifteen 1-day-old chicks were divided into

three groups of five each. Chicks of two groups were inoculated with $10^7$ $EID_{50}$ ($2^7$HAU) of rNDV and rNDV/IBV-codon optimized-S via oculonasal route. The five chickens of group three were inoculated with PBS. Three weeks after immunization, blood samples of all birds were collected for NDV antibody response analysis and challenged with one hundred 50% chicken lethal dose ($CLD_{50}$) of the highly virulent NDV strain GB Texas via oculonasal route. The chickens were observed daily for 10 days after challenge for mortality with clinical signs of disease (neurological signs included torticollis, paralysis, and prostration). We performed the experiment involving virulent NDV in our USDA approved Biosafety level-3 plus facility following the guidelines and approval of the Animal Care and Use Committee (IACUC), University of Maryland.

**Serological analysis**

The level of antibodies induced against NDV and IBV were evaluated. The serum samples were collected three weeks post-immunization. Hemagglutination inhibition (HI) assay using a standard protocol OIE was used to assess the level of antibody titer mounted against NDV in chickens immunized by rNDVs (3). The virus neutralization assay according to OIE was used to measure the level of neutralizing antibodies mounted against IBV1. Briefly, serum samples of three birds from the group immunized with rNDV expressing codon optimized S protein of IBV and serum samples of three birds from the group immunized with commercial IBV vaccine group were incubated at 56°C for 30 minutes. One hundred $EID_{50}$ of IBV strain Mass-41 was mixed with 2 fold dilutions of antiserum and incubated for 1 hour at 37°C. One hundred

µL of each serum and virus mixture was inoculated into three 10-day-old embryonated SPF chicken eggs. To confirm that at least 100 $EID_{50}$ of virus was inoculated into each egg, three eggs were inoculated with 100 µl of PBS containing 100 $EID_{50}$ of IBV. Three eggs were inoculated with 100 µL of PBS as negative control. Three eggs were inoculated with a mixture of 100 $EID_{50}$ of IBV and a dilution of 1:8 of a randomly selected serum sample collected from a bird immunized with rNDV strain LaSota as vector control. The eggs were incubated at 37°C and were observed daily for dead chicken embryos for 7 days post inoculation. The serum titers were calculated according to the method of Reed and Muench (121), based on mortality and IBV specific lesions on chicken embryos.

**Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)**

RNA was extracted using TRIzol Reagent (Invitrogen) from tracheal swab samples collected from chickens. The first strand cDNA was synthesized using Thermo Scientific RevertAid Reverse Transcriptase (RT). SYBR green RT-qPCR was performed using a specific primer pair set: a) N gene - 296 forward primer: 5' GACCAGCCGCTAACCTGAAT 3' and b) N gene - 445 reverse primer: 5' GTCCTCCGTCTGAAAACCGT 3' amplifying 150 nt of N gene of IBV strain Mass-41. PCRs were performed using a Bio-Rad CFX96 Cycler. Each 20 µl reaction was carried out using 5 µl of cDNA, 10 µl of iTaq Universal SYBR Green Supermix (Bio-Rad), 2 µl of forward and reverse primers and 3 µl of nuclease free water. Forty three cycles of PCR at 95°C for 10s (denaturation) and 58°C for 20s (annealing) followed by melting curve analysis that consisted of 95°C for 5s and 65 °C for 5s and 95°C for 5

min. A serial 10 fold dilution of cDNA synthesized from extracted RNA of allantoic fluid stock of a virulent IBV strain Mass-41 with $10^{7.5}$ $EID_{50}$/ml was used to establish the standard curve. The cDNA synthesized from extracted RNA of allantoic fluid stock of a virulent IBV strain Mass-41 and the cDNA synthesized from extracted RNA of swab sample solution were served as positive and negative controls, respectively. Melting point analysis was used to confirm the specificity of the test.

**Statistical analysis**

Data were analyzed among groups by One-Way-ANOVA test. The student t-test was used to compare two groups. To avoid bias, all animal experiments were designed as blinded studies.

*Result*

**Generation of rNDVs expressing S1, S2 or S protein of IBV**

The expression cassettes containing the codon optimized S1, S2, S and non-codon optimized S genes of IBV were cloned into the cDNA encoding the complete antigenome of NDV strain LaSota, using the PmeI site, between P and M genes (Fig.3.1). The correct sequences of genes cloned into full length cDNA of NDV were confirmed by nucleotide sequence analysis. Infectious recombinant NDVs containing S1, S2 and S genes of IBV were recovered from all cDNAs. The sequences of S1, S2 and S genes present in the rNDVs were confirmed by RT-PCR. To evaluate genetic stability of rNDV expressing codon optimized S protein, the viruses were passaged five times in 9-day-old embryonated specific pathogen free (SPF) chicken eggs. The

nucleotide sequence analysis of the S gene showed that the inserted ORF were maintained without any adventitious mutations.

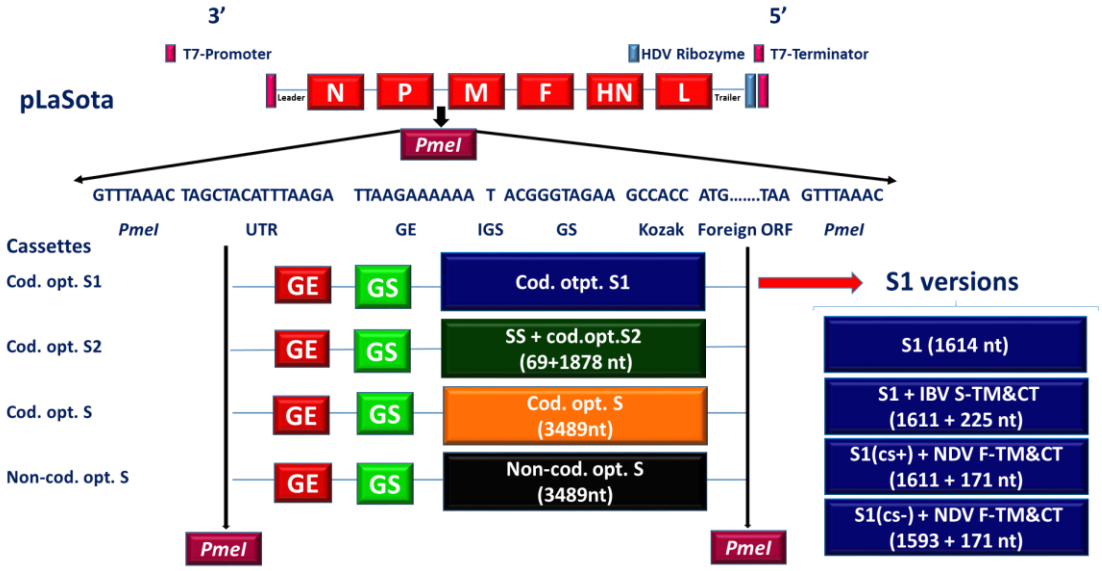


**Figure 9.** Schematic diagram of recombinant NDV constructs containing IBV genes. Seven transcription cassettes including; 1-4) Four versions of codon optimized S1 subunit of S gene of IBV strain Mass-41; namely, a) S1 subunit of S gene (1614 nt), b) S1 subunit of S gene (1611 nt) fused with N-terminus of transmembrane and cytoplasmic tail of S gene (255 nt), c) S1 subunit of S gene (1611 nt) containing five putative cleavage site residues of S gene fused with N-terminus of transmembrane and cytoplasmic tail of F gene of NDV (171 nt). In this construct, five C-terminus putative cleavage site residues of S1 gene (RRFRR) plus the first serine (S) residue of N-terminus of transmembrane and cytoplasmic tail of F gene of NDV provides six putative cleavage site residues of S protein of IBV strain Mass-41 (RRFRR/S). d) S1 gene (1593 nt) without cleavage site residues of S gene fused with N-terminus of

transmembrane and cytoplasmic tail of F gene of NDV (171 nt) , 5) the N-terminus of codon optimized S2 gene of IBV strain Mass-41 (1878 nt) fused with C-terminus of signal peptide sequence of S gene (69 nt), 6) the codon-optimized S gene (3489 nt) and 7) the non-codon optimized S gene of IBV strain Mass-41 (3489 nt) were flanked into individual plasmids containing cDNA of LaSota between P and M genes using *PmeI* site. Each transcription cassette contains the ORF of foreign gene with the addition of *PmeI* restriction enzyme site sequence, 15 nt of NDV UTR, GE signal of NDV, one T nucleotide as intergenic sequence, GS signal of NDV, nucleotides for maintaining the rule of six and Kozak sequence.

**Evaluation of the expression of the S1, S2 and S proteins of IBV**

The expression of codon optimized S2, and S proteins and non-codon optimized S protein of IBV strain Mass-41 by rNDV constructs was detected by Western blot analysis in DF-1 cells using a chicken polyclonal anti IBV serum (Fig.3.2A-upper panel &B). As the expression of non-codon optimized S was not detected clearly in the first attempt (Fig.3.2A), we detected it in another attempt (Fig.3.2B). The expression level of codon optimized S protein of IBV was significantly higher than that of the non-codon optimized S protein of IBV. For the codon optimized S protein of IBV expressed from rNDV (Fig.3.2A-Lane3 and Fig.3.2B - lane2), the two bands on top (~170-220 kDa) probably represent either uncleaved S protein (S0) or polymeric forms of S protein. The ~130 kDa, the ~95 kDa and the ~60 kDa band represent S1 or S2 subunit of cleaved S protein of IBV. In the case of non-codon optimized S protein of IBV expressed from rNDV (Fig.3.2A-lane2 and Fig.3.2B-

lane1), the two bands on top (~170-220 kDa) probably represent uncleaved S protein (S0) or polymeric forms of S protein. The ~ 95 kD band represents S2 or S1 subunit of cleaved S protein of IBV. In the case of rNDV /IBV- S2 strain (Fig.3.2A-lane 1), there are two bands (~170-220 kDa) on top, representing polymeric folded forms of S2 protein, a ~105 kDa band and a ~95 kDa band representing S2 subunit. The expression of S2 protein from a transcription cassette in which the signal peptide sequences of S protein was not fused with S2 gene was not detected (data not shown). Lane 4 of Fig.3.2A and lane 3 of Fig.3.2B represent rNDV as control. Lane 5 of panel A represents non-infected DF-1 cells. These results showed that codon optimized S and S2 proteins of IBV were expressed efficiently. The non-codon optimized S protein was also expressed from rNDV, but not efficiently and not consistently. A monoclonal anti-NDV/HN antibody was used to detect a ~70 kDa of HN protein of NDV in lysates, confirming similar level of NDV protein in each lane (Fig.3.2A-lower panel). We further evaluated incorporation of IBV S and S2 proteins into NDV virions. The rNDVs expressing codon optimized S and S2 proteins and rNDV expressing non-codon optimized S protein were inoculated into eggs, 3 days after inoculation, viral particles in infected allantoic fluid were partially purified and analyzed by Western blot (Fig. 3.2C-upper panel) . Two bands (~170-220 kDa) on top, representing S protein , a ~95 kDa band and a ~60 kDa band representing S2 or S1 subunit of cleaved S protein, were detected in purified particles of rNDV expressing codon optimized S protein by Western blot analysis (Fig. 3.2C-lane2). The lane 4 of Fig.3.2C shows two bands (~170-220 kDa) on top, representing polymeric folded of S2 protein, a ~105 kDa band

and a ~95 kDa band representing S2 subunit. The lane 1 of Fig.3.2C represents purified rNDV control and lane3 of Fig.3.2C shows purified rNDV expressing non-codon optimized S protein. These results suggested that the codon optimized S and S2 proteins of IBV expressed by rNDVs were incorporated into rNDV particles. A monoclonal anti-NDV/HN antibody was used to detect a ~70 kDa of HN protein of NDV in partially purified virions, confirming similar level of NDV protein in each lane (Fig.3.2C-lower panel).

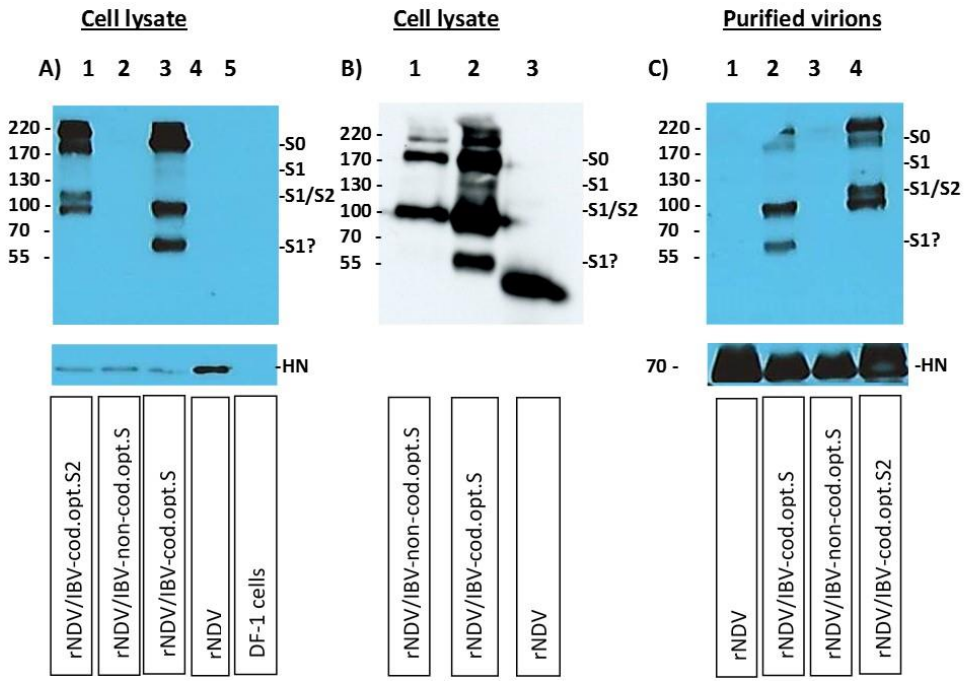


**Figure 10.** Western blot analysis of rNDVs expressing S or S2 protein of IBV. The expression of codon optimized S, and S2 proteins and non-codon optimized S protein of IBV were detected by Western blot analysis in infected DF-1 cell lysates, using a chicken polyclonal anti IBV serum (A-upper panel & B). The panel B was added to show the expression of non-codon optimized S protein clearly, since the expression of non-codon optimized S protein was not clear in panel A. For the codon optimized S

protein of IBV expressed from rNDV (A-lane 3 and B-lane 2) two bands on top (~170-220 kDa) represent uncleaved S protein (S0) or polymeric forms of S1 or S2 protein. The ~130 kDa band, ~95 kDa band and ~60 kDa band represent S2 or S1 subunit of cleaved S protein. In the case of non-codon optimized S protein expressed from rNDV (2A-lane 2 and B-lane 1), two bands on top (~170-220 kDa) represent uncleaved S protein (S0) or polymeric forms of S2 or S1 protein and ~ 95 kDa band represents S2 or S1 subunit of cleaved S protein. In the case of rNDV /IBV- S2 (A-lane 1), there are two bands (~170-220 kDa) on top, representing polymeric forms of S2 protein, the ~105 kDa and the ~95 kDa band representing S2 subunit. Lane 4 of panel A and lane 3 of panel B represent rNDV as control. Lane 5 of panel A represents non-infected DF-1 cells. The incorporation of codon optimized S2 and S proteins and non-codon optimized S protein of IBV in NDV particles were detected by Western blot(C-upper panel). Two bands (~170-220 kDa) on top represent uncleaved S protein (S0) or polymeric forms of S2 or S1 protein (C-lane 2). The ~95 kDa and the ~60 kDa band represent S2 or S1 subunit of cleaved S protein (C-lane 2). The two bands (~170-220 kDa) on top represent polymeric forms of S2 protein, the ~105 kDa band and the ~95 kDa band represent S2 subunit(C-lane 4). The lane 1 and 3 of panel C represent purified rNDV control and purified rNDV expressing non-codon optimized S protein, respectively. A monoclonal anti-NDV/HN antibody was used to detect the 70 kDa of HN protein of NDV in DF-1 cell lysates (A-lower panel); rNDV/S2 (lane 1), rNDV/IBV-non-cod.opt.S (lane 2), rNDV/IBV- cod.opt.S (lane 3), rNDV (lane 4) and

incorporated in NDV particles (C-lower panel); rNDV (lane 1), rNDV/IBV-cod.opt.S (lane 2), rNDV/IBV-non-cod.opt.S (lane 3) and rNDV/S2 (lane 4).

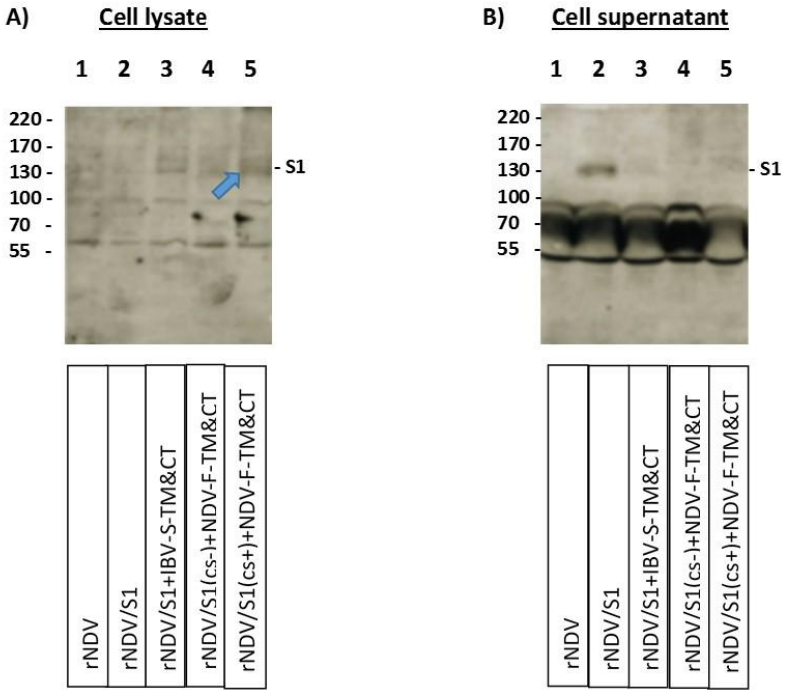


**Figure 11.** Western blot analysis of rNDV expressing S1 protein of IBV. The expression of codon optimized S1 protein of IBV expressed from four individual rNDVs expressing four different expression cassettes of S1 protein were detected using Western blot in cell lysates (A) and cell supernatant (B) of infected DF-1 cells infected with rNDVs, using a chicken polyclonal anti IBV serum . The lanes 1-5 represent cell lysates of rNDV, rNDV/S1, rNDV/S1 + IBV-S-TM&CT, rNDV/S1(cs-) + NDV-F-TM&CT, rNDV/S1(cs+) + NDV-F-TM&CT, respectively. A ~130 kD band represent expression of S1 protein by rNDV/S1+IBV-S-TM&CT, rNDV/S1(cs-)+NDV-F-TM&CT and rNDV/S1(cs+) + NDV-F-TM&CT in infected DF-1 cell lysate (A lanes

3-5) and rNDV/S1 in infected DF-1 cell supernatant ( B-lane 2). The full-length gel is presented in supplementary figure S1.

The expression of codon optimized S1 protein expressed from four individual rNDV constructs were detected by Western blot analysis in lysates (Fig.3.3A) and supernatant (Fig.3.3B) of infected DF-1 cells, using a chicken polyclonal anti IBV serum. The lanes 1-5 represent infected DF-1 cell lysates of rNDV, rNDV/S1, rNDV/S1+IBV-S-TM&CT, rNDV/S1(cs-)+NDV-F-TM&CT and rNDV/S1(cs+) + NDV-F-TM&CT, respectively. A ~130 kDa band representing expression of S1 by rNDV/S1 + IBV-S - TM&CT, rNDV/S1 (CS-) + NDV - F – TM & CT, and rNDV/S1(cs+)+NDV-F-TM&CT in lysate of DF-1 cells (Fig.3.3A-lanes 3-5) and rNDV/S1 in infected DF-1 cell supernatant (Fig.3.3B-lane 2) was observed. Our attempts to detect the incorporation of the S1 protein into NDV envelope were not successful, due to the difficulties in the detection of very low level of S1 protein by Western blot analysis (data not shown). Our results showed that the S1 protein was expressed at very low level by all the rNDVs based on Western blot analysis. Only the unmodified S1 protein was detected in the cell culture supernatant.

**Growth characteristics of rNDV constructs**

The recovered rNDVs were passaged in 9-day-old embryonated SPF chicken eggs. All the viruses were able to replicate well in eggs ($\geq 2^8$ HAU/ml). rNDV/S1, rNDV/S1(cs+)+NDV-F-TM&CT, rNDV/S2, rNDV/codon optimized-S and rNDV were evaluated in the presence of exogenous protease in DF-1 cells (Fig.3.4).

Compared to the parental virus, rNDV expressing codon optimized S protein of IBV grew slightly less efficiently. The maximum titer of parental virus reached 107.5 $TCID_{50}$/ml at 40 hours post infection, whereas the maximum titer of rNDV expressing codon optimized S gene of IBV reached 107.2 $TCID_{50}$/ml at 40 hours post infection. These results indicated that presence of S, S1 and S2 genes did not significantly affect the growth characteristics of rNDV.

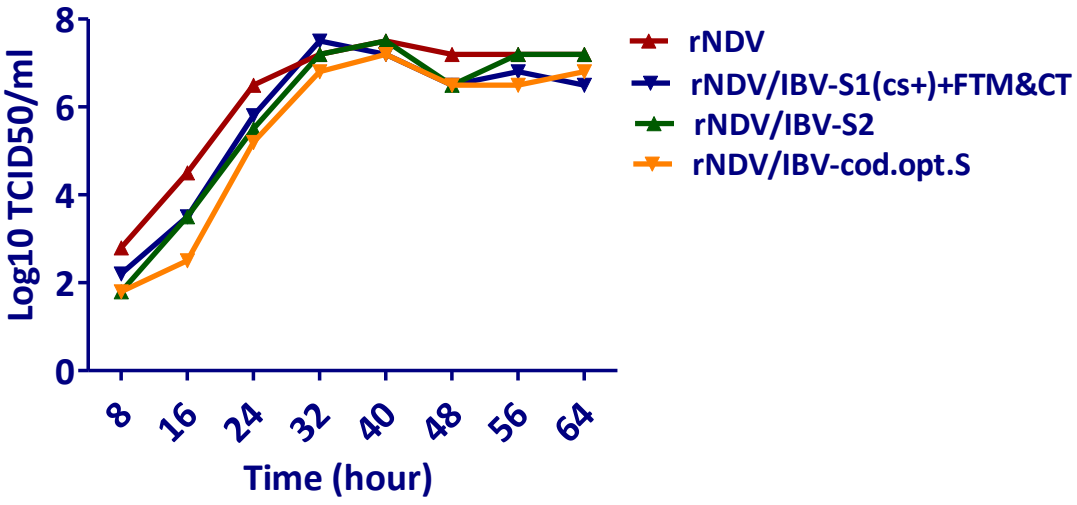


**Figure 12.** Multicycle growth kinetics of the rNDV constructs in DF-1 cells. DF-1 cells were infected with each recombinant virus at a MOI of 0.1. Two hundred µl of supernatant from infected cells were collected and replaced with fresh DMEM including 10% normal allantoic fluid at 8 hours intervals. The titer of the virus in harvested samples were determined by $TCID_{50}$ assay in DF-1cell.

**The protective efficacy of rNDVs expressing S1, S2 or S protein of IBV in chickens against a virulent IBV challenge**

IBV protection experiment 1. To evaluate the protective efficacy of rNDVs expressing S1, S2 or S protein of IBV, SPF chicks were immunized at 1-day-old age with each virus via oculanasal (ON) route. At three weeks post-immunization, chickens were challenged with virulent IBV strain Mass-41. The severity scores of IBV clinical signs were recorded twice a day for 10 days post-challenge (Fig.3.5A). Compared to chickens immunized with parental rNDV and chickens inoculated with PBS, chickens immunized with rNDVs expressing codon optimized S, S1 or S2 protein of IBV showed significantly less severe of clinical signs ($P<0.05$). Among groups of chickens immunized with rNDVs expressing codon optimized S1, S2 or S protein, the group immunized with rNDV expressing codon optimized S protein showed the least severity of clinical signs ($P<0.05$). In order to evaluate the efficacy of rNDVs expressing S1, S2 or S protein of IBV in preventing shedding of virulent IBV challenge virus in immunized chickens, on day five post-challenge , tracheal swab samples were collected from chickens of each group and were evaluated for the viral load by RT-qPCR. Our results did not show significant difference in virus shedding among groups of immunized chickens at day five post challenge (Fig.5B). However, the results of the inoculation of the tracheal swab samples into 10-day-old embrynated chicken eggs showed that 14 out of 15 (93.3%) chickens vaccinated with rNDV expressing codon optimized S protein of IBV and 0 out of 5 (0%) of non-infected chickens were shedding virus in trachea, respectively, whereas 15 out of 15 (100%) of chickens of all other groups were shedding virus in the trachea (data not shown).

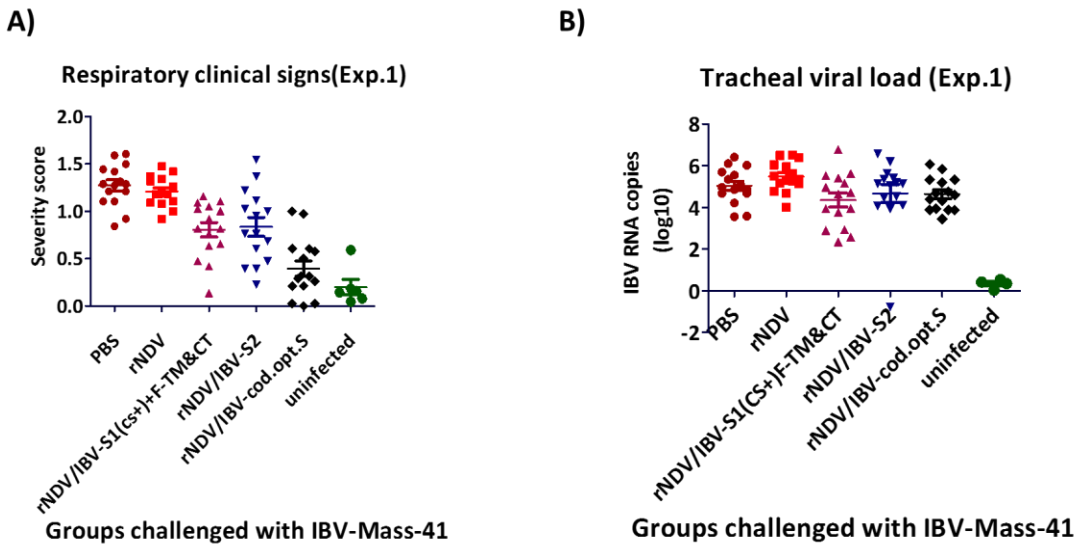


**Figure 13.** The protective efficacy of rNDV constructs in one-day-old immunized SPF chickens against virulent IBV challenge (IBV protection experiment 1) at 21 days post-immunization. A) Respiratory clinical signs of IBV following challenge with a $10^{3.1}$ $EID_{50}$ of virulent IBV strain Mass-41. The immunized chickens were challenged with a virulent IBV strain Mass-41. The severity scores of IBV clinical signs include; ocular discharge, nasal discharge and difficulty in breathing were recorded twice a day for each chicken for 10 days after challenge. The severity scores represent as average scores of clinical signs measured for each chicken over 10 days. B) Relative viral load determined by RT-qPCR in tracheal swab samples at day five following virulent IBV challenge. The relative viral load expressed as mean reciprocal ± SEM log 10.

IBV protection experiment 2. To evaluate the protective efficacy of rNDV expressing codon optimized S protein of IBV in adult chickens, SPF chickens were immunized at 4-week-old age. The protective efficacy of rNDV expressing codon optimized S gene of IBV was determined by challenging the immunized chickens with

the World Organization for Animal Health (OIE) recommended dose ($10^{3.1}$ $EID_{50}$) of virulent IBV strain Mass-41 at 3 week post-immunization1. The severity scores of IBV clinical signs were recorded twice a day for 10 days post-challenge (Fig.3.6A). Compared to chickens inoculated with PBS, chickens immunized with rNDV expressing codon optimized S protein of IBV and chickens immunized with a commercial live attenuated IBV vaccine showed significantly less severe clinical signs ($P<0.05$).

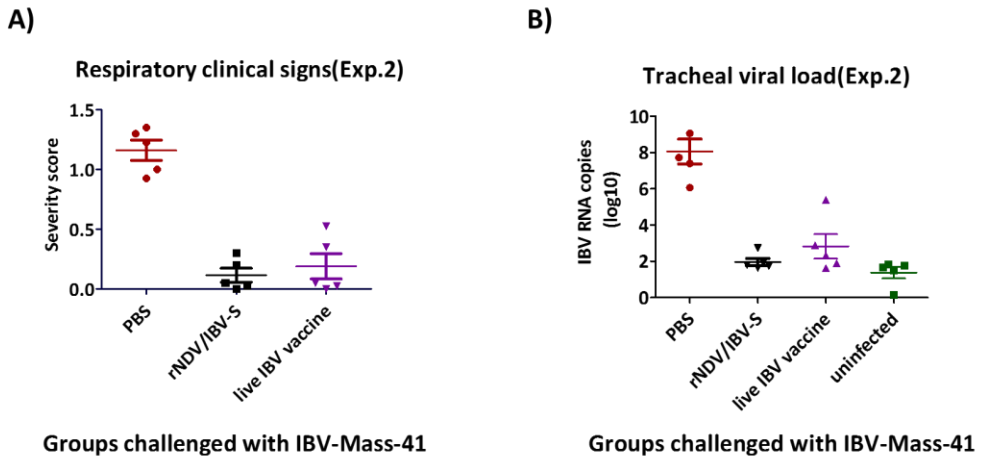


**Figure 14.** The protective efficacy of rNDV expressing codon optimized S protein of IBV against IBV challenge in immunized SPF chickens at 4-week-old age. A) Respiratory clinical signs of IBV following challenge with virulent IBV strain Mass-41. Three weeks after immunization, chickens were challenged with $10^{3.1}$ $EID_{50}$ of a virulent IBV strain Mass-41. The severity scores of IBV clinical signs were recorded twice a day for each chicken for 10 days after challenge. The severity score represents as average score of clinical signs measured for each chicken over 10 days. B) Relative

viral load determined by RT-qPCR in tracheal swab samples at day 5 following virulent IBV challenge. The relative viral load expressed as mean reciprocal ± SEM log 10.

IBV protection experiment 3. To evaluate the protective efficacy of rNDV expressing codon optimized S protein of IBV in adult chickens against a higher dose of virulent IBV challenge, SPF chickens were immunized at 4-week-old age. The protective efficacy of rNDV expressing codon optimized S gene of IBV was determined by challenging the immunized chickens with $10^{4.7}$ $EID_{50}$ virulent IBV strain Mass-41 at 3 week post-immunization. The severity scores of IBV clinical signs were recorded twice a day for 8 days post-challenge (Fig.3.7A). Compared to chickens immunized with rNDV and chickens inoculated with PBS, chickens immunized with rNDV expressing codon optimized S protein of IBV and chickens immunized with a commercial live attenuated IBV vaccine showed significantly less severe clinical signs ($P<0.05$).

In order to evaluate the efficacy of rNDV expressing S protein of IBV in preventing shedding of virulent IBV in immunized chickens, at day 5 following challenge with a virulent IBV, the tracheal swab samples collected from five chickens of each group were analyzed for the viral load by RT-qPCR. Our results showed that chickens vaccinated with rNDV expressing codon optimized S protein of IBV and chickens vaccinated with a commercial IBV vaccine showed very low levels of viral load in the trachea, whereas chickens inoculated with PBS showed high levels of viral load in the trachea ($P<0.05$). However, compared to chickens immunized with a commercial IBV vaccine, chickens immunized with rNDV expressing codon optimized S showed slightly less viral load in the trachea (Fig.3.6B).

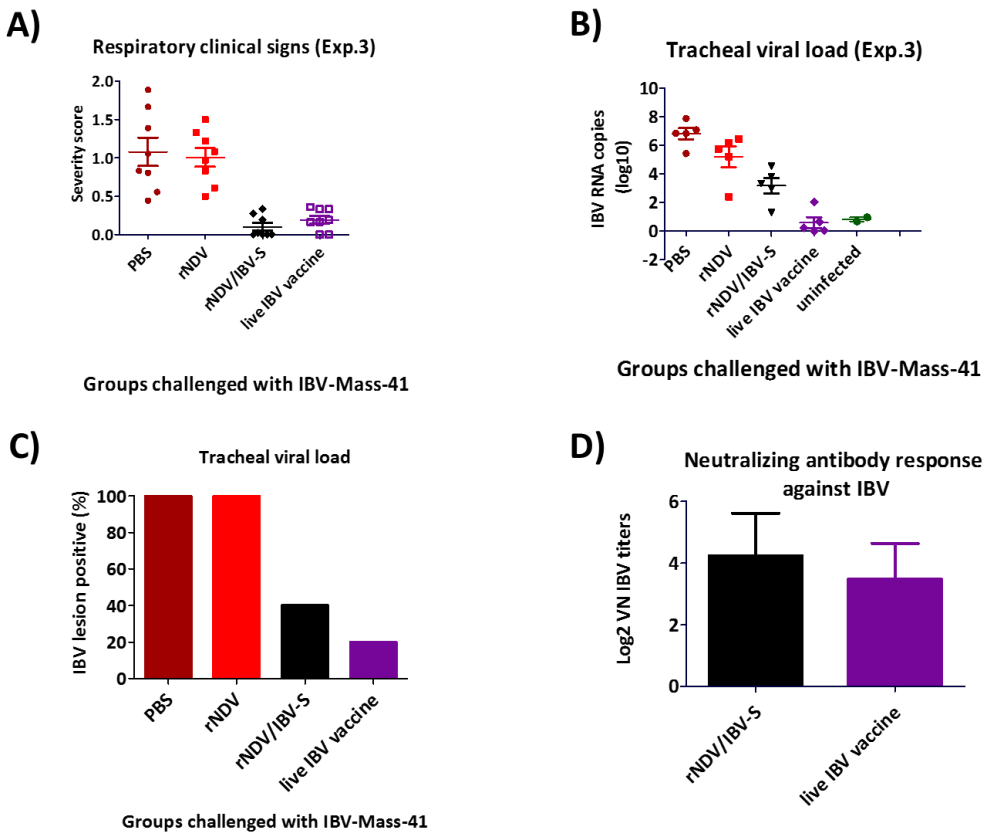


**Figure 15.** The protective efficacy of rNDV expressing codon optimized S protein of IBV and neutralizing antibody response against high dose of IBV challenge in immunized SPF chickens at 4-week-old age. A) Respiratory clinical signs of IBV following challenge with virulent IBV strain Mass-41. Three weeks after immunization, chickens were challenged with $10^{4.7}$ $EID_{50}$ of a virulent IBV strain Mass-41. The severity scores of IBV clinical signs were recorded twice a day for each chicken for 8 days after challenge. The severity score represents as average score of clinical signs measured for each chicken over the 8 days. C) Relative viral load determined by RT-qPCR in tracheal swab samples at day 4 following virulent IBV

challenge. The relative viral load expressed as mean reciprocal ± SEM log 10. D) Each tracheal fluid was tested for IBV specific lesions on chicken embryo by inoculation (0.1 ml) of one 10-day-old embryonated SPF chicken egg. D) Neutralizing antibody response against IBV. Antibodies induced against IBV were assessed using virus neutralization assay. Serum titers are expressed as reciprocals Log2 dilution.

In order to evaluate the efficacy of rNDV expressing S protein of IBV in preventing shedding of virulent IBV in immunized chickens, at days 4 following challenge with virulent IBV, the tracheal swab samples collected from five chickens of each group were analyzed for the IBV specific lesions in chicken embryo. Our results showed that 2 out of 5 (40%) chickens vaccinated with rNDV expressing codon optimized S protein of IBV and 1 out of 5 (20%) chickens vaccinated with a commercial IBV vaccine were shedding virus in trachea, respectively, whereas 5 out of 5 (100%) of chickens immunized with parental rNDV and 5 out of 5 (100%) of chickens inoculated with PBS were shedding virus in the trachea (Fig.3.7C). The tracheal swab samples collected from five chickens of each group were also analyzed for the viral load by RT-qPCR. Our results showed that chickens vaccinated with rNDV expressing codon optimized S protein of IBV showed low levels of viral load in the trachea and chickens vaccinated with a commercial IBV vaccine showed very low levels of viral load in the trachea, whereas chickens inoculated with PBS and rNDV showed high levels of viral load in the trachea. Compared to chickens immunized with rNDV expressing codon optimized S protein, chickens immunized with a commercial IBV vaccine showed less viral load in the trachea ($P<0.05$) (Fig.3.7B).

IBV protection experiment 4. To evaluate the effect of the route of inoculation of virulent IB challenge virus on the outcomes of the protective efficacy of rNDV expressing codon optimized S protein of IBV, SPF chicks were immunized at 1-day-old age. The protective efficacy of rNDV expressing codon optimized S gene of IBV was determined by challenging the immunized chickens with $10^4$ $EID_{50}$ virulent IBV strain Mass-41 by the intraocular route at 3 week post-immunization. This route of challenge has been specified in USDA-CFR-9 for IBV33. The severity scores of IBV clinical signs were recorded twice a day for 10 days post-challenge. Compared to chickens immunized with rNDV and unvaccinated chickens, chickens immunized with rNDV expressing codon optimized S protein of IBV and chickens immunized with a commercial live attenuated IBV vaccine showed significantly less severe clinical signs. However, compared to chickens immunized with commercial IBV vaccine, chickens immunized with rNDV expressing S protein showed less severe clinical signs ($P<0.05$) (Fig.3. 8A).

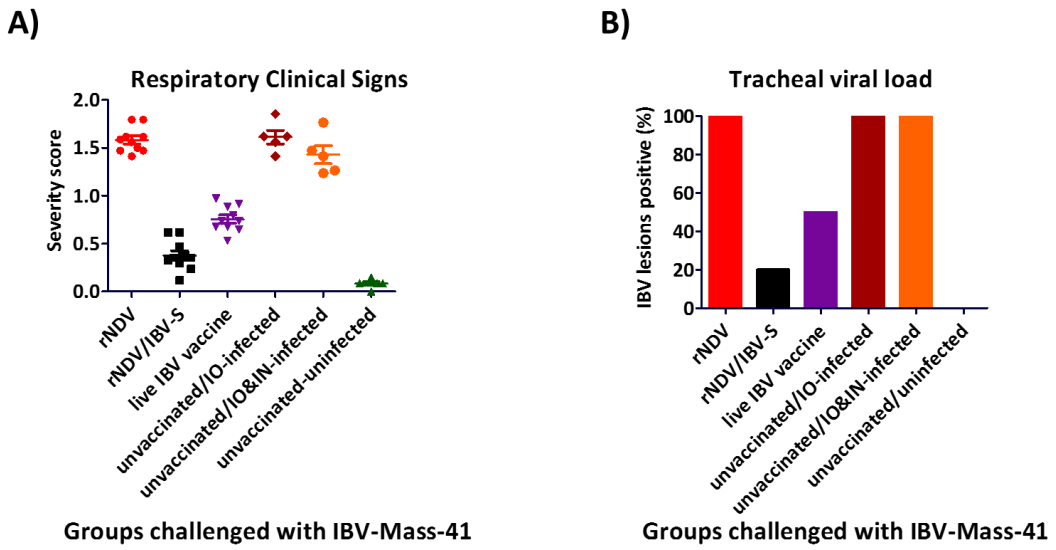

**Figure 16.** The protective efficacy of rNDV expressing codon optimized S protein of IBV against IBV challenge in immunized SPF chicks at 1-day-old age, by the intraocular challenge route. A) Respiratory clinical signs of IBV following challenge with virulent IBV strain Mass-41. Three weeks after immunization, chickens were challenged with $10^4$ $EID_{50}$ of a virulent IBV strain Mass-41 by the intraocular route. The severity scores of IBV clinical signs were recorded twice a day for each chicken for 10 days after challenge. The severity score represents as average score of clinical signs measured for each chicken over 10 days. B) Each tracheal fluid was tested for IBV specific lesions on chicken embryo by inoculation (0.2 ml) of five 10-day-old embryonated SPF chicken egg.

In order to evaluate the efficacy of rNDV expressing S protein of IBV in preventing shedding of virulent IBV in immunized chickens, at days 5 following challenge with virulent IBV, the tracheal swab samples collected from all chickens of each group were analyzed for the IBV specific lesions in chicken embryos. Our results showed that 2 out of 10 (20%) chickens vaccinated with rNDV expressing codon optimized S protein of IBV and 5 out of 10 (50%) chickens vaccinated with a commercial IBV vaccine were shedding virus in trachea, respectively, whereas 10 out of 10 (100%) of chickens immunized with parental rNDV and 5 out of 5 (100%) of unvaccinated chickens, infected with IBV, were shedding virus in the trachea (Fig.3.8B).

**The protective efficacy of rNDVs against a highly virulent NDV challenge**

To evaluate the protective efficacy of rNDV expressing S gene of IBV against a virulent NDV strain, groups of five 1-day-old chicks were immunized with rNDV, rNDV expressing codon optimized S protein and PBS. Three weeks after immunization, chickens were challenged with virulent NDV strain Texas GB in our BSL-3 plus facility. Our results showed that all chickens immunized with the rNDV and rNDV expressing codon optimized S gene of IBV survived after highly virulent NDV challenge, while all chickens in PBS group died at day 5 and 6 post-challenge (Fig.3.9A).

**Antibodies produced against IBV and NDV**

Hemagglutination inhibition (HI) assay using a standard protocol of OIE was used to assess the level of antibodies mounted against NDV in serum samples of chickens 21 days after immunization. The results showed that HI titers of NDV was detected in serum samples of all chickens immunized with rNDV and rNDV expressing codon optimized S protein. There was no significant differences observed among HI titers against NDV in serum samples of chickens from groups immunized with rNDV and rNDV expressing S protein (Fig.3.9B). Virus neutralization assay was performed according to a standard protocol of OIE to assess the level of neutralizing antibodies mounted against IBV strain Mass-41 in serum samples of chickens at 21 days after immunization. The results showed that the neutralizing antibodies against IBV were detected in serum samples of chickens immunized with rNDV expressing codon optimized S protein of IBV and with commercial live attenuated IBV vaccine (Fig.3.7D). Neutralizing antibodies against IBV were not detected in 1:8 dilution of a

serum sample from a chicken immunized with empty rNDV vector. This result showed that the rNDV expressing codon optimized S protein of IBV induces neutralizing antibodies against IBV.

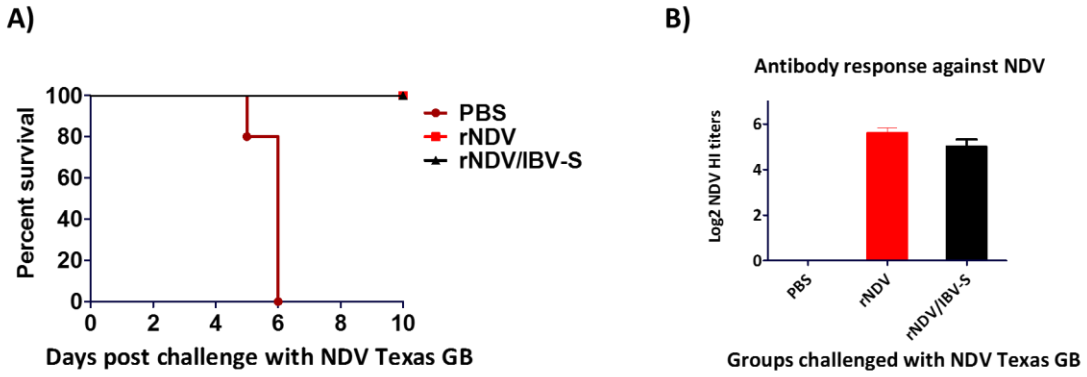


**Figure 17.** The protective efficacy of rNDVs against virulent NDV challenge and antibody responses against NDV in SPF chickens immunized at 1-day-old. A) The protective efficacy of rNDV and rNDV expressing codon optimized S gene of IBV strain Mass-41 against highly virulent NDV strain Texas GB challenge. B) HI antibody titers against NDV were assessed using HI assay. Serum titers are expressed as reciprocals of Log 2 dilution.

*Discussion*

This study was conducted to compare the protective efficacies of S1, S2, and S proteins of IBV using rNDV as a vaccine vector. The S1, S2, and S genes of IBV strain Mass-41 were individually inserted between the P and M genes of rNDV strain LaSota. This site was chosen because it has been identified as the optimal site for insertion of foreign genes into NDV genome (46,110,112,113,122). Four different versions of IBV

S1 gene were used to identify the version that is expressed at the highest level and incorporated into NDV particles. We were able to recover all the recombinant viruses and their growth characteristics were similar to rLaSota. However, the recombinant viruses containing IBV S gene grew slightly slowly than the parental virus. The viruses were stable after passages in SPF chicken embryos. Western blot analysis showed that chicken codon optimized S2 and S proteins were expressed at much higher levels and were incorporated into NDV particles. Whereas, all the four versions of S1 protein were detected at very low levels by Western blot analysis. It is noteworthy that the unmodified S1 protein was detected in the infected cell culture supernatant, indicating that the modification of S1 protein probably caused retention of the protein in the cell. These results suggest that the S2 protein acts as a chaperone to assist in the folding of the S1 protein. The S1 protein is folded incorrectly in the absence of S2 protein and the new structure probably causes loss of some conformational epitopes for IBV antibodies.

In the first IBV protection experiment, we found that 1-day-old chicks immunized with rNDV expressing the S protein of IBV conferred better protection from disease compared to 1-day-old chicks immunized with rNDVs expressing either S1 or S2 protein of IBV. Our results showed that the S protein, which contains both S1 and S2 proteins, is the best protective antigen of IBV. The S2 protein lacks major neutralizing epitopes which are present in the S1 protein, hence it is not an effective antigen. The S1 protein contains major neutralizing epitopes, but it losses some conformational epitopes when expressed separately. Although in the first study we

showed that rNDV expressing S protein provided enhanced protection, it could not reduce virus shedding, indicating that we needed to determine whether the elimination of virus shedding would probably require a much higher level of immune response than that is induced by rNDV expressing S protein or would require the optimization of the IBV protection study. In this study, our results support previous reports that rNDV vectored IBV vaccines prevent disease but do not stop virus shedding (88,89). These results also support the recent report that a spike ectodomain subunit vaccine protects chickens against IBV(124).

In the second IBV protection experiment, we investigated whether age at immunization influences the outcome of IBV challenge. Our results showed that a single immunization of 4-week-old chickens with rNDV expressing S protein completely protected chickens against IBV challenge based on disease and viral load in tracheas. Indeed, the level of protection conferred by rNDV expressing S protein was similar to that of a commercial IBV vaccine. However chickens immunized with either commercial live attenuated IBV vaccine or rNDV expressing IBV S protein showed very low levels of tracheal viral load. This showed that protection was greater when the chickens were immunized at an age when their immune system is relatively well developed.

In the third IBV protection experiment, we showed that rNDV expressing IBV S protein protects adult chickens against a higher dose of virulent IBV challenge. However, compared to standard challenge dose of virulent IBV, a higher challenge dose of virulent IBV caused higher levels of tracheal viral load in adult chickens

immunized with rNDV expressing IBV S protein and low levels of tracheal viral load in chickens immunized with commercial live attenuated IBV vaccine. Our result showed that although both the age of immunization and dose of challenge virus affect the results of IBV challenge, the influence of the age of immunization is greater than the effect of the dose of challenge virus.

Our results also showed that when we challenged adult immunized chickens with standard challenge dose of virulent IBV, rNDV expressing S protein showed slightly better protection than a commercial live IBV vaccine, based on disease and viral shedding in trachea, but when the adult immunized chickens were challenged with a higher dose of virulent IBV, commercial live IBV vaccine showed slightly better protection than rNDV expressing S protein. Hence to compare the efficacy of rNDV expressing S protein of IBV with the efficacy of live attenuated IBV vaccine, a large IBV protection study using commercial chickens is needed.

In the fourth IBV protection experiment, we showed that rNDV expressing IBV S protein protected young chickens against virulent IBV challenge by the intraocular route. The route of challenge has been recommended by USDA-CFR-9. Our results showed that compared to infection of chickens with virulent IBV by the oculanasal route, infection of chickens with virulent IBV by the intraocular route, caused much lower levels of tracheal viral load in young chickens immunized with rNDV expressing IBV S protein and low levels of tracheal viral load in chickens immunized with commercial live attenuated IBV vaccine. Our results showed that the route of the challenge virus inoculation affected the results of the tracheal virus

shedding in young chickens immunized by rNDV expressing S protein following IBV challenge; however, it did not affect the outcomes of the severity of clinical signs.

Although our studies showed that the rNDV expressing the S protein and commercial live IBV vaccine provided comparable protection, rNDV expressing the S protein has several advantages over live IBV vaccines in controlling IB in the field. i) NDV vectored IBV vaccine is highly safe in 1-day-old chicks, ii) it will not create new vaccine derived variant viruses, which is a major concern in using live modified IBV vaccines, iii) a single vaccine can be used to control both NDV and IBV, iv) we believe that the level of immunity induced by the NDV vectored vaccine against IBV is probably sufficient to completely stop IBV infection in field condition, and v) the immune response of NDV vectored vaccine can be enhanced by prime-boost vaccination strategy.

In summary, we have shown that although the S1 and S2 proteins of IBV are known to contain virus neutralizing epitopes, the presence of the whole S protein is necessary for eliciting a strong protective immune response. The S protein is the antigen of choice for any vectored IBV vaccine. NDV is an attractive vaccine vector for IBV, because it can be used as a bivalent vaccine. Our results suggest that a recombinant NDV vectored IBV vaccine is the vaccine of choice for controlling IBV infection in the field.

# Chapter 4

# Comparative protective efficacy of novel APMV-vectored vaccines against virulent IBV in chickens

*Abstract*

Infectious bronchitis is a highly contagious viral disease of poultry. It causes huge economic losses for the poultry industry worldwide. Vaccination is the most efficacious approach to control IB. However, currently used live attenuated vaccines are not safe and inactivated vaccines are not efficacious and affordable. Therefore, there is a need to develop a novel safe, efficacious and affordable vaccine against IBV. Viral vectored vaccines are desirable alternatives for conventional IBV vaccines. We have recently shown that a rAPMV-1 strain LaSota expressing the intact S protein of IBV was a promising vaccine candidate for IBV. Here, other than rLaSota vector, we sought to employ a novel chimeric rNDV/APMV-2 vector, with less efficient replication, and rLaSota-527 vector, with more efficient replication, to develop a likely improved viral vectored vaccine against IBV. Our result showed that other than rLaSota expressing S protein of IBV, the novel chimeric rNDV/APMV-2 expressing S protein, which is a better potential candidate for in ovo vaccination, provided protection against IBV in chickens. Most importantly, we also showed that compared to single immunization with rLaSota/IBV-S, over vaccination of chickens with rAPMVs /IBV-S or live IBV vaccines or single immunization of chickens with rLaSota-527/IBV-S,

with increased replication and immunogenicity, provided poorer protection against IBV. These results suggest that a single immunization of chickens with rLaSota/IBV-S, with optimal replication, induced balanced immune responses resulting to provide better protection.

*Introduction*

IB is a highly contagious viral disease of poultry (1). It is a predominantly highly infectious respiratory disease and subsidiary affects renal and reproductive tracts. Respiratory distress, poor carcass weight, substandard egg quality and decreased egg production resulting from IB cause huge economic losses for the poultry industry worldwide (1,6).

IBV, a member of the family *Coronaviridae*. The genome of IBV is nonsegmented positive-strand RNA virus of about 27.6 kilobase (kb) in length. The genome encodes five basic and four accessory genes in the constant order 5'-leader-replicase-UTR-S-M-E-N-UTR-polyA-3'. IBV is an enveloped virus and contains S, M, E and N proteins (8,9).

Vaccination is the most efficacious approach to control IB. Currently, live attenuated and inactivated vaccines are used against IB (1). However, inactivated vaccines do not elicit mucosal and strong cell mediated immunity (15,16). The costly production and application methods are other limiting factors for inactivated vaccines (7,8). The following limiting factors also are counted for live-attenuated IBV vaccines; (i) Live attenuated IBV vaccines may revert back to their origin virulence via

circulation in the field (17,18). (ii) They create new variant viruses through recombination and mutations (82,83). (iii) They may cause pathological signs in immunized birds, particularly, in young chicks (84, 85). (iv) The protective efficacy of live attenuated vaccines is not guaranteed (80). Therefore, the development of a novel safe, efficacious and affordable vaccine against IBV is required.

Viral-vectored vaccines are desirable alternatives for current IBV vaccines. Although different vectors have been used to develop novel vaccines against IBV, each viral vector poses its own advantages and disadvantages (86-90). Avian paramyxoviruses (APMVs) belong to the family *Paramyxoviridae*. To date, twenty classified serotypes and one unofficially classified serotype of paramyxoviruses have been reported (7). APMV-1 belongs to the genus *Orthoavulavirus* (7). APMV-1 viruses are classified into velogenic (highly virulent), mesogenic (moderately virulent) and lentogenic (avirulent) strains based on their pathogenicity in chickens. Velogenic and mesogenic strains cause Newcastle disease (ND) and are called NDV. APMV-1 strain LaSota is a naturally avirulent virus with restricted replication in respiratory tract (46). Not only lentogenic strains have been used as safe live vaccines against over 60 years (3), but also they have been used as vaccine vectors against several pathogens (38). In previous study (chapter3), we have shown that a rAPMV-1 strain LaSota expressing the intact S protein of IBV was a promising vaccine candidate for IBV. Like live attenuated IBV vaccines, rLaSota expressing IBV S protein infects chickens through respiratory tract resulting in eliciting strong immunity against IBV, but unlike live attenuated IBV vaccines, it is a highly safe vaccine which can overcome the

complexities and dilemmas associated with live attenuated IBV vaccines. Recently a novel chimeric NDV/APMV-2 vector in which the ectodomains of F and HN protein of NDV were replaced with ectodomains of corresponding proteins of APMV-2 was developed in our lab (51). Due to the differences in amino acids of APMV-2 and APMV-1 strain LaSota F proteins, rLaSota, rNDV/APMV-2 caused less fusion activity compared to and was less efficient in replication in vitro. It was also shown that compared to rLaSota, rNDV/APMV-2 was less pathogenic for chicken eggs embryos, due to the differences between F and HN proteins of the two viruses (51, 125). Hence these features make rNDV/APMV-2 as a potential viral vector for *in ovo* administration. Earlier another improved rAPMV-1 strain LaSota in which a Y527A mutation in the F protein cytoplasmic tail (rLaSota-527) resulted in increased fusion activity, replication and immunogenicity was developed previously (52). Therefore, in this study, other than rLaSota vector, we sought to employ rNDV/APMV-2 vector, with less efficient replication, and rLaSota-527 vector, with more efficient replication, to develop a likely improved viral vectored vaccine against IBV. We generated rNDV/APMV-2 or rLaSota-527 expressing the intact S protein of IBV. We evaluated the protective efficacy of rNDV/APMV-2, rLaSota-527 or rLaSota expressing S protein of IBV in chickens against IBV challenges. Our result showed that immunization of chicks with rNDV/APMV-2 or rLaSota expressing S protein provided comparable protection against IBV challenge. We also illustrated that compared to prime-boost immunization regimens using rNDV/APMV-2, rLaSota expressing S protein and/or live IBV vaccine, single immunization of chickens with rLaSota

expressing S protein or live attenuated IBV vaccine provided better protection against IBV challenge. Furthermore, we showed that compared to rLaSota-527 expressing S protein, immunization of chickens with rLaSota expressing S protein provided better protection against IBV challenge. Collectively, our result showed that over immunization or immunization chickens with an increased replication rLaSota expressing S protein did not provide improved protection against IBV. Whereas a single immunization with a wild type rLaSota expressing S protein elicited a balanced immune response resulting in better protection.

*Materials and Methods*

**Cells and viruses**

HEp-2 cells and DF1 cells were obtained from the ATCC (Manassas, VA) to recover recombinant viruses by reverse genetics and to in vitro characterization of recovered viruses, respectively. They were grown in DMEM containing 10% FBS. Recombinant viruses were propagated in allantoic fluids of 10-day-old embryonated SPF chicken eggs (Charles Rivers, MA). The virulent IBV strain Mass 41 was propagated in 10-day-old embryonated SPF chicken eggs. The allantoic fluid containing IBV were harvested five days after infection, centrifuged with low speed to sediment debris and the supernatant were aliquated and stored at -70°C. The titer of the stored virus was determined by $EID_{50}$. This virus stock was used to infect chickens in IBV challenge experiments.

**Generation and growth characteristics of rNDV/APMV-2 or rLaSota-527 expressing S protein of IBV**

A plasmid containing full anti-genomic cDNA of NDV strain Beaudette C (BC) in which the F and HN ectodomains of NDV were replaced with corresponded F and HN ectodomain of APMV-2 was constructed previously (51). A plasmid containing full anti-genomic cDNA of APMV-1 strain LaSota with a Y527A mutation in cytoplasmic tail of F protein also was constructed previously (52). Here we inserted a transcription cassette containing the intact S gene of IBV strain Mass-41 (GenBank Accession no. AY851295.1) between P and M genes of NDV/APMV-2 and LaSota-527 using *PmeI* restriction enzyme site. The correct sequence of flanking region was confirmed by sequence analysis. The transcription cassette contained the following sequences in a 3' to 5' order; *PmeI* restriction enzyme site, 15 nucleotides of NDV UTR, GE of P gene of NDV, one T nucleotide as IGS, GS, Kozak, ORF of the S gene of IBV and *PmeI* restriction enzyme site. The ORF of S gene of IBV strain Mass-41 was codon optimized for higher expression in chicken cells (GenScript; optimization on Gallus Gallus codons using OptimumGene TM PSO algorithm). The rNDV-APMV-2 or rLaSota-527 expressing S protein of IBV were recovered from cDNAs using reverse genetics as described previously (109). The recovered rNDV-APMV-2 expressing S protein of IBV and rLaSota-527 expressing S protein of IBV were named rNDV/APMV-2/IBV-S and rLaSota-527/IBV-S, respectively. The rNDV-APMV-2/IBV-S and rLaSota-527/IBV-S were passaged in 10-day-old embryonated eggs. The viruses with high HA titers were harvested and stored at -70°C in vials of aliquots. A

rLaSota expressing S protein of IBV (rLaSota/IBV-S) which was generated previously (chapter 3) also was used in this study (126). In two separate experiment, the plaque morphology of rNDV-APMV-2/IBV-S or rLaSota-527/IBV-S in comparison with rLaSota/IBV-S were assayed in DF1 cells under DMEM containing 0.8% methylcellulose and 10% fresh allantoic fluid over layer.

**Expression of S protein by rNDV-APMV-2/IBV-S and rLaSota-527/IBV-S**

The expression of S protein by rNDV-APMV-2/IBV-S or rLaSota-527/IBV-S in comparison with the expression of S protein by rLaSota/IBV-S were assessed by two separate experiments. DF1 cells were infected with rLaSota, rLaSota/IBV-S, rLaSota-527/IBV-S or rNDV-APMV-2/IBV-S in the presence of 10% allantoic fluid. The DF-1 cell lysates at similar level of infections were collected 30 hours after infection. The expression of S protein in cell lysates were detected by Western blot analysis using a chicken polyclonal anti IBV serum.

**The protective efficacy of rNDV-APMV-2 or rLaSota expressing S protein against IBV challenge in SPF chicke**ns

Total number of 34 day old SPF chicks, obtained from Charles Rivers (Charles Rivers MA) were divided in three groups (groups 1-3) of ten chicks and one group of four chicks (group 4). The chicks of groups one and two were immunized with $10^6$ $EID_{50}$, 200 µl in volume, of rNDV-APMV-2/IBV-S and rLaSota/IBV-S through oculonasal route, respectively. The chicks of group three were inoculated with 200 µl phosphate normal saline (PBS) through oculonasal route and chicks of group four were

left uninfected. Four weeks after immunization, all birds of groups one to three were infected with $10^4$ $EID_{50}$ ,200 µl in volume, of virulent IBV strain Mass-41 through eye drop route and birds of group four were left uninfected. The severity scores of clinical signs of IBV including, nasal discharge, ocular discharge and difficulty in breathing (0 = normal, 1= presence of mild ocular discharge, mild nasal discharge and or sneezing 2= presence of heavy ocular discharge and or heavy nasal discharge with mild tracheal rales and mouth breathing and or coughing 3= heavy ocular discharge and heavy nasal discharge with sever tracheal rales and mouth breathing, gasping, dyspnea and or severe respiratory distress) were recorded twice a day for 10 days post-challenge. At day five post-challenge, tracheal swab samples were collected from all chickens and placed into 3 ml cold DMEM containing 10X antibiotics and amphotericin B. The viral RNA were quantified in swab samples by an IBV-N gene-specific RT-qPCR, as described previously (126).

**The protective efficacy of rNDV-APMV-2 or rLaSota expressing S protein against IBV challenge in broilers**

Total number of 48 day-old broilers were divided in eight groups, six each. In a single or prime-boost immunization (prime at day one and boost at week three of age), chicks were immunized with $2^7$ HAU, 200µl in volume, of rNDV-APMV-2/IBV-S, rLaSota/IBV-S expressing S protein and/or recommended dose, 200µl in volume, of a live attenuated IBV vaccine by the oculonasal route based on the immunization regimen for each group listed in table 1. At week six of age (six weeks-post prime immunization and three weeks post-boosting), birds were infected with $10^{5.5}$ $EID_{50}$ of

virulent IBV strain Mass-41 by the oculonasal route. The severity scores of clinical signs of IBV and post-challenge viral load were assessed as described above.

**The protective efficacy of rLaSota-527 or rLaSota expressing S protein against IBV challenge in SPF chicke**ns

Total number of 20 4-week-old SPF chicks, obtained from Charles Rivers (Charles Rivers MA) were divided in four groups with five chickens. Chickens of groups one and two were immunized with $2^7$ HAU, 200 µl in volume, of rLaSota-527/IBV-S and rLaSota/IBV-S, respectively, through oculonasal route. Chickens of group three were immunized with recommended dose, 200 µl in volume, of a live attenuated IBV vaccine through oculonasal route. Chicks of group four were inoculated with 200 µl PBS through oculonasal route. Three weeks after immunization, all birds were infected with $10^{3.1}$ $EID_{50}$, 200 µl in volume, of virulent IBV strain Mass-41 through oculanasal route. The severity scores of clinical signs of IBV and post-challenge viral load were assessed as described above. The tracheal swab samples taken from uninfected SPF chickens involved in another IBV protective experiment were used in this experiment as uninfected controls.

All animal experiments involving were performed in our USDA approved Biosafety level-2 and Biosafety level-2+ facilities following the guidelines and approval of the Animal Care and Use Committee (IACUC), University of Maryland.

**Statistical analysis**

Data were analyzed among groups by One-Way-ANOVA test. The t-test was used to compare two groups. To avoid bias, IBV animal experiments were designed as blinded studies.

**Table 1.** The groups of broilers immunized in single or prime-boost regimens.

| **Groups** | **Prime-immunization** | **Boost-immunization** |
|---|---|---|
| **1** | PBS | PBS |
| **2** | rNDV-APMV-2/IBV-S | - |
| **3** | rLaSota/IBV-S | - |
| **4** | rNDV-APMV-2/IBV-S | rLaSota/IBV-S |
| **5** | rLaSota/IBV-S | rNDV-APMV-2/IBV-S |
| **6** | rLaSota/IBV-S | rLaSota/IBV-S |
| **7** | - | rLaSota/IBV-S |
| **8** | - | live IBV Vaccine |

*Result*

**Generation and growth characterization of rNDV/APMV-2 or rLaSota-527 expressing S protein of IBV**

The transcription cassette containing S gene of IBV was inserted between P and M genes in two individual plasmids containing cDNA of full length antigenomic RNA of NDV-APMV-2 or LaSota-527. rNDV-APMV-2 expressing IBV S protein (rNDV-APMV-2/IBV-S) and rLaSota-527 expressing IBV S protein (rLaSota-527/IBV-S)

were recovered from cDNAs successfully. A rLaSota expressing S protein of IBV (rLaSota/IBV-S) which we generated previously also was used in the in vitro and in vivo experiments of this study (126). The recovered viruses replicated in 10-day-old embryonated SPF chicken eggs efficiently. The plaque morphology assay showed that either rNDV-APMV-2 or rNDV-APMV-2/IBV-S did not form visible plaque in DF1under DMEM containing 0.8% methyl cellulose over layer (Fig. 4.1C). Compared to rLaSota/IBV-S, rLaSota-527/IBV-S formed slightly larger plaques in DF1 cells (Fig.4.1D).

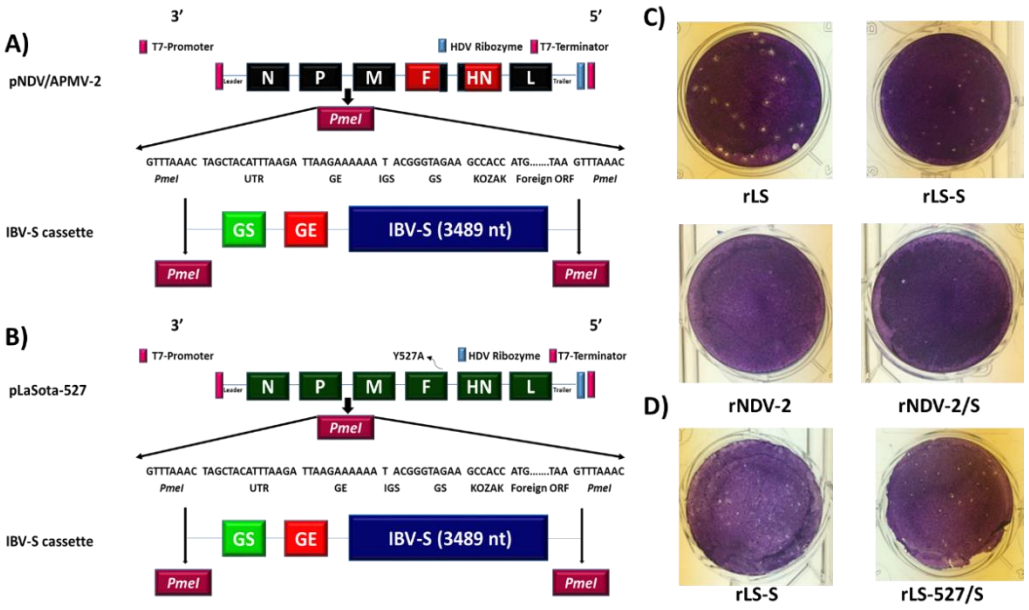


**Figure 18.** The schematic diagram for the construct and plaque morphology of rNDV-APMV-2 or rLaSota expressing the S protein of IBV. A plasmid containing cDNA of full genomic RNA of NDV strain Beaudette C (BC) in which its F and HN ectodomains were replaced with corresponded F and HN ectodomain of APMV-2 was constructed previously. A transcription cassette containing codon optimized S gene of IBV strain

Mass-41 was inserted between P and M genes of NDV using *PmeI* restriction enzyme site (A). A plasmid containing cDNA of full genomic RNA of APMV-1 strain LaSota with Y527A mutation in cytoplasmic tail of its F gene was constructed previously. The above transcription cassette containing codon optimized S gene of IBV strain Mass-41 was inserted between P and M genes of LaSota-527 using same strategy (B). The transcription cassette of S gene contains the following sequences in a 3' to 5' constant order: *PmeI* restriction enzyme site, 15 nucleotides of NDV UTR, GE of NDV P gene, one T nucleotide IGS, GS of NDV M gene for S gene, Kozak, IBV S gene ORF and *PmeI* restriction enzyme site. The plaque morphology of rNDV-APMV-2/IBV-S in comparison with plaque morphology of rLaSota/IBV-S in DF1cells (C). The plaque morphology of rLaSota-527/IBV-S in comparison with plaque morphology of rLaSota/IBV-S in DF1cells (D).

**Expression of the S protein of IBV by rNDV-APMV-2 or rLaSota-527**

The expression of S protein of IBV by rNDV-APMV-2/IBV-S or rLaSota-527/IBV-S was detected comparatively with the expression of S protein by rLaSota/IBV-S using Western blot analyses. Similar to rLaSota/IBV-S, either rNDV-APMV-2/IBV-S (Fig.4.2A) or rLaSota-527/IBV-S (Fig.4.2B) expressed the S protein at high level. For the S protein of IBV expressed from rNDV-APMV-2/IBV-S (Fig.4.2A lane 2), rLaSota-527/IBV-S (Fig.4.2B lane 4) or rLaSota/IBV-S (Fig.4.2A lane1 and Fig.4.2B lane3) the bands on top (~170-220 kDa) represent either uncleaved S protein (S0) or polymeric forms of S protein. The ~130 kDa, the ~95 kDa and the ~60 kDa band represent S1 or S2 subunit of cleaved S protein of IBV.

**The protective efficacy of rNDV-APMV-2/IBV-S and rLaSota/IBV-S against IBV challenge in SPF chickens**

In order to evaluate the comparative protective efficacy of rNDV-APMV-2/IBV-S and rLaSota/IBV-S against IBV, two-day old SPF chicks were immunized with $10^6$ $EID_{50}$ of rNDV-APMV-2/IBV-S or rLaSota/IBV-S and four weeks post-vaccination they were challenged with $10^4 EID_{50}$/bird. Compared to non-immunized chicks, both rNDV-APMV-2/IBV-S and rLaSota/IBV-S provided significant and comparable protection against clinical signs of IBV ($P<0.05$) (Fig.4.3A). However neither rNDV-APMV-2/IBV-S nor rLaSota decreased the post-challenge viral load in trachea (Fig.4.3B).

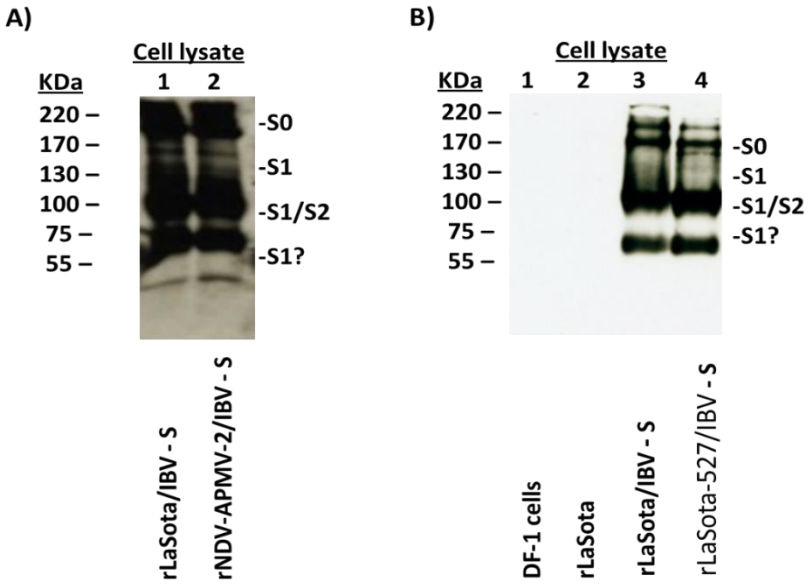


**Figure 19.** The expression of S protein of IBV by rNDV-APMV-2/IBV-S or rLaSota-527/IBV-S. The expression of S protein by rNDV-APMV-2/IBV-S in comparison with rLaSota/IBV-S was detected in DF1 cells by Western blot analysis using a chicken

polyclonal anti IBV serum (A). Lanes 1 and 2 represent rLaSota/IBV-S and rNDV-APMV-2/IBV-S, respectively. The expression of S protein by rLaSota-527/IBV-S in comparison with rLaSota/IBV-S also was detected in DF1 cells by Western blot analysis using above serum (B). Lanes 1, 2, 3 and 4 represent DF1 cells, rLaSota, rLaSota/IBV-S and rLaSota-527/IBV-S, respectively.

The antibodies against LaSota and rNDV-APMV-2 were assessed by HI assay using the protocol of OIE (3). The rNDV-APMV-2/IBV-S and rLaSota/IBV-S induced antibodies against rNDV-APMV-2 and rLaSota, respectively. However compared to rNDV-APMV-2/IBV-S, rLaSota/IBV-S induced slightly higher HI titers (Fig.4.3C)

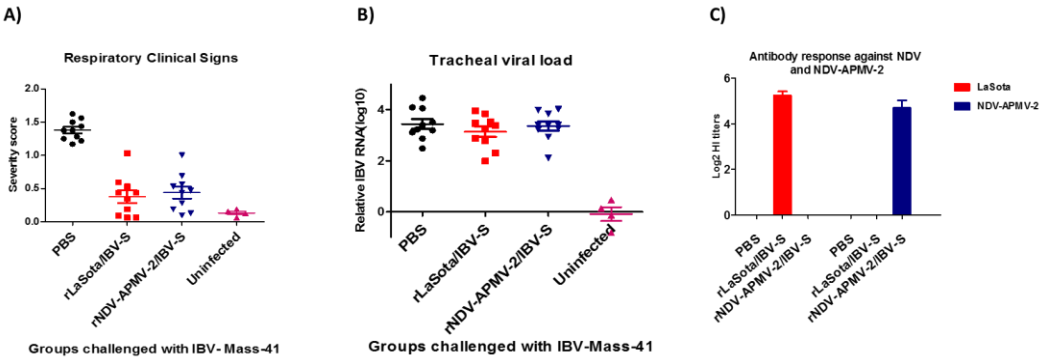


**Figure 20.** The protective efficacy or rNDV-APMV-2/IBV-S or rLaSota/IBV-S against IBV challenge in SPF chickens. Two-day old chicks were immunized and four weeks after immunization, they were infected with IBV. The clinical signs of IBV were recorded twice a day for 10 days post-challenge. Each symbol represents mean scores of 10 days clinical signs for individual birds and horizontal lines represent mean scores of each group, respectively (A). At day five post challenge tracheal swabs were collected and the IBV RNA load in each sample were quantified by RT-qPCR. Each

symbol represents the number of IBV RNA copies for individual tracheal swab samples and horizontal lines represent mean RNA load of each group, respectively (B). Four weeks after immunization, the sera were collected from all chickens and the antibodies against rNDV-APMV-2 and LaSota were detected by HI assay. Serum titers are expressed as reciprocals of Log2 dilution.

**The protective efficacy of rNDV-APMV-2/IBV-S and rLaSota/IBV-S against IBV challenge in broilers**.

Eight groups of broilers were immunized with single or prime-boost immunization regimens listed in table 1. At the age of six weaks, chickens were infected with $10^{5.5}$ $EID_{50}$/bird of virulent IBV. The results showed that the immunization of chickens with rLaSota/IBV-S, rNDV-APMV-2/IBV-S or live IBV vaccine, no matter which regimens of immunization, reduced the post-challenge IBV clinical signs, significantly ($P<0.05$). However, immunization of broilers with rLaSota/IBV-S or live IBV vaccine at week three of age provided better protection against IBV than the other groups. Compared to rLaSota/IBV-S, single immunization of chicks with rNDV-APMV-2/IBV-S at day one of age provided less protection.

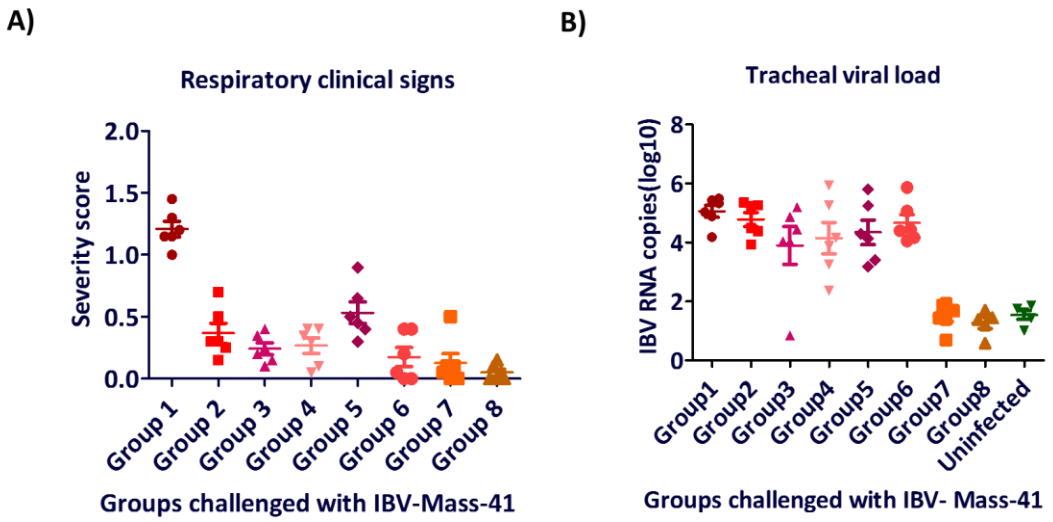


**Figure 21.** The protective efficacy of rNDV-APMV-2/IBV-S and rLaSota/IBV-S against IBV challenge in broilers. Chickens were immunized at day one and/or week three of age based on the groups listed in table-1 and were infected with IBV at week six of age. The clinical signs were recorded twice a day for 10 days post-challenge. Each symbol represents mean scores of the clinical signs for individual birds and horizontal lines represent mean scores of each group (A). At day five post challenge tracheal swabs were taken and the IBV RNA load in each sample were quantified by RT-qPCR. Each symbol represents the number of IBV RNA copies for individual tracheal swab samples and horizontal lines represent mean RNA load of each group (B).

A booster immunization with rNDV-APMV-2/IBV-S following prime immunization with rLaSota/IBV-S provided less protection compared to reverse prime-boost regimen of immunization (Fig.4.4A). Single immunization of chickens with

rLaSota/IBV-S or live IBV vaccine at week three of age reduced post-challenge tracheal viral load significantly($P<0.05$), whereas single immunization of chickens at day one of age and prime-boost immunization regimens did not decreased post-challenge viral load (Fig.4.4B). Antibody titers against rNDV/APMV-2 and/or rLaSota were detected in corresponding immunized groups of chickens by HI assay using the protocol of OIE (3). However homologous prime-boost immunization of chickens with rLaSota/IBV-S induced the highest HI titers against rLaSota (Fig.4.5).

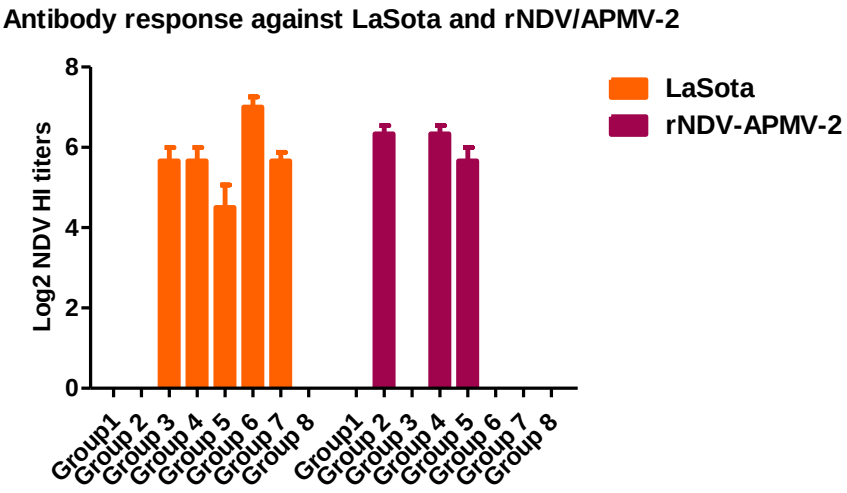


**Figure 22.** The antibodies induced against rNDV-APMV-2 and rLaSota in broilers. Chickens were immunized at day one and/or week three of age based on the groups listed in table1 and were infected with IBV at week six of age. At week six of age the sera were collected from all chickens and the antibodies induced against rNDV-APMV-2 and LaSota were detected by HI assay. Serum titers are expressed as reciprocals Log2 dilution.

**The protective efficacy of rLaSota/IBV-S and rLaSota-527/IBV-S against IBV challenge in SPF chickens**

In order to evaluate the comparative protective efficacy of rLaSota-527/IBV-S and rLaSota/IBV-S against IBV, four week-old SPF chickens were immunized with rLaSota-527/IBV-S, rLaSota/IBV-S or a live IBV vaccine. Three weeks post-immunization, chickens were infected with virulent IBV. Immunization of chickens with rLaSota/IBV-S, rLaSota-527/IBV-S or live IBV vaccine were provided protection against clinical signs of IBV ($P<0.05$) (Fig.4. 6A). The viral genome in trachea of unimmunized chickens were detected at high level at days 4, 5 and 6 post-challenge and low level at day three post challenge. Compared to unimmunized chickens, immunization of chickens with rLaSota-527/IBV-S, decreased 2 log10 viral genome load in trachea at day 4 and 5, and 6 log 10 at day 6 after IBV challenge. Whereas a low or very low (negligible) levels of IBV genome were detected at day five post-challenge in trachea of chickens immunized with a live IBV vaccine or rLaSota/IBV-S, respectively ($P<0.05$) (Fig.4.6B). The antibodies induced against LaSota were assessed by haemagglutination inhibition (HI) assay using the protocol of OIE (3). Compared to rLaSota/IBV-S, rLaSota-527/IBV-S induced slightly higher antibody HI titers against LaSota (Fig.4.6C).

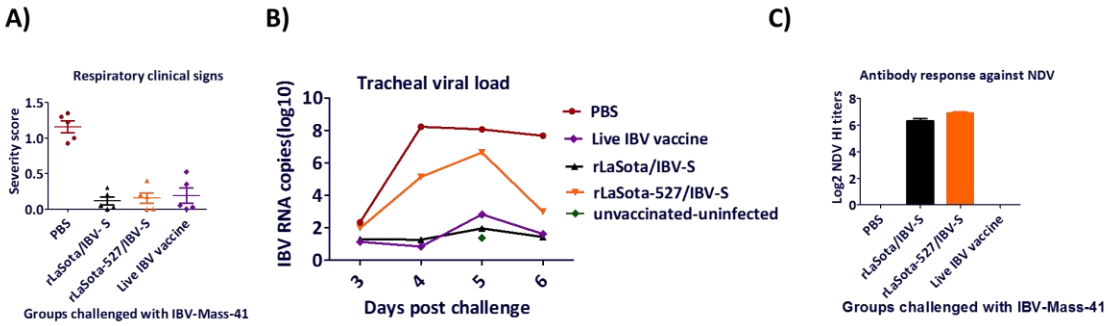


**Figure 23.** The protective efficacy of rLaSota-527/IBV-S or rLaSota/IBV-S against IBV challenge in SPF chickens. Four week-old chickens were immunized and three weeks after immunization, they were infected with IBV. The clinical signs of IBV were recorded twice a day for 10 days post-challenge. Each symbol represents mean scores of 10 days clinical signs for individual birds and horizontal lines represent mean scores of each group, respectively (A). At days three to six post- challenge tracheal swabs were taken and the IBV RNA load in each sample were quantified by RT-qPCR. Each symbol represents mean Log10 IBV RNA of each group (B). Three weeks after immunization the sera were collected from all chickens and the antibodies induced against LaSota were detected by HI assay. Serum titers are expressed as reciprocals of Log 2 dilution (C).

*Discussion*

The control of IBV without vaccination is not effective (1). The currently used IBV vaccines are not safe and efficacious (127). Therefore, there is a need to develop improved vaccines. Viral vectored vaccines are desirable alternatives for current IBV vaccines. Several viral vectors have been investigated to develop vaccines against IBV

(86-90). However, to develop viral vectored vaccines against IBV, the advantages and disadvantages of each vector should be taken in consideration. In our previous study, we showed that a recombinant APMV-1 strain LaSota expressing the intact S protein of IBV protects chickens against IBV challenge (126). Therefore, APMVs are promising vectors to develop vaccines against IBV. Here we employ a chimeric NDV/APMV-2 vector, with decreased replication in chicken, and a modified rLaSota, with increased replication in chicken, to express the intact S protein of IBV. We evaluated the protective efficacy of rNDV-APMV-2/IBV-S or rLaSota-527/IBV-S in comparison with rLaSota/IBV-S in chickens against IBV challenge.

Our result showed that rNDV/APMV-2/IBV-S did not develop plaque in DF1 cells and compared to rLaSota/IBV-S, rLaSota-527/IBV-S formed a larger plaque size in DF1 cells. This result was not unexpected, because the monobasic cleavage site motif of F protein of APMV-2 caused rNDV/APMV-2 not to form visible plaque in DF1cells (46, 51, 125). Previous study also showed that compared to rLaSota, rLaSota-527 formed a larger plaque size in DF1 cells (52). Similar to rLaSota/IBV-S, both rNDV-APMV-2/IBV-S and rLaSota-527/IBV-S expressed S protein at high levels suggesting that the intact S protein of IBV strain Mass-41 is expressed very well from APMVs (using the same transcription cassette strategy), and it is not affected by the changing of APMV vector, significantly.

Immunization of SPF chicks with rNDV-APMV-2/IBV-S and rLaSota/IBV-S provided comparable protection against IBV clinical signs. It was shown by Cavangah et al (1988) that amino acids with in hypervariable region (S1 subunit) of IBV spike

protein are responsible for neutralizing epitopes (13). We previously showed that although S1 subunit contain major neutralizing epitopes, the interaction of S1 and S2 subunits in intact S protein is required for expressing the neutralizing epitopes in correct conformation. The neutralizing epitopes within S1 subunit were expressed in correct conformation only in the intact S protein (126). Therefore, immunization of chickens with rAPMV-1 strain LaSota or rNDV-APMV-2 expressing S protein was sufficient to protect chickens from IBV disease, in consistent with our previous study (126). In this IBV protective experiment, neither rLaSota/IBV-S nor rNDV-APMV-2/IBV-S decreased the post-challenge viral load, significantly. However, compared to unimmunized chickens, immunization of chicks with rLaSota/IBV-S decreased post-challenge viral load slightly, but it was not significant. This was because of the non-optimized immunization/challenge IBV study: (i) Immunization of day-old chicks with low dose of vaccines, then infecting the immunized birds with high dose of virulent IBV challenge at four weeks post-immunization, and/or (ii) detection of challenge virus genome in trachea with a highly sensitive method (RT-qPCR), compared to re-isolation of challenge virus in eggs are the possible reasons for detection of high level of post-challenge virus. Compared to rLaSota/IBV-S, rNDV-APMV-2/IBV-S induced slightly lower HI titers against corresponded backbone virus. In consistent with previous studies that showed that APMV-2 or rNDV/APMV-2 replicated less efficiently than LaSota, this result also suggests that compared to rLaSota/IBV-S, rNDV-APMV-2/IBV-S replicates less efficiently (49,51).

Similar to live IBV vaccine, single immunization of broilers at three weeks of age reduced post-challenge IBV genome in trachea of chickens, significantly. But single immunization or prime-boost immunization (heterologous or homologous regimens) with rLaSota/IBV-S or/and rNDV-APMV-2/IBV-S did not reduced post-challenge tracheal IBV RNA load at week six of age. These results suggest that not only over vaccination with rAPMV/IBV-S did not improve the challenge outcomes, but, surprisingly, increased the post-challenge clinical signs and tracheal viral load. We also observed that compared to single immunization, prime-boost immunization of SPF chickens with a live IBV vaccine provided poorer protection (unpublished). This phenomenon was likely happened because of the high titer of antibodies induced against non-neutralizing epitopes within the S protein of IBV and/or because of the negative effect of the over infection of respiratory tract on the development of balanced immune responses against IBV. Previously it was shown that coronaviruses infections may cause antibody dependent enhancement (ADE) (128). A study also showed that the spike protein of sever acute respiratory syndrome (SARS) coronavirus triggers ADE (129). Here, prime-boost immunization with rLaSota/IBV-S or live IBV vaccine induced most likely higher titers of antibodies against both neutralizing and non-neutralizing epitopes of S protein. Although a higher neutralizing antibodies were produced by prime-boost immunization, blocking a high dose of highly infectious IBV particles by neutralizing antibodies was not feasible easily. Therefore, on the other hand, the high titers of non-neutralizing antibodies enhanced the infection of respiratory tract cells by the free IBV particles. It also was likely that compared to

prime-boost immunization, a single immunization through respiratory tract route provided much balanced and appropriate activation of immune compartments leading to an efficient prevention of post-challenge viral transmission. Therefore, these results suggest that prime boost immunization with rAPMVs/IBV-S or live IBV vaccines induces higher immune responses, but not necessarily provides better protection outcomes. Over vaccination of chickens with rAPMVs/IBV-S or live IBV vaccines may cause ADE and/or may elicit an imbalance immune response in respiratory pathway.

Single immunization of broilers with rNDV-APMV-2/IBV-S or rLaSota/IBV-S at day one of age or week three of age provided protection against a high dose of IBV challenge. However compared to single immunization at day one of age, immunization of three week-old broilers with rLaSota/IBV-S provided better outcomes, in protecting from disease and preventing viral shedding. This results suggest that i) in consistent with our previous study (24), compared to young chicks, immunization of adult chickens with rLaSota/IBV-S provided better protection, due to maturation of immune system, ii) the longer interval was between immunization and challenge, the poorer protection was provided post challenge. The interval between immunization and challenge is a factor that affects the challenge outcomes (13). By the time the level of humoral antibodies against IBV may increase, however other compartments of immune responses may decrease. The antibodies also may increase the infectivity of virulent virus, due to ADE. Therefore, by increasing the time distant between immunization and challenge, the rLaSota/IBV-S provided less protection.

Moreover, our results showed that compared to rLaSota-527/IBV-S, with increased replication and immunogenicity, immunization of SPF chickens with rLaSota/IBV-S, with optimal replication and immunogenicity, provided better protection. Compared to rLaSota/IBV-S, immunization of chickens with rLaSota-527/IBV-S, similar to prime-boost vaccination (described above), may cause ADE and/or may induce imbalanced immune responses resulting to provide poorer protection.

In conclusion, in this study we showed that other than rLaSota expressing S protein of IBV, the novel rNDV/APMV-2 expressing S protein, which is a better potential candidate for in ovo vaccination, provided protection against IBV in chickens. Most importantly, we also showed that compared to single immunization with rLaSota/IBV-S, over vaccination of chickens with rAPMVs /IBV-S or live IBV vaccines or single immunization of chickens with rLaSota-527/IBV-S, with increased replication and immunogenicity, surprisingly, provided poorer protection against IBV. These results suggest that a single immunization of chickens with rLaSota/IBV-S, with optimal replication, induced much balanced immune responses in respiratory tract against IBV.

# Chapter 5

# Contributions of HA1 and HA2 subunits of HPAIV in induction of neutralizing antibodies and protection in chickens

*Abstract*

Highly pathogenic avian influenza virus subtype H5N1 causes a devastating disease in poultry. Vaccination is an effective method of controlling avian influenza virus infection in poultry. The HA protein is the major determinant recognized by the immune system of the host. Cleavage of the HA precursor HA0 into HA1 and HA2 subunits is required for infectivity of the avian influenza virus. We evaluated the individual contribution of HA1 and HA2 subunits to the induction of HPAIV serum neutralizing antibodies and protective immunity in chickens. Using reverse genetics, rNDVs were generated, each expressing HA1, HA2, or HA protein of H5N1 HPAIV. Chickens were immunized with rNDVs expressing HA1, HA2, or HA. Immunization with HA induced high titers of serum neutralizing antibodies and prevented death following challenge. Immunization with HA1 or HA2 alone neither induced serum neutralizing antibodies nor prevented from death following challenge. Our results suggest that interaction of HA1 and HA2 subunits is necessary for display of epitopes involved in the induction of neutralizing antibodies and protection on intact HA molecule. These epitopes are lost when the two subunits are separated. Therefore, vaccination with either HA1 or HA2 may not provide protection against HPAIV.

*Introduction*

Avian influenza (AI) is an important disease of poultry. Avian influenza virus (AIV) belongs to genus *Alphainfluenzavirus* in the family *Orthomyxoviridae* (7). AIVs are divided into subtypes based on antigenic differences of the two major surface glycoproteins, HA and NA (19). Currently, 16 H subtypes (H1-H16) and 9 N subtypes (N1-N11) in different combinations have been found in avian populations. Of the 16 H subtypes, H5 and H7 subtypes have high pathogenicity in poultry (2,19). Furthermore, zoonotic transmissions of AIV subtypes H5 and H7 have caused multiple outbreaks of severe disease in humans (21,22).

Vaccination is considered a practical method to control AI in endemic countries (2,23). However, live vaccines against AIV are not recommended for use in poultry, because of the potential risk of genome segment reassortment between vaccine and field strains that can lead to emergence of viral variants with different antigenic properties (2). The vast majority of AIV vaccines (95.5%) currently used in the field are inactivated vaccines (23). However, they do not elicit strong immune responses. The process of production and administration of this type of vaccines also are not economical and practical. Furthermore, vaccination programs with inactivated vaccines cause difficulty to differentiate vaccinated birds from infected ones that can result in trade restrictions (24,25). Therefore, viral vectored vaccines containing the HA protein of HPAIV have been developed and licensed in some countries (2,31).

The HA is an important multifunctional protein of influenza virus. The HA protein mediates binding of the virus to host cell receptor. It is the major antigen against

which neutralizing antibodies are induced during infection (19). Each HA monomer is synthesized as an inactive precursor protein HA0. The cleavage of HA0 protein into HA1 and HA2 subunits by a cellular protease is required for viral infectivity (34,35). Following cleavage, the HA1 and HA2 subunits are held together by disulfide bonds. The HA1 binds to sialic acid receptors on cell surface and initiate virus entry by endocytosis. Due to acidic pH-induced conformational change of HA2 in endosome, the fusion of viral and endosomal membranes occurs (19). The HA1 subunit, which forms the globular head, is highly variable among subtypes and contains major neutralizing epitopes. The HA2 subunit, which forms most of the stalk domain, is considerably conserved among subtypes and contains minor but cross-reactive neutralizing epitopes (19,36,37).

The presence of subtype-specific neutralizing epitopes on HA1 subunit and the presence of broad neutralizing epitopes on HA2 subunit make them potential candidates to develop subtype-specific and universal vaccines against influenza viruses, respectively (19,37). To date, several head-based and stalk-based vaccines against human influenza viruses have been evaluated in mice and/or ferret models, but an improved vaccine is not yet available (12,41,43, 130-133). Some promising outcomes from mice and/or ferret model challenge studies keep these approaches attractive. Although mice and ferrets represent invaluable animal models to study human influenza virus pathogenesis, they may not accurately reflect the disease pathogenesis in humans (44,45). Therefore, there is a need to clearly evaluate the contributions of HA and HA2 subunits in a natural host model. Chickens are highly

susceptible to H5N1 HPAIV infection and play a critical role in the spread of the virus. Hence, chickens provide an excellent model to determine the contributions of HA1 and HA2 subunits of H5N1 HPAIV in induction of neutralizing antibodies and protection. Viral vectored vaccines containing the intact HA protein of H5N1 HPAIV have been investigated in chickens in several studies (33,38,39). However, the individual contributions of HA1 and HA2 subunits of HPAIV HA protein in induction of neutralizing antibodies and protection has not been investigated in chickens.

NDV(26,38,39), turkey herpesvirus (HVT)(27), fowl pox virus (FPV)(28), adenovirus(29,131), infectious laryngotracheitis virus (ILTV) (30)and Marek's disease virus (MDV)(31)have been used as live recombinant vectors against HPAIV in chickens. Among these vectors NDV is most promising, because it infects chickens through respiratory tract resulting in eliciting strong local and systemic immune responses (25,46). NDV belongs to the genus *Orthoavulavirus* in the family *Paramyxoviridae* (7). NDV strain LaSota has been used as a safe and effective live vaccine for more than 60 years (46). In this study, we have used reverse genetics techniques to generate rNDVs expressing HA1, HA2, or HA protein of H5N1 HPAIV. The individual contributions of HA1 and HA2 subunits of HPAIV HA protein in induction of neutralizing antibodies and protection were evaluated in chickens. Our results showed that the HA1 and HA2 subunits when separated, neither provided protection nor induced neutralizing antibodies. In the absence of stalk, the globular head of HA protein was not expressed efficiently by the rNDV vector and in the absence of transmembrane (TM) and cytoplasmic tail (CT), it was not incorporated into

NDV envelope. These results suggest that the important epitopes for induction of protective and neutralizing antibodies are formed by interaction of HA1 and HA2 subunits. These epitopes are lost when the two subunits are separated. The disulfide bond between HA1 and HA2 subunits is necessary to maintain the native conformations of both head and stalk domains. Therefore, rNDVs expressing HA1 or HA2 subunit were not protective in chickens against HPAIV challenge, while rNDV expressing the intact HA protein provided complete protection.

*Materials and Methods*

**Cells and viruses**

HEp-2 cells, DF1cells and MDCK cells purchased from the ATCC (Manassas, VA), were used to recover rNDVs, to determine in vitro characteristics of rNDVs, and to assess neutralizing antibodies induced against avian influenza virus, respectively. HEp-2 cells and DF1 were grown in DMEM containing 10% FBS. MDCK cells were grown in DMEM containing 5% FBS. The rNDV strain LaSota and rNDVs expressing HA1, HA2 or HA protein of H5N1 HPAIV, recovered by reverse genetic technique, were grown in 10-day-old embryonated SPF chicken eggs (Charles Rivers, MA) at 99.5°F. The HPAIV strain A/Vietnam/1203/2004 (H5N1) and the LPAIV strain A/Mallard/Pennsylvania/10218/1984 (H5N2) viruses were propagated in 10-day-old SPF embryonated chicken eggs in Biosafety level-3 plus and Biosafety level-2 facilities, respectively. The 50% endpoint titers of H5N1 HPAIV and H5N2 LPAIV in allantoic fluid harvested from eggs were determined by $EID_{50}$ and tissue culture $TCID_{50}$ methods, respectively. The titers of viruses were calculated using a formula derived

from Reed and Muench and Spearman-Karber methods(134) .The modified vaccinia virus strain Ankara expressing T7 RNA polymerase (MVA-T7) was propagated using primary chicken embryo fibroblast cells.

**Generation of rNDVs expressing HA1, HA2, or HA protein of HPAIV (H5N1)**

A plasmid BR322 containing full-length antigenomic cDNA of NDV strain LaSota has been constructed previously (109). Here, transcription cassettes containing HA1 and HA2 were introduced between P and M genes of NDV strain LaSota using PmeI site. A previously constructed plasmid of full-length antigenomic cDNA of NDV strain LaSota containing the transcription cassette of HPAIV A/Vietnam/1203/2004 (H5N1) HA gene between NDV P and M genes also was used in this study (135). The HA1, HA2, and HA genes were codon optimized for higher level of expression. Four transcription cassettes were designed; (i) a transcription cassette containing the HA1 subunit (1,020 nt) to determine the contribution of HA1 subunit in induction of neutralizing antibodies and protection, (ii) a transcription cassette containing HA1 subunit (1,020 nt) fused with TM and CT of NDV F gene (171 nt) for incorporation into NDV envelope, (iii) a transcription cassette containing signal sequence of HA gene (48 nt) fused with HA2 gene (660 nt) to determine the contribution of HA2 subunit in induction of neutralizing antibodies and protection, and (iv) a transcription cassette containing the HA gene to evaluate the contribution of HA1 and HA2 subunits in comparison with intact HA in induction of protection and immunity. The polybasic cleavage site (PQRERRRKKR'G) of HPAIV strain A/Vietnam/1203/2004 (H5N1) was modified to monobasic cleavage site (PQRETR'G) of LPAIV strain

A/Mexico/31381/94 (H5N2). The transcription cassettes of codon optimized HA1 and HA2 genes were amplified from the plasmid containing codon optimized HA gene of HPAIV A/Vietnam/1203/2004 (H5N1). The amplified fragments were cloned into individual pGEM®-T Easy Vectors (Promega Corporation). The correct sequence of the genes introduced into the pGEM®-T vectors was confirmed by nucleotide sequence analysis. Then they re-cloned into complete individual plasmids containing cDNA of rLaSota at P and M gene junction using *PmeI* site, following digestion of transcription cassettes from shuttle vectors using *PmeI* restriction enzyme (Fig. 1). The correct sequences of the flanking regions were confirmed by nucleotide sequence analysis. NDV genome contains six genes arranged in order 3'-N-P-M-F-HN-L-5' with the GS and GE transcriptional sequences at upstream and downstream of each gene, respectively. The transcription cassette of a foreign gene can be introduced between any two genes of NDV at intergenic region. However, it has been found that the optimal site for expression of most foreign genes is intergenic region between P and M genes (38, 110,112,113). Transcription cassettes of H5N1 HPAIV genes contain; PmeI restriction enzyme site sequence at two ends of the transcription cassette plus in the constant order; 3'-GE signal of NDV, one T nucleotide as IGS, GS signal of NDV, Kozak sequence and foreign gene ORF sequence-5'. rNDV LaSota strains containing the HA1 gene, the HA1 gene fused with TM and CT of NDV F gene, signal peptide fused with the HA2 gene and the intact HA gene were recovered by reverse genetics as described previously (109). The presence of transcription cassettes containing HPAIV genes in rNDVs genomes was confirmed by RT-PCR.

**Expression of HA1, HA2, and HA proteins of H5N1 HPAIV**

The expression of HA1, HA2 and HA proteins in DF1 cells by Western blot analysis. DF1 cells were infected with rLaSota, rLaSota/HA1, rLaSota/HA1+NDV-FTM&CT, rLaSota/HA2 and rLaSota/HA at a multiplicity of infection (MOI) of 0.1 in presence of 10% fresh allantoic fluid, harvested from embryonated SPF chicken eggs. DF1 cell lysates and supernatants were collected 30 hours post-infection. Cell supernatants were concentrated. A polyclonal chicken anti H5N1 HPAIV and a monoclonal anti NDV HN protein sera were used to detect the expression of HA1, HA2, and HA proteins of HPAIV and HN protein of NDV by Western blot analysis, respectively. The surface expression of HA1, HA2 and HA proteins by NDV vectors was confirmed by indirect immunofluorescence assay (IFA) using the above polyclonal chicken anti H5N1 HPAIV serum. The incorporation of HA1, HA2 and HA proteins into NDV envelope were assessed by Western blot analysis. Ten-day-old embryonated SPF chicken eggs were infected with rLaSota, rLaSota/HA1, rLaSota/HA1+NDVFTM&CT, rLaSota/HA2 or rLaSota/HA. Allantoic fluids were harvested from eggs three days after infection and ultra-centrifuged at 1500 rpm for 10 min. Then allantoic fluids containing rNDVs were centrifuged through 30% sucrose cushion (SW28 rotor) at 28000 rpm for 2:30 hours at 4°C. The settled pellets were dissolved in 50 µl PBS, lysed and analyzed by Western blot using the two above antisera.

**Growth characteristics of rNDVs expressing HA1, HA2, or HA protein of H5N1 HPAIV**

rNDVs expressing HPAIV HA1, HA2, or HA protein were passaged in eggs. The allantoic fluids containing high titers ($2^8$ HAU/$_{50}$µl) of each recombinant virus were aliquoted in vials and stored at -70 °C. The titer of each virus was determined by plaque forming unit (PFU) assay on DF1 cells in presence of 10% fresh allantoic fluid. Monolayer DF1 cells in 6-well tissue culture plates were washed with DMEM and infected at a MOI of 0.1 with parental rLaSota, rLaSota strains expressing HA1, ,HA1+NDVFTM&CT, HA2 or HA protein. After 90 minutes adsorption at 37°C, cells were washed with DMEM, then incubated with DMEM containing 10% fresh SPF chicken egg allantoic fluid at 37°C in presence of 5% CO2. At intervals of 8 hours until 64 hours post-infection, volumes of 200 µl of supernatant from infected cells were collected and replaced with equal volumes of fresh DMEM containing 10% fresh allantoic fluid. The titer of rNDVs in collected supernatants was determined by TCID50 method on DF1 cells. The titers were calculated using a formula derived from Reed and Muench and Spearman-Karber methods (134).

**The protective efficacy of rNDVs expressing HA1, HA2 or HA protein against H5N1 HPAIV challenge**

The protective efficacy of rNDVs expressing HA1, HA2 or HA protein of H5N1 HPAIV was evaluated in SPF chicks immunized at day one of age and challenged against H5N1 HPAIVat three weeks after immunization. A total of fifty 1-day-old SPF White Leghorn chicks, obtained from Charles Rivers, were divided into five groups of ten each in Biosafety level-2. Chicks of groups one to five were immunized with $10^6$ PFU, 200 µl in volume, of rLaSota, rLaSota/HA1,

rLaSota/HA1+NDVF TM&CT, rLaSota/HA2 and rLaSota/HA, respectively, via the oculonasal route. Three weeks after immunization, all chickens were moved into the Biosafety level-3 plus facility and were challenged with $10^6$ $EID_{50}$, 200 µl in volume, of HPAIV A/Vietnam/1203/2004 (H5N1) via the oculonasal route (2). The infected birds were observed for ten days after challenge. The mortality rate was recorded for each group daily. We conducted all experiments involving avirulent NDV strain LaSota in our USDA approved Biosafety level-2 and Biosafety level-2 plus facilities and the experiment involving HPAIV in our USDA approved Biosafety level-3 plus facility following the guidelines and approval of the IACUC, University of Maryland.

**Serological assays**

The sera were collected from all chickens three weeks after immunization. The humoral antibodies induced against NDV and HPAIV in sera of immunized chickens were assessed. HI assay using the protocol of OIE was used to assess the level of antibody titers mounted against a LPAIV A/Mallard (H5N2) in chickens immunized with rNDVs (2). To confirm the result, we repeated the HI assay four times and we calculated the titers as mean of four experiments. The virus neutralization assay was used to measure neutralizing antibodies induced against a LPAIV A/Mallard (H5N2) in chickens immunized by rNDVs. Briefly, serum samples of all immunized chickens were incubated at 56°C for 30 minutes. The titer of LPAIV (H5N2) was determined by $TCID_{50}$ on MDCK cells in presence of DMEM containing 1µg/ml N-tosyl-l-phenylalanine chloromethyl ketone (TPCK)-treated trypsin. One hundred $TCID_{50}$ of H5N2 LPAIV was mixed with 2 fold dilutions of antiserum and incubated for 90

minutes at 37°C. One hundred µl of each serum and virus mixture was added into monolayer of MDCK cells, three wells of 96-well tissue culture plate per dilution and incubated at 37°C. One hour after incubation, the supernatant of wells were removed and wells were washed with DMEM. One hundred DMEM containing 1µg/ml TPCK-treated trypsin were added to each well. The plates were incubated at 37°C in presence of 5% CO2. Three days after infection 50 µl of supernatant of each well were transferred to haemagglutination (HA) assay plate. The presence of H5N2 LPAIV in supernatants were detected by HA assay using 1% chicken RBC. The serum titers were calculated using a formula derived from Reed and Muench and Spearman-Karber methods (134). HI assay using the protocol of OIE was used to assess the level of antibody titers mounted against NDV strain LaSota in chickens immunized with rNDVs (3).

**Statistical analysis**

Data were analyzed among groups by One-Way-ANOVA test. The student t-test was used to compare two groups. To avoid bias, HPAIV challenge experiment was designed as blinded study.

*Results*

**Generation rNDVs expressing HA1, HA2, or HA protein of H5N1 HPAIV**

The expression cassettes of HPAIV HA gene subunits were constructed and inserted between P and M genes of NDV in individual plasmids containing full length antigenomic cDNA of NDV strain LaSota (Fig.5.1). The correct sequence of transcription cassettes containing HPAIV HA subunits and flanking regions was

confirmed by sequence analysis. Infectious rNDVs containing the HA1 subunit, HA1 subunit fused with NDV-F TM and CT domains, the HA2 subunit and the HA gene of HPAIV were recovered from all cDNAs by reverse genetics technique using HEp-2 cells. They were named rLaSota/HA1, rLaSota/HA1+NDVF-TM&CT, rLaSota/HA2 and rLaSota/HA, respectively. The presence of HA1, HA2 and HA genes in rNDVs were confirmed by RT-PCR.

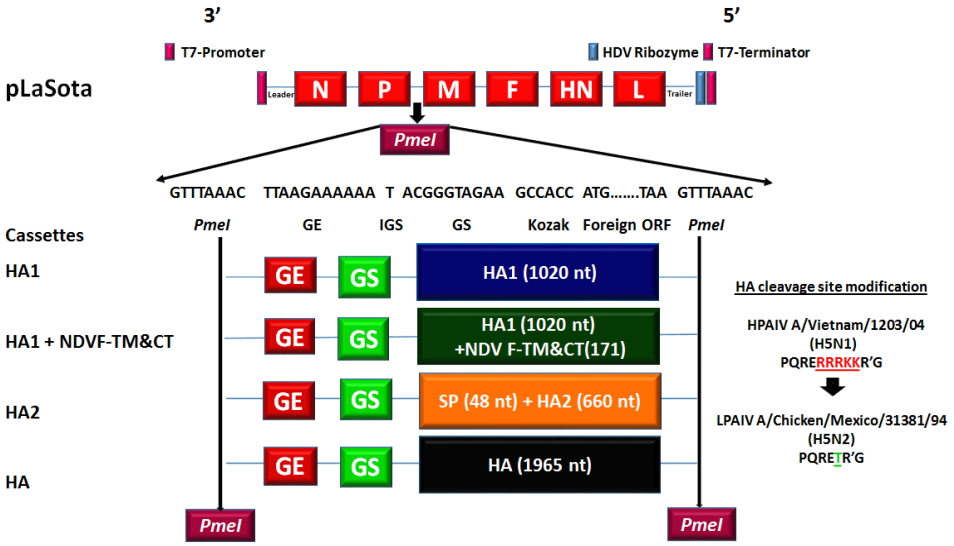


**Figure 24.** Construction of recombinant NDVs containing H5N1 HPAIV HA1, HA2, or HA gene. Following four transcription cassettes were inserted into individual plasmids containing cDNA of LaSota between P and M genes using *PmeI* site. i) codon optimized HA1 subunit of HA gene of H5N1 HPAIV (1,020 nt), ii) codon optimized HA1 subunit of HA gene of H5N1 HPAIV (1,020 nt) fused with N-terminus of transmembrane and cytoplasmic tail of F gene of NDV (171 nt), iii) the N-terminus of codon optimized HA2 subunit of HA gene of H5N1 HPAIV (660 nt) fused with C-terminus of signal peptide sequence of HA gene of H5N1 HPAIV(48 nt) and iv) the

codon optimized HA gene of H5N1 HPAIV (1;695 nt). Each transcription cassette contains; *PmeI* restriction enzyme site sequence at two ends of the transcription cassette plus in the constant order; 3'-GE signal of NDV, one T nucleotide as intergenic sequence (IGS), GS signal of NDV, Kozak sequence (In order to follow the rule of six ACC and GCCACC were used for HA construct and other constructs, respectively.) and foreign gene ORF sequence-5'.

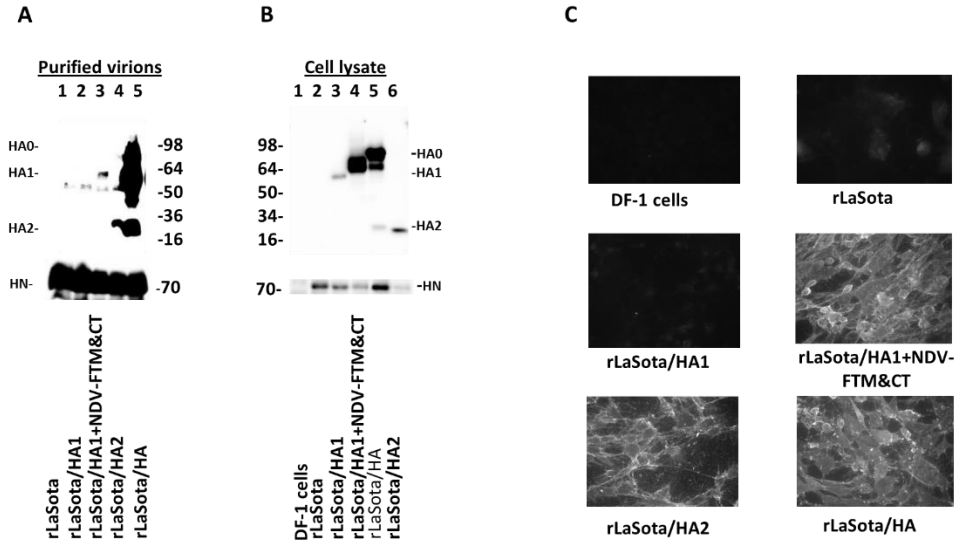


**Figure 25.** The incorporation of HA1, HA2 and HA proteins of HPAIV into NDV particles and the expression of HA1, HA2 and HA proteins by rNDVs. A) The incorporation of HA1, HA2 and HA proteins of HPAIV into NDV particles was detected by Western blot using a chicken polyclonal anti H5N1 HPAIV serum (A upper panel); rLaSota (lane1), rLaSota/HA1 (lane2), rLaSota/HA1+NDVF TM&CT (lane3), rLaSota/HA2 (lane4) and rLaSota/HA (lane5). A monoclonal anti NDV HN antibody was used to detect the 70 kDa HN protein of NDV in partially purified rNDVs (A lower panel); rLaSota (lane1), rLaSota/HA1 (lane2), rLaSota/HA1+NDVF TM&CT (lane3),

rLaSota/HA2 (lane4), rLaSota/HA (lane5). B) The expression of HA1, HA2 and HA proteins were detected in infected DF1 cell lysates by Western blot analysis using a chicken polyclonal anti H5N1 HPAIV serum (B upper panel); DF1 cells (lane1), rLaSota (lane2), rLaSota/HA1 (lane3), rLaSota/HA1+NDVF TM&CT (lane4), rLaSota/HA (lane5) and rLaSota/HA2 (lane 6). A monoclonal anti NDV HN antibody was used to detect the 70 kDa HN protein of NDV in DF1 cell lysates (B lower panel); DF1 cells (lane1), rLaSota (lane2), rLaSota/HA1 (lane3), rLaSota/HA1+NDVF TM&CT (lane4), rLaSota/HA (lane5) and rLaSota/HA2 (lane 6). The full length of cropped gels are presented in S1. C) DF1 cells were infected with rNDVs at MOI of 0.1. The cells were fixed with 3% paraformaldehyde 24 hours post-infection, then were blocked with 3% goat serum. The florescence signals on the surface of cells were observed by immunofluorescence microscope after interacting the cells with a primary chicken polyclonal anti H5N1 HPAIV serum and a secondary FITC conjugated goat anti chicken antibody.

**Expression of the HA1, HA2 and HA proteins of H5N1 HPAIV**

The expression of HA1, HA2 and HA proteins of HPAIV was detected by Western blot analysis in DF1 cells lysates (Fig.5.2B upper panel). The HA protein was expressed efficiently (Fig. 5.2B-lane5), a band ~ 70 kDa band represents uncleaved HA protein (HA0). A~60 kDa and a ~ 25 kDa bands represent HA1 and HA2 subunits of cleaved HA0, respectively. The expression of HA1 subunit fused with NDV F protein TM and CT was much higher than that of HA1 subunit alone, a ~60 kDa band represents HA1 subunit fused with NDV F protein TM and CT (Fig.5.2B, lane4) and a

band ~ 55 kDa band represents HA1 subunit (Fig.5.2B, lane3). In the case of HA2 expressed by rLaSota/HA2 there is a band (~25 kDa) on bottom (Fig.5.2B lane 6). Lanes 1 and 2 of Fig. 2 B represent DF1 cells and rNDV, respectively, as controls. A monoclonal anti NDV HN antibody was used to detect a ~ 70 kDa of HN protein of NDV in lysates, indicating similar level of NDV protein in each lane (Fig.5.2B - lower panel). We also evaluated incorporation of HPAIV HA1, HA2 and HA proteins into NDV envelope (Fig.5.2A). Our results showed that the HA protein was incorporated into NDV envelope, properly. The ~ 70 kDa, ~ 60 kDa and ~ 25 kDa bands represent HA0, HA1 and HA2 subunits, respectively (Fig.5.2A, lane5). The HA1 subunit fused with NDVF protein TM and CT was incorporated into NDV envelope. A ~ 60 kDa band represents HA1 subunit fused with NDV F protein TM and CT incorporating into the NDV envelope (Fig.5.2A lane 3). Lane 2 of Fig. 2A shows that HA1 without TM and CT did not get incorporated into the NDV envelope. The HA2 subunit was incorporated into the NDV envelope. A ~ 25 kDa band shows HA2 subunit expression and a faint ~ 75 kDa band on top shows polymorphic form of HA2 subunit (Fig.5.2A lane 4). Lane 1 of Fig. 2A represents partially purified NDV particles as control. A ~55 kDa band in all lanes was a result of nonspecific interaction of antibody with NDV proteins. A monoclonal anti NDV HN antibody was used to detect the level of ~ 70 kDa HN protein of NDV. Our results showed similar levels of HN protein in all rNDVs (Fig.5.2A lower panel). The surface expression of HA1, HA2 and HA proteins by NDV vectors were detected by IFA. HA1 fused with NDV-F TM and CT domains, HA2 and

HA proteins were expressed at the same level on the surface of DF1 cells, whereas HA1 alone was not expressed on the cells surface (Fig.5.2C).

**Growth characteristics of rNDVs expressing HA1, HA2, or HA protein of H5N1 HPAIV.**

The rNDVs expressing HA1, HA2, or HA protein were passaged in 10-day-old embryonated SPF chicken eggs. All rNDVs replicated in eggs efficiently with titers of $2^8$ HAU/$_{50}$ µl (parental rNDV strain LaSota usually replicates in eggs with titer of 29 HAU/$_{50}$µl). The multicycle growth kinetics of rNDVs in presence of fresh allantoic fluid, as a source of protease (136), in DF1 cells showed that rNDVs expressing HA1, HA2, or HA protein grew at similar level compared to the parental rNDV. However, in the first 32 hours of growth cycle, compared to parental rNDV, other rNDVs grew slightly slowly (Fig.5.3).

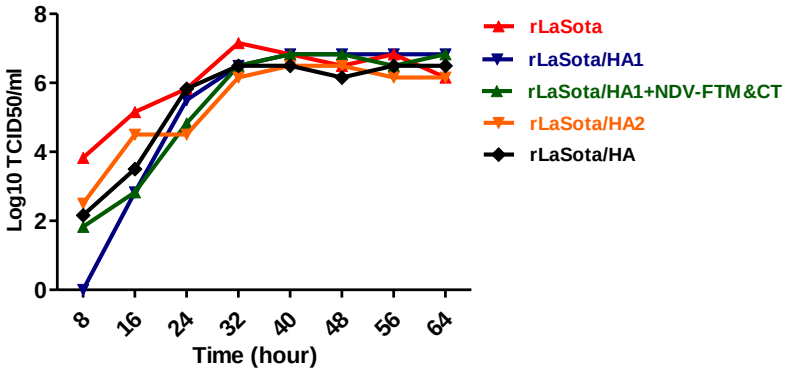


**Figure 26.** Growth kinetics of rNDVs expressing HA1, HA2 or HA protein in DF1 cells. The monolayers of DF1 cells in 6-well plate tissue culture were infected rNDVs at MOI of 0.1. The over layered media containing virus were removed after one hour adsorbtion and replaced with fresh DMEM containing 10% allantoic fluid. The titer of

viruses in volumes of supernatants collected in 8 hours intervals were detected by $TCID_{50}$ using DF1 cells.

**The protective efficacy of rNDVs expressing HA1, HA2 or HA protein in chickens against H5N1 HPAIV challenge**

The protective efficacies of rNDVs expressing HA1, HA2, or HA protein were evaluated in 1-day-old SPF chicks immunized at day one of age and challenged with H5N1 HPAIV at three weeks post immunization. The survival rates against HPAIV were recorded daily for ten days after challenge (Fig.5.4). The results showed 100% survival rate for chickens immunized with rLaSota/ HA until ten days post-challenge, while 100% mortality was observed for unimmunized chickens and chicks immunized with rLaSota/HA1 at day two post-challenge.

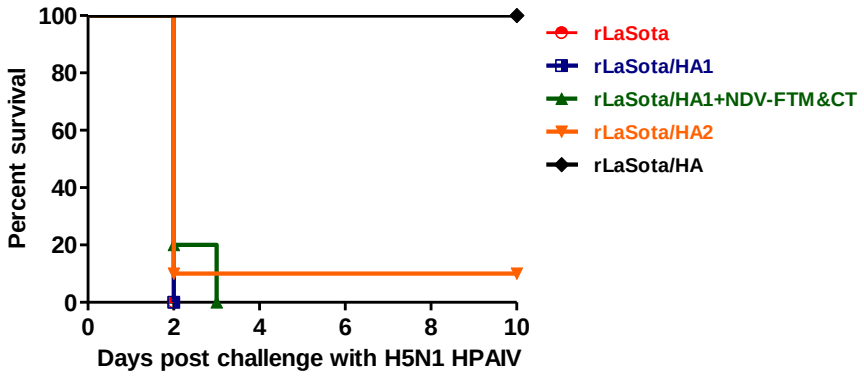


**Figure 27.** Survival rate of immunized chickens with rNDVs expressing HA1, HA2, or HA protein of HPAIV against H5N1 HPAIV challenge. Groups of ten SPF chicks were immunized with $10^6$ PFU of rLaSota, rLaSota/HA1, rLaSota/HA1+NDV-FTM&CT, rLaSota/HA2 or rLaSota/HA via oculonasal route at day one of age. Three weeks after immunization, chickens were challenged with $10^6$ $EID_{50}$ of HPAIV

A/Vietnam/1203/2004 (H5N1) through oculanasal route. Chickens were observed daily for ten days after challenge. The percent of survival rate was recorded for each group.

The mortality rate for chickens immunized with rLaSota/HA2 was nine out of ten chickens at day two post-challenge. One out of ten chickens immunized with rLaSota/HA2 survived until ten days post-challenge. In the case of chickens immunized with rLaSota/HA1+NDV F TM&CT, results showed eight out of ten chickens died at day two post-challenge and the remaining two chickens died at day three post-challenge.

**Antibody response against AIV**

Neutralizing antibodies induced against LPAIV (H5N2) in serum samples of chickens at 21 days after immunization were assessed by a micro-virus neutralization assay on MDCK cells. The results showed that the neutralizing antibodies against LPAIV (H5N2) were detected only in serum samples of chickens immunized with rLaSota expressing intact HA protein. However, compared to rLaSota group, insignificant titers of neutralizing antibodies against LPAIV (H5N2) were detected in serum samples of chickens immunized with rLaSota/HA2 and rLaSota/HA1+NDVFTM&CT ($P>0.05$) (Fig.5.5A).

The HI titers of antibodies induced against a heterologous H5N1 LPAIV were assessed in sera of chickens 21 days after immunization by HI assay using the protocol of OIE (repeated four times). The results showed that HI titers against H5N1 LPAIV was detected only in serum samples of chickens immunized with rLaSota expressing

intact HA protein. However, compared to rLaSota and rLaSota/HA2 groups, insignificant HI titers were detected against H5N1 LPAIV in sera of chickens immunized with rLaSota/HA1 or rLaSota/HA1+NDVFTM&CT (P>0.05) (Fig.5.5B).

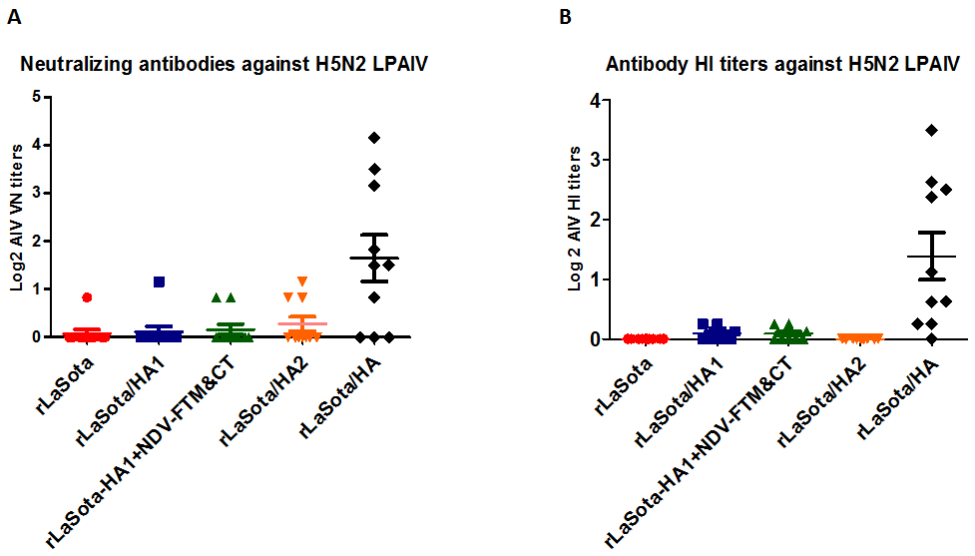


**Figure 28.** Antibody response against AIV. SPF chicks were immunized at day one of age and sera were collected at day 21 after immunization. Neutralizing antibodies against a heterologous H5N2 LPAIV in collected sera were assessed by a micro-virus neutralization assay on MDCK cells using a standard protocol (A). Symbols and horizontal lines represent individual titers of each sample and mean titers of each group, respectively. HI antibody titers were assessed against a heterologous H5N2 LPAIV by HI assay (repeated four times) using the protocol of OIE (B). Each symbols represents mean titers of four HI assays for individual samples and horizontal lines represent mean titers of each group, respectively. Serum titers were expressed as reciprocals Log2 dilution.

The results showed that comparable HI titers against NDV strain LaSota were detected in sera collected from chickens of five groups (Fig.6) (P>0.05).

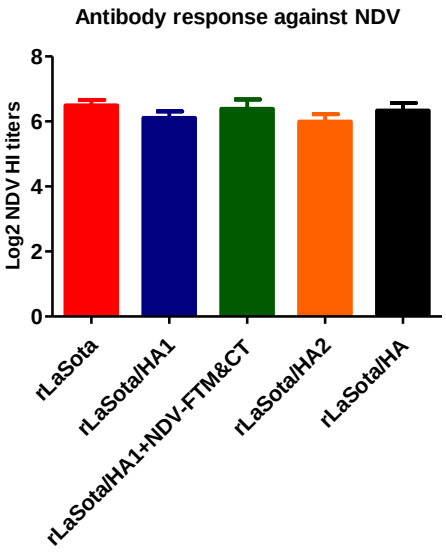


**Figure 29.** Antibody response against NDV. SPF chicks were immunized at day 1 of age and sera were collected at day 21 after immunization. HI antibody titers induced against NDV strain LaSota in collected sera were assessed by HI assay using the protocol of OIE. Serum titers are expressed as reciprocal of Log2 dilution.

*Discussion*

Development of a broadly protective vaccine against influenza virus has been an elusive target. Isolation of broadly protective monoclonal antibodies directed against conserved portions of the HA protein suggests that it is possible to design new immunogens that may serve as universal influenza virus vaccines (41,137,138,139). This study aimed to determine the individual contributions of HA1 and HA2 subunits of H5N1 HPAIV HA protein in induction of neutralizing antibodies and protection in

chickens. rNDV was used as the expression vector, because NDV infects the respiratory tract as AIV and has been previously used to express AIV HA protein (26,38,140). Chickens are highly susceptible to HPAIV; therefore, an ideal system for this study. Our results showed that the intact HA protein induced neutralizing antibody response and provided full protection against death following a lethal homologous challenge. Whereas, HA1 or HA2 subunits neither induced significant level of neutralizing antibody response nor provided protection following a lethal homologous challenge. These data suggest that the epitopes responsible for induction of protective immunity are present on the head and stalk domains in their native conformation, but their native conformation is lost when the two subunits are separated.

It was observed that the expression level of HA1 subunit fused with NDV F protein TM and CT was higher than that of HA1 subunit alone. This result suggests that the presence of TM and CT is required for high level expression of HA1 subunit. We also evaluated incorporation of HPAIV HA1, HA2, or HA protein into NDV particles. Among the four constructs, HA1 without TM and CT was the only protein that did not incorporate into NDV envelope, while HA1 subunit fused with NDV F protein TM and CT and HA and HA2 subunits, which contain their own TM and CT, incorporated into NDV envelope. It suggests that the presence of TM and CT is required for incorporation of HA1 subunit into NDV envelope, which can be substituted by heterologous TM and CT domains. The polymeric folded forms were observed in the case of HA2 subunit, confirming that the HA2 is in charge of the trimer formation of the HA protein through triple-stranded coiled coil of α-helixes (19).

The result of the protective efficacy of rNDVs expressing HA1, HA2 or HA protein in 1-day-old immunized chicks challenged with H5N1 HPAIV at three weeks post immunization showed that rNDV expressing the intact HA protein provided complete protection against HPAIV in chickens, while rNDV expressing HA1 or HA2 subunit were not protective. Our finding that the HA provided complete protection from lethal challenge was not unexpected, because it is well established that the HA protein contains the major protective antigens of HPAIV. The HA protein is highly immunogenic and therefore induced high neutralizing antibody titers (19,37). Previous to this study, the individual contributions of HA1 and HA2 subunits of HPAIV HA protein in induction of neutralizing antibodies and protection in chickens were not known. Our results demonstrated that when separated the HA1 and HA2 subunits do not provide either protection or induce neutralizing antibody response in chickens. This may be due to change in the conformation of the HA1 and HA2 subunits. It suggests that the native conformations of the HA1 and HA2 subunits are maintained when the two subunits are held together by disulfide bonds. The important protective epitopes are only present on these subunits in their native conformation. They are not present on individually expressed HA1 or HA2 subunit. It is also possible that some protective epitopes on HA protein are formed by residues from both HA1 and HA2 subunits and hence these epitopes are lost when the two subunits are separated.

The neutralizing antibodies against a H5N2 LPAIV was detected only in serum samples of chickens immunized with rNDV expressing HA protein. These results showed that the HA1 and HA2 subunits, when separated also failed to induce

neutralizing antibodies. Although no study was performed to determine the structure of HA1 or HA2 expressed by the rNDV vector, our results provide an indirect evidence that disulfide bond between HA1 and HA2 or some unknown interactions between key residues of HA1 and HA2 is necessary to maintain the native conformation of HA protein (19,141). This is in consistent with outcomes of an earlier study which showed that interaction between HA1 and HA2 subunits affects HA protein stability and AIV infectivity (139). Interestingly, rNDV expressing HA1 subunit fused with NDV F TM and CT also did not induced neutralizing antibodies or provide protection, but somehow resulted in high level of expression of HA1 detected by IFA and Western blot analysis. This result suggests that insertion of HA1 into NDV envelope lead to conformational change of HA1 and expose of non-neutralizing epitopes, which were recognized by AIV antibodies in IFA and Western blot. However, the presence of NDV F TM and CT was not able to compensate the lack of HA2 subunit required for correct folding of the HA1 subunit. Previous studies have shown that HA1 and HA2 subunits of H1N1 provided protection and induced antibodies in mouse and/or ferret models (12,43, 131) .Although mice and ferrets are excellent models to study human influenza virus pathogenesis and transmission, they may not be a sufficiently permissive to evaluate the protective efficacy of human influenza virus vaccines (44,45). Therefore, the results obtained from mice and ferrets may not hold true in humans. The vaccine should be evaluated in the most rigorous system possible. Our results suggest that evaluation of stalk-based vaccines in chickens against H5N1 HPAIV challenge will provide more accurate results.

The antibodies against NDV were detected at similar levels in immunized chickens of all groups, indicating that all the recombinant viruses grew at same level in chickens. The level of antibodies induced against NDV backbone was higher than antibodies detected against HA protein, because a heterologous LPAIV (H5N2), with about 90% amino acid identity with the HA protein of recombinant vaccine, was utilized to detect HI and neutralizing antibody titers against HPAIV. Previous studies have been shown that the antibody titers assayed against a heterologous H5 were lower compared to the homologous virus (142,143).

In summary, this study suggest that a H5N1 vaccine construct incorporating either HA1 or HA2 subunit would likely not provide protection against HPAIV challenge in chickens. To be effective the vaccine must incorporate the intact HA protein.

# Chapter 6

# A recombinant APMV-3 expressing the HA protein protects chickens against H5N1 HPAIV challenge

*Abstract*

HPAI is a devastating disease of poultry and a serious threat to public health. Vaccination with inactivated virus vaccines has been applied for several years as one of the major policies to control HPAIV infections in chickens. Viral-vectored HA protein vaccines are a desirable alternative for inactivated vaccines. However, each viral vector possesses its own advantages and disadvantages for the development of a HA-based vaccine against HPAIV. rNDV strain LaSota expressing HA protein vaccine has shown promising results against HPAIV; however, its replication is restricted only to the respiratory tract. Therefore, we thought to evaluate avian paramyxovirus serotype 3 (APMV-3) strain Netherlands as a safe vaccine vector against HPAIV, which has high efficiency replication in a greater range of host organs. In this study, we generated rAPMV-3 expressing the HA protein of H5N1 HPAIV using reverse genetics and evaluated the induction of neutralizing antibodies and protection by rAPMV3 and rNDV expressing the HA protein against HPAIV challenge in chickens. Our results showed that immunization of chickens with rAPMV-3 or rNDV expressing HA protein provided complete protection against HPAIV challenge. However, immunization of chickens with rAPMV-3 expressing HA protein induced higher level of neutralizing antibodies compared to that of rNDV expressing HA protein. Our study also showed

that rAPMV-3 vector vaccine can overcome maternally derived antibodies (MDA) to NDV. These results suggest that a rAPMV-3 expressing HA protein might provide better protection against H5N1 HPAIV in field conditions.

*Introduction*

Avian influenza viruses (AIVs) are members of the genus *Alphainfluenzavirus* in the family *Orthomyxoviridae* (7). They can cause a wide range of clinical disease in chickens. Alphainfluenzaviruses are classified into combinations of 18 H (H1-H18) and 11 N (N1-N11) subtypes, based on antigenic differences of their HA and NA proteins surface glycoproteins. H1-H16 and N1-N9 subtypes have been found in avian species (19). AIVs are divided into LPAIVs that cause restricted respiratory and/or intestinal disease without mortality in SPF chickens, and HPAIVs that cause high mortality following an acute systemic infection in SPF chickens (2,20). Avian influenza is a devastating disease of poultry and a serious threat to public health, which is caused by H5 or H7 subtype of HPAIV (21,22).

Nationwide vaccination with inactivated virus vaccines and/or viral vectored HA-based vaccines is one of the major policies that is currently implemented against HPAIV in chickens in many countries (23). Although the greater part of currently used vaccines are inactivated vaccines, this type of vaccines are not the best choice to combat HPAIV infection in poultry. Because not only the effectiveness of these vaccines is suboptimal, but also the processes of production and administration of these vaccines are expensive, time consuming and labor intensive. Furthermore, the inability to easily

serologically differentiate infected birds from vaccinated ones is a concern of vaccination with inactivated vaccines containing virus particles (24,25). On the contrary, viral-vectored HA protein vaccines are a desirable alternative for inactivated vaccines (26-31, 144). However, the advantages and disadvantages of each virus should be considered when it is chosen as a vector for the development of a HA-based vaccine against HPAIV (33). Among several viral-vectored HA protein vaccine candidates that have been extensively studied in chickens, rNDV expressing HA protein of HPAIV has shown highly promising results and has been licensed to use in the field (2,26,140,145-147).

NDV is a member of genus *Orthoavulavirus* in the family *Paramyxoviridae* (7). NDV strains are classified into three pathotypes based on the severity of disease which they cause in chickens: lentogenic (low virulent), mesogenic (moderately virulent) and velogenic (highly virulent). The major viral factor that determines the pathotypes of NDV is the fusion protein cleavage site (FPCS) sequence. Cleavage of precursor F0 to F1 and F2 subunits is a prerequisite for NDV infection. Lentogenic NDV strains contain a mono or dibasic amino acids within the FPCS such that the F0 protein can be cleaved into F1 and F2 subunits only by a trypsin-like protease that is present extracellularly in the respiratory and intestinal tracts. In contrast, mesogenic and velogenic NDV strains have multibasic amino acids at the FPCS that can be cleaved intracellularly by the ubiquitous furin-like protease, resulting in systemic infection (148). Although both lentogenic and mesogenic NDV strains have been evaluated as human and veterinary vaccine vectors, the mesogenic NDV strain

Beadette C was found to be highly effective because of its systemic replication (149). However, mesogenic NDV strains are Select Agents and therefore cannot be used as vaccine vectors. Hence, we thought to evaluate another avian paramyxovirus serotype (APMV) as a vaccine vector against HPAIV, which has high efficiency replication in a greater range of host organs, in comparison to NDV strain LaSota, which is restricted only to the respiratory tract(46,47).

All strains of NDV belong to APMV-1, but officially nineteen (APMV-2 to APMV-20) and unofficially one (APMV-21) serotypes have been identified (7). Of these serotypes, APMV-3 strain Netherlands, which belongs to the genus *Paraavulavirus*, has a multibasic FPCS, allowing it to replicate in wide range of host tissue (49,50). APMV-3 is highly safe in chicken and turkey and it is not a Select Agent. Therefore, it can be used as a vaccine vector in chickens and turkeys. Furthermore, APMV-3 is an antigenically distinct APMV serotype; therefore, it can circumvent pre-existing immunity to NDV better than rNDV vector. In this study, we generated recombinant APMV-3 (rAPMV3) strain Netherlands expressing the HA protein of H5N1 HPAIV using reverse genetics. We evaluated the induction of neutralizing antibodies and protection by rAPMV3 strain Netherlands and rNDV strain LaSota expressing the HA protein against HPAIV and highly virulent NDV challenges in chickens. Our results showed that compared to rNDV expressing HA protein, immunization of chickens with rAPMV-3 expressing HA protein induced higher level of neutralizing antibodies against HPAIV. Moreover, a prime vaccination of broilers with rAPMV-3 expressing HA protein followed by a booster immunization with rNDV

expressing HA protein induced even high level of humoral antibody response against HPAIV. Both rAPMV-3 expressing HA protein and rNDV expressing HA protein provided protection in chickens from death following HPAIV challenge. Although the both vectors expressing the HA protein induced protective immunity against HPAIV challenge in immunized chickens, rAPMV-3 expressing the HA protein induced higher levels HI and neutralizing antibody titers than the rNDV expressing the HA protein. These results suggest that rAPMV-3 might be a better vector for HPAIV vaccination in the field.

*Materials and Methods*

**Cells and viruses**

HEp-2 cells and DF1 cells were obtained from the ATCC (Manassas, VA) to recover recombinant viruses by reverse genetics and for in vitro characterization of recovered viruses, respectively. The MDCK cells was also obtained from ATCC to perform virus-neutralization test against LPAIV strain A/Mallard/Pennsylvania/10218/1984 (H5N2). HEp-2 and DF1 cells were grown in DMEM containing 10% FBS; and MDCK cells were grown in DMEM containing 5% FBS. Recombinant viruses were propagated in allantoic fluids of 10-day-old embryonated SPF chicken eggs (Charles Rivers, MA). HPAIV strain A/Vietnam/1203/ 2004 (H5N1) was propagated in 10-day-old embryonated SPF chicken eggs, aliquoted and stored at -70°C in Biosafety Level-3 plus facilities. The stored virus stock was used as challenge virus and its end point titer was determined by $EID_{50}$. The H5N2 LPAIV was propagated in 10-day-old embryonated SPF chicken eggs, aliquoted and stored at

-70°C in Biosafety level-2 facility. The stored virus stock was used in micro-neutralization assay and its end point titer was determined by $TCID_{50}$ in MDCK.

**Construction of rAPMV-3 expressing HA protein of H5N1 HPAIV**

A plasmid containing full-length antigenomic cDNA of APMV-3 strain Netherlands was constructed previously (150,151) and later the viral protein 2 (VP2) gene of infectious bursal disease virus (IBDV) was flanked between P and M genes of APMV-3 using this plasmid. Here we replaced the VP2 gene with HA gene of HPAIV strain A/Vietnam/1203/2004 (H5N1) using SacII restriction enzyme site. The HA gene was codon optimized for higher level of expression. A transcription cassette containing ORF sequence of HA gene of H5N1 HPAIV was amplified from a plasmid of full length antigenomic cDNA of NDV containing codon optimized HA gene of HPAIV strain A/Vietnam/1203/2004 (H5N1). The polybasic cleavage site motifs (PQRERRRKKR'G) of HPAIV strain A/Vietnam/1203/2004 (H5N1) was modified to monobasic cleavage site motifs (PQRETR'G) of LPAIV strain A/Mexico/31381/94 (H5N2). The HA gene transcription cassette contained nucleotide sequences of following genetic elements arranged in 3′ to 5′ order; SacII restriction enzyme site, GC nucleotides for rule of six, Kozak , HA ORF, GE of P gene of APMV-3 (here it serves as GE for HA gene), 31 nucleotides of APMV-3 IGS, GS of APMV-3 M gene and *SacII* restriction enzyme site. Following the cloning of the amplified fragment of HA gene transcription cassette into pGEM®–T Easy vector (Promega Corporation), it was digested by *SacII* restriction enzyme, and re-cloned between P and M genes of APMV-3 instead of VP2 gene which was inserted between P and M genes previously. The

recombinant APMV-3 expressing HA protein was recovered by co-transfection of the plasmid of full-length anitigenomic cDNA of APMV-3 containing HA gene and supporting plasmids containing N, P and L proteins of APMV-3 strain Netherlands into HEp-2 cells using reverse genetic techniques as described previously(42). The rAPMV-3 expressing HA protein and rNDV strain LaSota expressing HA protein were named rAPMV-3/HA and rLaSota/HA, respectively.

**Expression of HA protein by rAPMV-3 or rNDV expressing HA protein of H5N1 HPAIV**

The monolayer of DF1 cells were infected with rLaSota, rLaSota/HA and rAPMV-3/HA in presence of 10% allantoic fluid. The DF1 cell lysates were collected 30 hours after infection. A polyclonal chicken anti H5N1 serum was used to detect expression of HA protein. In order to detect incorporation of HA protein into rAPMV-3 or rNDV particles, the recombinant viruses were inoculated in 10-day-old embryonated SPF chicken eggs. Three days after inoculation the allantoic fluids were harvested and centrifuged at 1500 rpm for 10 minutes to remove the cell debris. The allantoic fluids containing recombinant particles were ultra-centrifuged at 28000 rpm (rotor SW28) for 2:30 hours at 4°C through 30% sucrose cushion. The sedimented pellets containing partially purified virus particles were dissolved in 50 µl phosphate buffer saline (PBS). The Western blot analysis using above serum was utilized to detect the incorporation of HA protein into rAPMV-3 or rNDV particles.

**Growth characteristics of rAPMV-3 or rNDV expressing HA protein of H5N1 HPAIV**

The recombinant viruses were passaged in 10-day-old embryonated SPF chicken eggs. The viruses with high HA titers were harvested and stored at -70°C in aliquots. The titer of each stored virus was determined by plaque assay in presence of 10% allantoic fluid. Then the monolayers of DF1 cells in a 6-well tissue culture plate were infected with rLaSota, rLaSota/HA, rAPMV-3 and rAPMV-3/HA at 0.1 MOI. The over layered medium was removed after one hour adsorption and replaced with fresh DMEM containing 10% allantoic fluid following washing the monolayers with DMEM. Volumes of 200µl were collected from each well and replaced with fresh medium containing 10% allantoic fluid in 8 hours intervals. The collected supernatants were stored at -70°C and the titer of viruses in each sample was detected by $TCID_{50}$ using DF1 cells. The titer of each sample was calculated using a formula derived from Reed and Muench and Spearman-Karber methods (134).

**The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV challenge in SPF chickens**

H5N1 HPAIV protective experiment 1. Total number of 30 four-day-old white leghorn SPF chicks, purchased from Charles Rivers, were divided in three groups, each group ten chicks. The chicks of first and second groups were immunized with $10^{6.5}$ $TCID_{50}$/bird, 160 µl in volume, of rLaSota expressing HA protein and rAPMV-3 expressing HA protein, respectively. The birds of the third group were inoculated with

160µl PBS through oculonasal route. Three weeks after immunization, birds were transferred to the Biosafety level-3 plus facility and were infected with $10^5$ $EID_{50}$ of HPAIV strain A/Vietnam/1203/2004 (H5N1) by the oculonasal route. The infected chickens were observed daily for death and clinical signs of H5N1 HPAIV. At day four post-challenge, oropharyngeal swabs were taken from chickens and were kept in three ml cold DMEM containing 10 X antibiotics and amphotericin B. Each swab sample was centrifuged at 1500 rpm for 10 minutes and 200µl of supernatant of each swab sample was inoculated into one 10-day-old embryonated SPF chicken egg. Three days post-inoculation the allantoic fluids were tested by HA assay for the presence of challenge virus.

H5N1 HPAIV protective experiment 2. Total number of 30 day-old white leghorn SPF chicks, purchased from Charles Rivers, were divided in three groups, each group ten chicks. The chicks of the first and the second groups were immunized with 106 PFU/bird, 200µl in volume, of rLaSota expressing HA protein and rAPMV-3 expressing HA protein, respectively. The birds of the third group were inoculated with 200µl PBS through oculonasal route. Three weeks after immunization, 29 birds (one bird from PBS group died during the immunization time) were transferred to the Biosafety level-3 facilities and were infected with $10^6$ $EID_{50}$ of HPAIV strain A/Vietnam/1203/2004 (H5N1) by the oculonasal route. The post-challenge mortality recorded, and viral load was assessed as described in experiment 1.

**The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV challenge in broilers**

Total number of 30 day-old broilers were divided into six groups, five each. In a single or prime-boost immunization regimen (prime at day one and boost at week three of age), chicks were immunized with $10^6$ $TCID_{50}$/bird, 200µl in volume, of rLaSota expressing HA protein or rAPMV-3 expressing HA protein by the oculonasal route based on the immunization regimen for each group listed in table 1. At week five of age (five weeks-post prime immunization and two weeks post-boosting), birds were transferred to the Biosafety level-3 plus facilities and were infected with $10^6$ $EID_{50}$ of HPAIV strain A/Vietnam/1203/2004 (H5N1) by the oculonasal route. The post-challenge mortality was recorded, and viral load was assessed as described in experiment 1.

**The protective efficacy of rAPMV-3 or rNDV expressing HA protein against virulent NDV challenge in SPF chickens**

Total number of 15 four-day-old white leghorn SPF chicks, purchased from Charles Rivers, were divided into three groups, five each. The chicks of the first and the second groups were immunized with $10^{6.5}$ $TCID_{50}$/bird, 160µl in volume, of rNDV expressing HA protein and rAPMV-3 expressing HA protein, respectively. The birds of the third group were inoculated with 200µl PBS through oculonasal route. Three weeks after immunization, birds were transferred to the Biosafety level-3 facility and were infected with 100 $CLD_{50}$ of virulent NDV strain GB Texas by the oculonasal route. The infected chickens were observed daily for death and clinical signs of NDV.

**Serological analysis**

The antibodies induced against the HA protein of H5N1 virus in sera collected from immunized chickens were assessed by hemmagglutinin inhibition (HI) assay using an antigenicaly related H5N2 LPAIV (3). The LPAIV strain A/Mallard/Pennsylvania/10218/1984 (H5N2) was used instead of H5N1 HPAIV, because the experiment can be conducted at biosafety level-2 facility. The neutralizing antibodies induced against the LPAIV H5N2 in sera were assessed by micro-virus neutralization assay using a standard protocol. Briefly, sera were incubated at 56°C for 30 minutes. Serial two-fold dilution of each sera were prepared. 25µl of each dilution and 25µl of H5N2 LPAIV stock containing $10^2$ $TCID_{50}$ of virus were mixed and incubated at 37°C for 90 minutes. The titer of H5N2 LPAIV was determined by TCID50 on MDCK cells in presence of DMEM containing 1µg/ml TPCK-treated trypsin. The supernatant of monolayer of MDCK cell in 96-well plates were removed and cells were washed with DMEM. First 50 µl of DMEM were added to each well. Then 50 µl mixture of serum and virus was added to each well, three wells per each dilution of serum, and incubated at 37°C. After one hour adsorption, the supernatant of wells were removed and cells were washed with DMEM. One hundred µl DMEM containing 1µg/ml TPCK-treated trypsin was added to each well and incubated at 37°C. Three days after incubation, 50 µl of supernatant of each well were assessed for presence of LPAIV strain H5N2 virus by HA assay using 1% chicken RBC. The titer of neutralizing antibodies in each serum were calculated using a formula derived from Reed and Muench and Spearman-Karber methods (134). The antibodies induced against NDV strain LaSota and rAPMV-3 strain Netherlands in sera of immunized

chickens also were assessed by HI assay using the protocol of OIE (8 HAU antigen was used in HPAIV protective experiment-1 HI assay and 4 HAU antigen was used in other HI assays) (44).

All animal experiments involving rAPMV-3 and avirulent NDV strain LaSota were performed in our USDA approved Biosafety level-2 and Biosafety level-2 plus facilities and all animal experiments involving HPAIV and virulent NDV were conducted in our USDA approved Biosafety level-3 plus facilities following the guidelines and approval of the Animal Care and Use Committee (IACUC), University of Maryland.

**Table 6.1.** The groups of broilers immunized in single or prime-boost regimens.

| **Groups** | **Prime-immunization** | **Boost-immunization** |
|---|---|---|
| **A** | PBS | PBS |
| **B** | rLaSota/HA | rAPMV-3/HA |
| **C** | rLaSota/HA | - |
| **D** | rAPMV-3/HA | rLaSota/HA |
| **E** | rAPMV-3/HA | - |
| **F** | - | rAPMV-3/HA |

**Statistical analysis**

Data were analyzed among groups by One-Way-ANOVA test. The t-test was used to compare two groups. To avoid bias, HPAIV and NDV challenge experiments were designed as blinded studies.

*Result*

**Generation of rAPMV-3 expressing HA protein of H5N1 HPAIV and expression of HA protein by rAPMV-3 or rNDV**

A transcription cassette containing codon optimized HA gene of H5N1 HPAIV was inserted between P and M gene of rAPMV-3 (Fig.6.1). The rAPMV-3 expressing HA protein was recovered from the full length antigenomic cDNA plasmid and propagated in allantoic fluid of embryonated SPF chicken eggs. The full length antigenomic cDNA of LaSota containing HA gene plasmid that was constructed previously (135) also was recovered previously and was used in parallel with rAPMV-3 expressing HA protein for in vitro and in vivo comparisons. The rAPMV-3 expressing HA protein and rNDV strain LaSota expressing HA protein were named rAPMV-3/HA and rLaSota/HA, respectively.

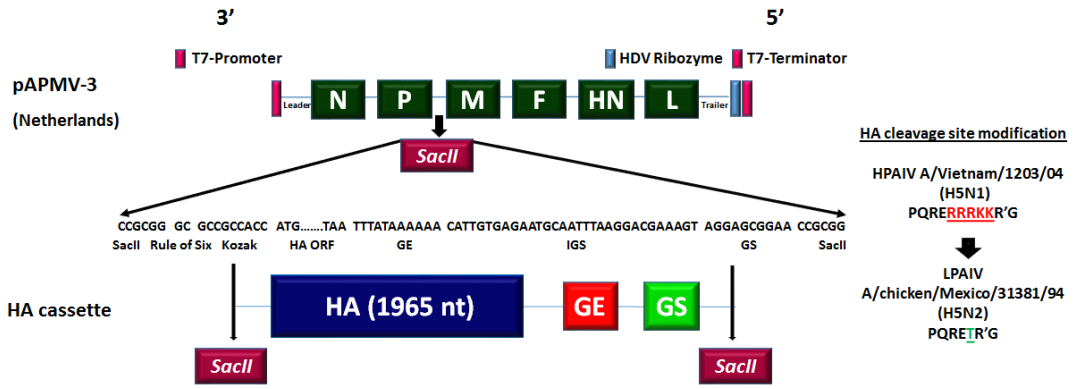


**Figure 30.** The schematic diagram for the construct of recombinant avian paramyxovirus serotype 3 (rAPMV-3) containing HA gene of HPAIV. The full- length antigenomic cDNA of APMV-3 strain Netherlands was constructed into a plasmid

BR322 previously. A transcription cassette containing ORF sequence of HA gene of H5N1 HPAIV was flanked between P and M genes of rAPMV-3. The HA gene transcription cassette contains sequences of following elements arranged in 3′ to 5′ order; SacII restriction enzyme site, GC nucleotides for rule of six, Kozak, HA gene ORF, GE of APMV-3 P gene that here it serves as GE for HA gene, IGS, GS of APMV-3 M gene and SacII restriction enzyme site. The polybasic cleavage site motifs (PQRERRRKKR'G) of HPAIV strain A/Vietnam/1203/2004 (H5N1) was modified to monobasic cleavage site motifs (PQRETR'G) of LPAIV strain A/Mexico/31381/94 (H5N2).

The expression of HA protein by rAPMV-3/HA and rLaSota/HA was detected in chicken embryo fibroblast (DF1) cell lysates by Western blot analysis (Fig.6.2A). Either rAPMV-3/HA or rLaSota/HA expressed HA protein efficiently. However, the expression of HA protein by rLaSota/HA in DF1 cells was detected at slightly higher level than that by rAPMV-3/HA. A ~60 kDa band in Fig.6.2A lane 3 represents HA1 subunit of cleaved HA0 protein expressed by rAPMV-3/HA. A ~70kDa band and a ~60 kDa band in Fig. 2A lane 4 represent HA1 protein and uncleaved HA0 protein expressed by rLaSota/HA, respectively. Lanes 1 and 2 of Fig. 2A represent DF1 cells and rLaSota as controls. The incorporation of HA protein into rAPMV-3 or rNDV particles also was detected by Western blot analysis (Fig.6.2B). The HA protein was incorporated into rAPMV-3/HA (Fig.6.2B lane 4) and rLaSota/HA (Fig.6.2B lane 2) particles. Three ~ 70, ~ 60 and ~25 kDa bands represent uncleaved HA protein (HA0), HA1 subunit and HA2 subunit, respectively.

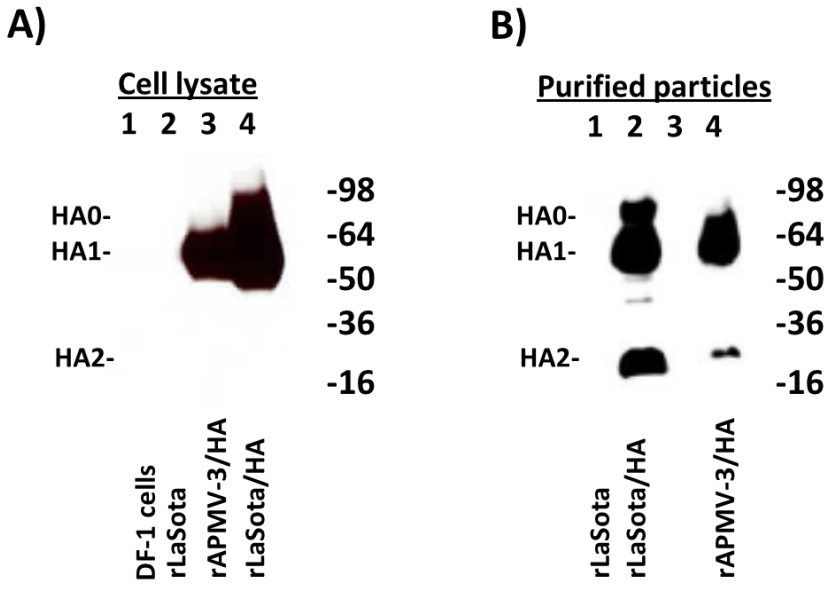


**Figure 31.** The expression of HA protein by rAPMV-3 or rNDV and incorporation of HA protein into rAPMV-3 or rNDV particles. The monolayer of DF1 cells were infected with rLaSota, rLaSota/HA or rAPMV-3/HA at 0.1 MOI. The cell lysates were collected 30 hours after infection. The expression of HA protein was detected by Western blot analysis using a polyclonal chicken anti H5N1 serum (A). Lanes 1-4 represents; DF1 cells, rLaSota, rAPMV-3 HA and rLaSota/HA, respectively. The recombinant viruses were inoculated in 10-day-embryonated SPF chicken eggs, the infected allantoic fluids were collected three days post-inoculation and were partially purified. The incorporation of HA protein into rAPMV-3 or rNDV particles were detected by Western blot analysis using the mentioned serum (B). The lanes 1-4 represent; rLaSota, rLaSota/HA, empty lane and rAPMV-3/HA, respectively.

**Growth Characteristics of rAPMV-3 or rNDV expressing HA protein of H5N1 HPAIV**

The rAPMV-3/HA and rLaSota/HA were passaged in 10-day-old SPF embryonated chicken eggs. Both recombinant viruses replicated efficiently in eggs. However, rAPMV-3/HA replicated slightly more efficiently in eggs with titer of about $2^8$-$2^9$ HAU/$_{50}$ µl than rLaSota/HA with titer of about $2^7$-$2^8$ HAU/$_{50}$ µl. Compared to rLaSota/HA, rAPMV-3/HA developed larger plaques in monolayers of DF1 cells in presence of DMEM containing 0.8% methylcellulose and 10% fresh allantoic fluid over layer (Fig.6.3C). The multicycle growth kinetics of recombinant viruses showed that rLaSota/HA and rAPMV-3/HA grew slightly slowly in DF1 cells than their corresponding recombinant parental viruses (Fig.6.3A and B).

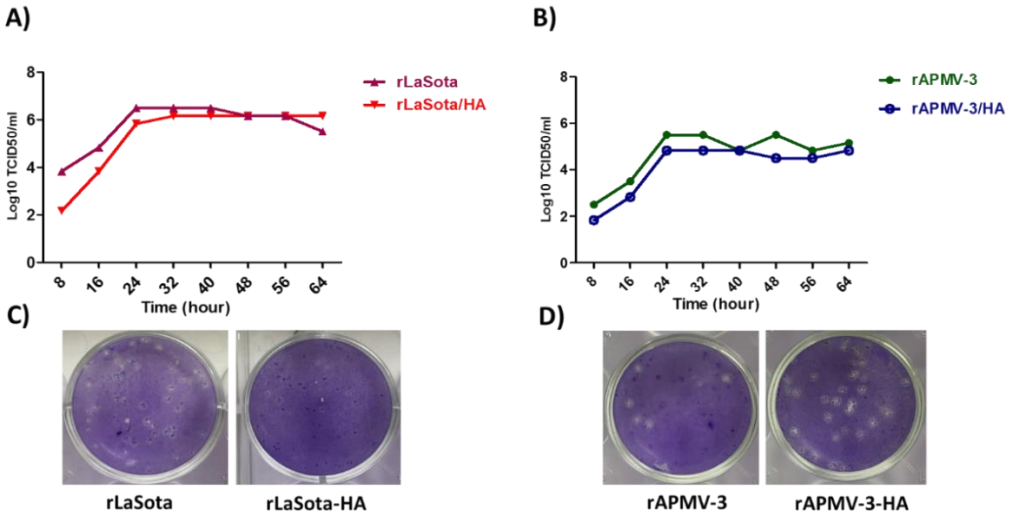


**Figure 32.** Multicycle growth kinetics and plaque morphology of rNDV or rAPMV-3 expressing HA protein in DF1 cells. The monolayers of DF1 cells were infected with rLaSota, rLaSota/HA, rAPMV-3 and rAPMV-3/HA at 0.1 MOI. The supernatant media containing virus were removed after one hour adsorbtion. The cells were washed and DMEM containing 10% allantoic fluid was added to cells. The titer of viruses in equal

volumes of supernatants collected from infected cells in 8 hours intervals were detected by $TCID_{50}$ using DF1 cells (A and B). The plaque morphology of rLaSota, rLaSota/HA, rAPMV-3 and rAPMV-3/HA in presence of 10% allantoic fluid were evaluated in DF1 monolayers (C).

**The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV in SPF chickens**

HPAIV protective experiment 1. In this study four-day-old SPF chicks were immunized by the oculonasal route with rAPMV-3/HA or rLaSota/HA and infected with $10^5$ 50% egg infectious dose ($EID_{50}$)/bird of H5N1 HPAIV by the oculonasal route at three weeks after immunization. The results showed that all chickens immunized with rAPMV-3/HA or rLaSota/HA were protected from mortality of HPAIV challenge until ten days post-challenge, whereas all unimmunized chickens died at day two after challenge ($P<0.05$)(Fig.6.4A). At day four post-challenge, the challenge virus was re-isolated from three out of ten chickens immunize with rAPMV-3/HA, while it was re-isolated from only one out of ten chickens immunized with rLaSota/HA (Fig.6.4B). Compared to rLaSota/HA, immunization of chickens with rAPMV-3/HA induced higher neutralizing antibodies ($P<0.05$) (Fig.6.4C) and higher HI titers ($P<0.05$) (Fig.6.4D) against HPAIV and corresponding backbone vector (Fig.6.4E).

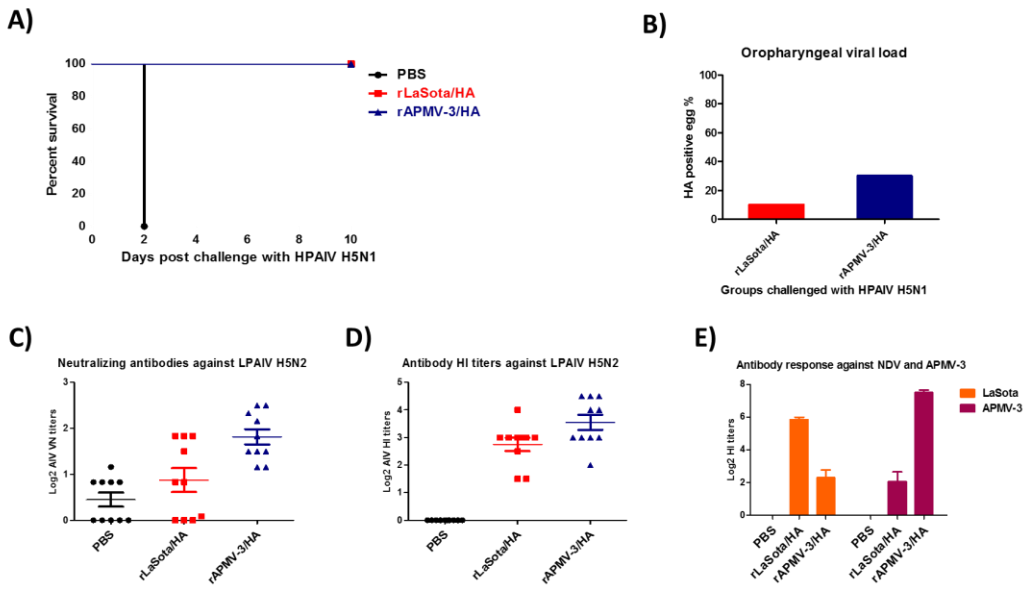


**Figure 33.** The induction of neutralizing antibodies and protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV in SPF chickens (experiment 1). Day old SPF chicks were immunized with $10^{6.5}$ $TCID_{50}$/bird of rLaSota/HA or rAPMV-3/HA through oculonasal route. Three weeks after immunization, chickens were infected with $10^5$ $EID_{50}$ of H5N1 HPAIV. The infected birds were observed for 10 days post-challenge and the mortality rate for each group were recorded daily (A). At day four post-challenge oropharyngeal swabs were taken from survived birds and each swab sample were inoculated in one 10-day-old embryonated eggs for re-isolation of challenge virus. The infected eggs were detected by HA assay (B). Three weeks post-immunization the sera were collected from all chickens and the level of antibodies induced against a H5N2 LPAIV were detected by micro-virus neutralization assay (C) and HI assay (D) and the antibody HI titers against NDV and APMV-3 also were detected by HI assay (E). Serum titers are expressed as reciprocals Log2 dilution.

HPAIV protective experiment 2. In this study day old SPF chickens were immunized with rAPMV-3/HA or rLaSota/HA and infected with a higher dose of $10^6$ $EID_{50}$/bird of H5N1 HPAIV at three weeks after immunization. The results showed that all chickens immunized with rAPMV-3/HA survived from HPAIV challenge. Nine out of ten chickens immunized with rLaSota/HA were protected from death of HPAIV until ten days post-challenge and one chicken died at day four after challenge. Whereas all non-vaccinated chickens died at day two after challenge ($P<0.05$) (Fig.6.5A). At day four post-challenge, the challenge virus was re-isolated from five out of ten chickens immunize with rAPMV-3/HA and four out of ten chickens immunized with rLaSota/HA (Fig.6.5B). Compared to rLaSota/HA, immunization of chickens with rAPMV-3/HA induced higher neutralizing antibodies (Fig.6.5C) and higher HI titers ($P<0.05$) (Fig.6.5D) against HPAIV and corresponding backbone vector (Fig.6.5E).

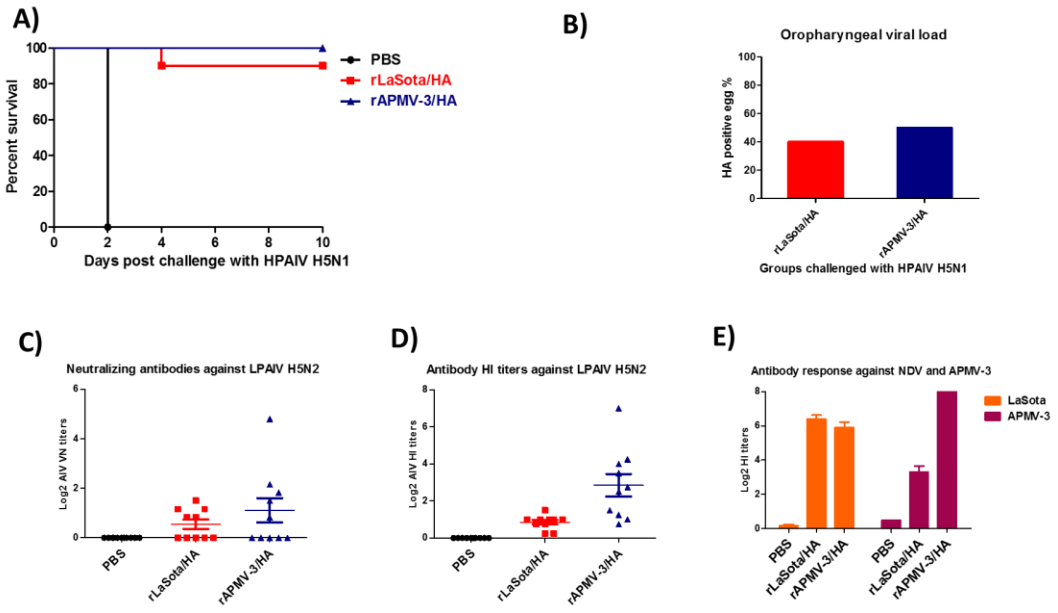

**Figure 34.** The induction of neutralizing antibodies and protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV in SPF chickens (experiment 2). Day old SPF chicks were immunized with $10^6$ PFU/ml of rLaSota/HA or rAPMV-3/HA through oculonasal route. Three weeks after immunization, chickens were infected with $10^6$ $EID_{50}$ of H5N1 HPAIV. The infected birds were observed for 10 days post-challenge and the mortality rate for each group were recorded daily (A). At day four post-challenge oropharyngeal swabs were taken from survived birds and each swab sample were inoculated in five 10-day-old embryonated eggs for re-isolation of challenge virus. The infected eggs were detected by HA assay (B). Three weeks post-immunization the sera were collected from all chickens and the level of antibodies induced against the H5N2 LPAIV were detected by micro-virus neutralization assay (C) and HI assay (D). The humoral antibody responses against NDV and APMV-3 also were detected by HI assay (E). Serum titers are expressed as reciprocals Log2 dilution.

**The protective efficacy of rAPMV-3 or rNDV expressing HA protein against H5N1 HPAIV in broilers**

Groups of broilers were immunized with either single or prime-boost regimens (prime immunization at day one of age and booster immunization at week three of age) of rAPMV-3/HA and/or rLaSota/HA listed in table1 and infected with $10^6$ $EID_{50}$/bird of H5N1 HPAIV at week five of age. The results showed that all immunized chickens with rAPMV-3/HA and/or rLaSota/HA, no matter which immunization regimen, survived from HPAIV challenge. Whereas all non-vaccinated broilers died at day one

after challenge (P<0.05) (Fig.6.6A). The challenge virus was re-isolated from no immunized chicken at day four after challenge.

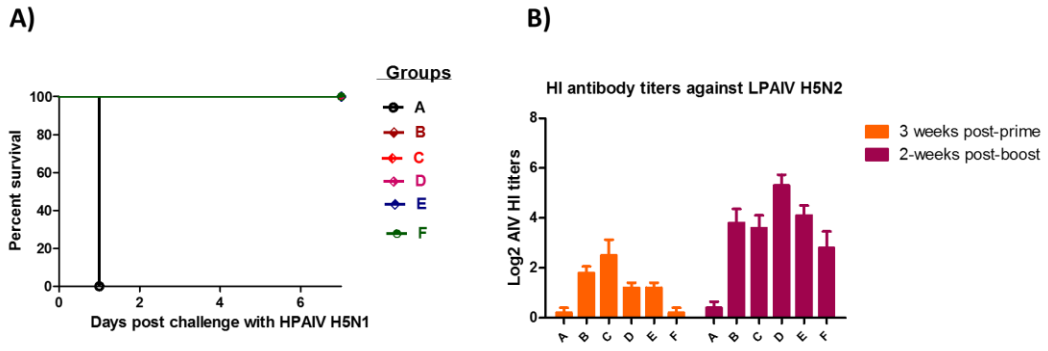


**Figure 35.** The protective efficacy of rAPMV-3 or rNDV expressing HA against H5N1 HPAIV in broilers. The groups of five broiler chickens were immunized with $10^6$ PFU/bird of rLaSota/HA and/or rAPMV-3/HA based on regimens of vaccination for each group mentioned in table 1 through oculonasal route. Chickens were infected with $10^6$ $EID_{50}$ of H5N1 HPAIV by the oculonasal route at week five of age. The infected birds were observed for 10 days post-challenge and the mortality rate for each group were recorded daily (A). Three weeks after prime-immunization and two weeks after boost-immunization the sera were collected from all chickens and the antibodies induced against a H5N2 LPAIV were detected by HI assay (B). Serum titers are expressed as reciprocals of Log2 dilution.

Single immunization of day old broilers with either rAPMV-3/HA or rLaSota/HA induced humoral antibodies against HPAIV (Fig.6.6B) and corresponding backbone vector (Fig.6.7A and 6.7B) and over time, antibody titers increased at five weeks after immunization (HI titers at five weeks post-immunization were higher than

those at three weeks post-immunization) (Fig,6.6B, 6.7A and 6.7B). Prime immunization of broilers with rAPMV-3/HA followed by a booster immunization with rLaSota/HA induced higher HI titers against HPAIV compared to the prime immunization of broilers with rLaSota-3/HA followed by a booster immunization with rAPMV-3/HA (Fig. 6.6B). The maternal derived antibodies (MDAs) HI titers against NDV strain LaSota was detected in non-infected control broilers at day two, weeks 1, 2, 3, 4 and 5 of age, and the titers decreased by the age (Fig.6.7D). The MDAs HI titers also was detected against rAPMV-3 at day two and week one of age (Fig.6.7C).

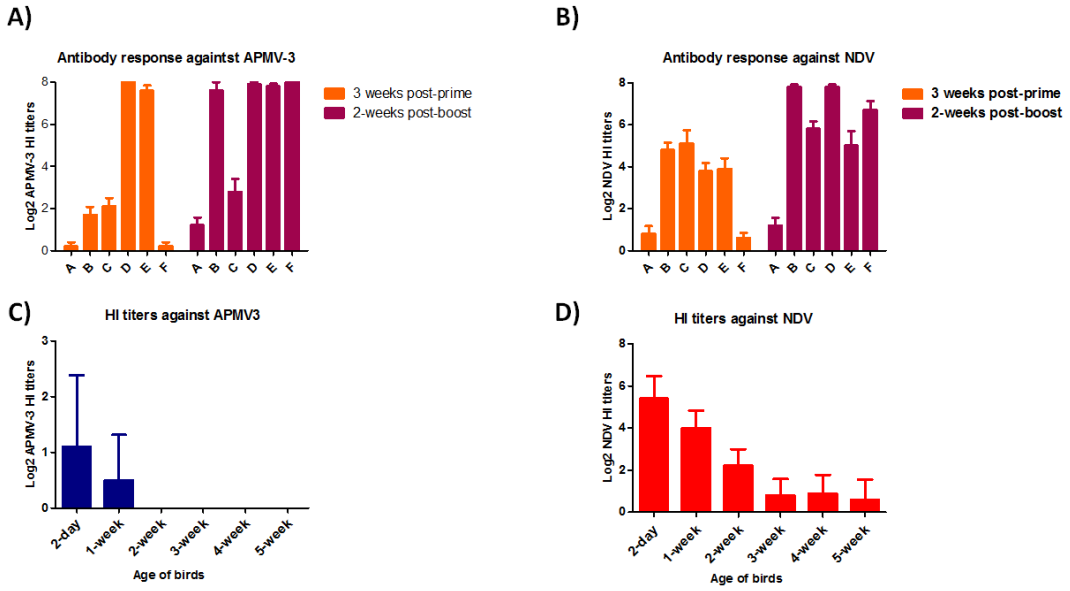


**Figure 36.** The antibody responses against APMV-3 and NDV by rAPMV-3 or rNDV expressing HA in broilers. The groups of broilers were immunized with $10^6$ $TCID_{50}$/bird of rLaSota/HA or rAPMV-3/HA based on regimen of vaccination for each group mentioned in table 1 through oculonasal route. Three weeks after prime-immunization and two weeks after boost-immunization the sera were collected from

all chickens and the antibodies induced against rAPMV-3 strain Netherlands (A) and rNDV strain LaSota (B) were detected by HI assay. The level of MDAs against rAPMV-3 strain Netherlands (C) and rNDV strain LaSota (D) in non-infected broilers also were detected by HI assay. Serum titers are expressed as reciprocals Log2 dilution.

**The protective efficacy of rAPMV-3 or rNDV expressing HA protein against virulent NDV challenge**

Four-day-old SPF chicks were immunized with rAPMV-3/HA or rLaSota/HA and infected with 100 50% chicken lethal dose ($CLD_{50}$)/bird of H5N1 HPAIV at three weeks after immunization.

The results showed that all chickens immunized with either rAPMV-3/HA or rLaSota/HA were protected from mortality and clinical signs of virulent NDV challenge until ten days post-challenge, whereas all non-immunized chickens died at days five and six after challenge (Fig. 6.8A). Compared to rLaSota/HA, immunization of chickens with rAPMV-3/HA induced higher HI titers (Fig.6.8B) against corresponding backbone vector. The cross-reactions were observed between antibodies induced by rLaSota/HA and rAPMV-3/HA against rAPMV-3 and LaSota, respectively (Fig.6.4B).

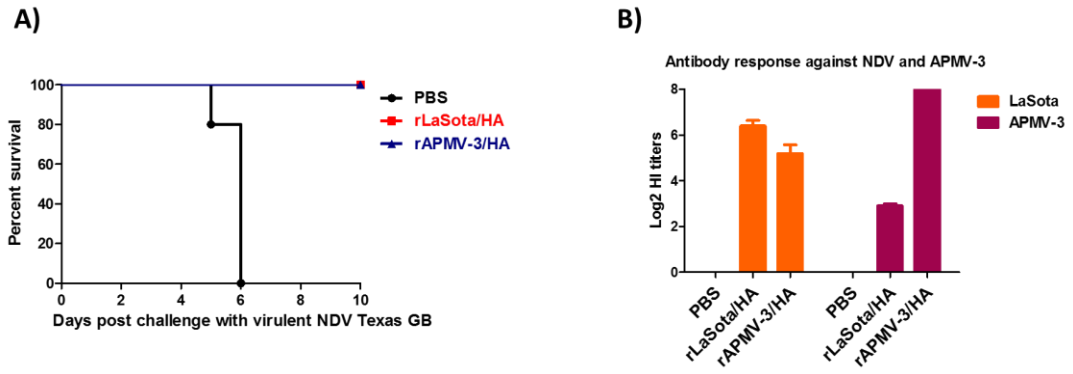


**Figure 37.** The antibody responses and the protective efficacy rAPMV-3 or rNDV expressing HA against NDV strain GB Texas in immunized SPF chickens. Four-day-old SPF chickens were immunized with $10^{6.5}$ $TCID_{50}$/ml of rAPMV-3/HA or rLaSota/HA through oculonasal route. Three weeks after immunization, chickens were infected with 100 $CLD_{50}$ of NDV strain GB Texas. The infected birds were observed for 10 days post-challenge and the mortality rate clinical signs of NDV were recorded daily (A).Three weeks post-immunization the sera were collected from all chickens and the level of antibodies induced against APMV-3 and NDV were detected by HI assay (B). Serum titers are expressed as reciprocals of Log2 dilution.

*Discussion*

rNDV strain LaSota has been found as a highly successful vaccine vector against HPAIV in chickens (26,32,33, 10,145-147). Although rLaSota vector induces good local and systemic responses, it replicates only in the respiratory tract. We feel that the immune response to the HA antigen can be enhanced by using another avirulent avian paramyxovirus vector that not only replicates in the respiratory tract, but also systemically. We chose to evaluate APMV-3 as a vaccine vector, because it does not

cause disease in chickens, replicates well in chickens and is not a Select Agent. APMV-3 possesses a multibasic amino acid motif at the FPCS, which allows it to replicate systemically resulting in induction of robust immune responses (46-50). A recent study showed that rAPMV-3 vector expressing the Ebola virus glycoprotein induced high levels of mucosal and humoral neutralizing antibodies against Ebola virus in guinea pigs (135). Both APMV-3 FPCS (A-R-P-R-G-R↓L) and virulent NDV strains FPCS (R-R-Q-K-R↓F) contain multibasic amino acids which is recognized by furin like proteases (47). But the FPCS of virulent NDV strains contain a F residue at the first position of F1 subunit and the FPCS of avirulent NDV strains contain a L residue at this position. It has been suggested that the presence of F residue versus L residue as the first residue of F1 subunit is associated with virulence (107). APMV-3 FPCS contains a L residue at this position. We think that although APMV-3 FPCS contains the furin recognition site (R-X-(R/K)-R↓), its lack of F residue at the first position of F1 subunit that maybe the reason why it is avirulent in chickens. Therefore, APMV-3 can replicate systemically, but does not cause disease in chickens. Hence, rAPMV-3 is an attractive vector to generate a recombinant HA-based vaccine against HPAIV. In this study, by employing reverse genetics techniques, we generated rAPMV3 strain Netherlands expressing the HA protein of H5N1 HPAIV. We evaluated the efficacy of rAPMV3 strain Netherlands expressing HA protein in comparison with the rNDV strain LaSota expressing HA protein in induction of neutralizing antibodies and protection against HPAIV and highly virulent NDV challenges in chickens.

Both rAPMV-3 and rNDV expressed HA protein at high levels in DF1 cells and the HA protein was found to be incorporated into both rAPMV-3 and rNDV particles. However the expression level of HA protein by rLaSota was slightly higher than that by rAPMV-3 in DF1 cells. The higher level of HA expression by the rNDV vector maybe due to the high level of replication of the vector in vitro in presence of exogenous protease. rAPMV-3 or rNDV expressing HA grew slightly slowly compared to their corresponding parental viruses in DF1 cells, which may be due to insertion of a foreign gene into the viral genome (152). Compared to rNDV vector, rAPMV-3 vector developed large size plaques in DF1 cells and grew in embryonated chicken eggs about 1log higher titer, as a result of the difference between FPCS of the two viruses (46,47).

Our results showed that compared to rLaSota expressing HA protein, rAPMV-3 expressing HA protein induced higher levels of neutralizing antibodies against HPAIV and against the corresponding backbone vector in SPF chickens. These results are in consistent with the result of a previous study indicating that rAPMV-3 replicates more efficiently than rLaSota in chickens leading to induction of stronger humoral antibody response (153). We also showed that prime immunization of broilers with rAPMV-3 expressing HA protein followed by a booster immunization with rNDV expressing HA compared to immunization of broiler chickens with rNDV expressing HA protein followed by a booster immunization with rAPMV-3 expressing HA protein resulted in higher levels of humoral antibody induced against HPAIV. These results are in agreement with the humoral antibodies outcomes from previous prime-boost

immunization studies in broilers using rNDV strain LaSota and a novel rNDV/APMV-2 vector expressing HPAIV HA protein (39,154). These results suggest that rAPMV-3 vectored vaccine was able to overcome MDA against NDV in one-day-old broiler chicks and was able to induce high level of HA-specific antibodies. Overcome of pre-existing antibodies has also been reported for other paramyxovirus vectors (155,156)

Immunization of chickens with rAPMV-3 expressing HA protein or rNDV expressing HA protein provided complete protection from death following HPAIV challenge. These results suggest that the immunity provided by rAPMV-3 or rNDV expressing HA protein was sufficient to provide protection against HPAIV challenge in chickens, in consistent with previous reports (2, 32,33, 10,145-147). Immunization of SPF chickens with either rAPMV-3 expressing HA or rNDV expressing HA decreased the post-HPAIV challenge oropharyngeal viral load, significantly. Despite the higher levels of neutralizing antibodies induced against HPAIV by rAPMV-3 expressing HA protein and by rNDV expressing HA protein, neither rAPMV-3 nor rNDV vector provided sterilizing protection against HPAIV in SPF chickens in two experimental challenges. In the case of broilers, single immunization or prime-boost immunization of chickens with rAPMV-3 or rNDV expressing HA protein resulted in eliciting sterilizing immunity. The absent of viral shedding following HPAIV challenge in broilers could be due to a low dose of challenge, the higher age of chickens at time of challenge, and/or the low number of eggs per swab sample for re-isolation of challenge virus. These results support the generally accepted view that it is difficult to achieve sterilizing protection against HPAIV by vaccination (23). However, in a very

well optimized challenge experiment a very low level of challenge virus may be isolated from chickens immunized with a highly efficacious vaccine that may be considered as sterilizing protection.

Both rAPMV-3 and rNDV vectors expressing the HA protein did not completely stopped challenge virus shedding. We do not consider this to be a major deficiency of these vector vaccines, because H5N1 HPAIV is a fast replicating and a highly infectious virus; therefore, complete cessation of replication of $10^6$ $EID_{50}$ of H5N1 HPAIV delivered directly into the nasal tract of a chicken will require very high levels of local and systemic immunity. In field conditions, vaccinated chickens will not encounter such high dose of infectious HPAIV. Therefore, we think that the levels of neutralizing antibody titers elicited by both vectors will be sufficient to provide complete protection against HPAIV in the field. Surprisingly, a slightly higher challenge virus shedding was found in chickens vaccinated with rAPMV-3 expressing HA than with rNDV expressing HA. The minor difference in shedding could be due to swab collection or swab processing. We feel that the level of neutralizing antibody titer in vaccinated chickens is a better indicator of protective immunity than determination of virus shedding by egg inoculation, which can give false negative results.

Our NDV challenge results showed that immunization of SPF chickens with rAPMV-3 or rNDV expressing HA protein provided complete protection from death against highly virulent NDV strain GB Texas challenge. Interestingly, there are only 31% and 34% amino acid identities between F and HN proteins of APMV-3 and NDV, respectively, but they provide cross-protection (46). This protection was likely

provided by some cross-reactive neutralizing epitopes in surface glycoproteins of APMV-3, which has been reported previously (157).

In summary, the results of this study showed that immunization of chickens with rAPMV-3 expressing HA protein or rNDV expressing HA protein provided complete protection from disease following HPAIV challenge. But the results showed that immunization of chickens with rAPMV-3 expressing HA protein induced significantly higher levels of neutralizing antibodies than the levels induced by rNDV expressing HA protein. Furthermore, the rAPMV-3 vector vaccine can overcome pre-existing antibodies to NDV. Taken together these results suggest that under field conditions, mass-vaccination with a rAPMV-3 expressing HA protein might provide better protection against H5N1 HPAIV than a rNDV expressing HA protein.

# Chapter 7

# Conclusion

HPAI, IB and ND are three most important contagious viral respiratory diseases in poultry. They cause huge economic losses in poultry industry around the world (1,2,3,4). HPAI is a devastating disease in poultry and is a serious threat to public health (2,21,22). IB causes huge economical losses due to the respiratory disease, poor carcass weight, poor egg quality and decreased egg production (6). Virulent NDV strains may cause up to 100% mortality in poultry populations (3,46). In addition to direct economic

losses of HPAI, IB and ND, the trade restrictions associated to theses contagious diseases also cause major economic losses in poultry industry(5). Vaccination is the major policy which is implemented to combat HPAIV, IBV and NDV worldwide. In contrary to inactivated vaccines, live vaccines are affordable, practical and more efficacious (17). However, among HPAIV, IBV and NDV, only NDV possesses naturally avirulent strains which have been used as highly safe vaccines for more than 60 years (3). Furthermore, the nature of NDV genome causes that the recombination is rare and the mutation rate is very low in NDV genome (46). On the other side, the reassortment and mutation rates in HPAIV genome and recombination and mutation rates in IBV genome are very high(8,9,19). The reverse genetics has made it possible to employ avirulent NDV strains as vaccine vectors to develop vaccines against different pathogens. Therefore, in this multistep study we have employed NDV vector and novel APMV vectors to develop improved IBV and HPAIV vaccines.

The first study was conducted to investigate the protective efficacies of the S, S1, and S2 proteins of IBV using rNDV strain LaSota as a vaccine vector and to develop a safe and efficacious rNDV vectored IBV vaccine. It was shown that amino acids within S1 and S2 subunits of IBV spike protein are responsible for major and minor neutralizing epitopes, respectively. Here we showed that although S1 subunit

contain major neutralizing epitopes, the interaction of S1 and S2 subunits in intact S protein is required for expressing the neutralizing epitopes in correct conformation. The neutralizing epitopes within S1 subunit were expressed in correct conformation only in the intact S protein. The results suggest that S2 protein acts as a chaperone to assist in the folding of the S1 protein. The S1 protein is folded incorrectly in the absence of S2 protein and the new structure probably causes loss of major conformational epitopes for IBV antibodies. Our results showed that the S protein, which contains the S1 and S2 neutralizing epitopes in correct confirmation is the best protective antigen of IBV. We showed that immunization of chickens with rNDV expressing S protein was sufficient to provide protection against virulent IBV based on USDA-CFR-9 and OIE criteria for live IBV vaccines. However, our result showed that the age at immunization, the IBV challenge dose and the route of challenge affects the challenge outcomes. Furthermore, the results showed immunization of chicks with rNDV expressing IBV S protein provided complete protection against virulent NDV challenge. Taken together, we have shown that the S protein is the antigen of choice for vectored IBV vaccines. NDV is an attractive vaccine vector for IBV, because it can be used as a bivalent vaccine. Our results suggest that a recombinant NDV vectored IBV vaccine is the vaccine of choice for controlling IBV infection in the field.

In the second study, other than r NDV strain LaSota vector, we employed a novel chimeric rNDV/APMV-2 vector, with less efficient replication, and rLaSota-527 vector, with more efficient replication, to develop a likely improved viral vectored vaccine against IBV. We generated rNDV/APMV-2 or rLaSota-527 expressing the intact S protein of IBV. Our results showed that other than rLaSota expressing S protein of IBV, the novel chimeric rNDV/APMV-2 expressing S protein, which is a better potential candidate for *in ovo* vaccination, provided protection against IBV in chickens. Most importantly, our results showed that compared to single immunization with rLaSota expressing S protein, over vaccination of chickens with rNDV or rNDV/APMV-2 expressing S protein and/or live IBV vaccines or single immunization of chickens with rLaSota-527 expressing S protein, with increased replication and immunogenicity, provided poorer protection against IBV. These results suggest that a single immunization of chickens with wild type rNDV strain LaSota expressing S protein induced balanced immune responses resulting to provide better protection against IBV.

The third study was conducted to evaluate the individual contributions of HA1 and HA2 subunits of HPAIV HA protein in induction of neutralizing antibodies and protection in chickens as a highly susceptible natural host model for HPAIV. We have used reverse genetics techniques to generate recombinant rNDVs expressing HA1, HA2, or HA protein of H5N1 HPAIV. Our results showed that the HA1 and HA2 subunits when separated, neither provided protection nor induced neutralizing antibodies. In the absence of stalk, the globular head of HA protein was not expressed

efficiently by the rNDV vector and in the absence of transmembrane (TM) and cytoplasmic tail (CT), it was not incorporated into NDV envelope. These results suggest that the important epitopes for induction of protective and neutralizing antibodies are formed by interaction of HA1 and HA2 subunits. These epitopes are lost when the two subunits are separated. The disulfide bond and unknown interactions between HA1 and HA2 subunits is necessary to maintain the native conformations of head and stalk domains. Altogether, this study suggest that a H5N1 vaccine construct incorporating either HA1 or HA2 subunit would likely not provide protection against HPAIV challenge in chickens. To be effective the vaccine must incorporate the intact HA protein. These results also highlight the importance of using chickens as highly permissive natural model in HPAIV vaccine studies.

In the fourth study, other than rNDV strain LaSota, we employed APMV-3 strain Netherlands as a vaccine vector, which has high efficiency replication in a greater range of host organs, to generate an improved vaccine against HPAIV. Our results showed that immunization of chickens with either rAPMV-3 expressing HA protein or rNDV expressing HA protein provided complete protection from disease following HPAIV challenge, however, compared to chickens immunized with rNDV expressing HA protein, the post-challenge viral load in chickens immunized with rAPMV-3 expressing HA protein was slightly higher. But the results showed that immunization of chickens with rAPMV-3 expressing HA protein induced significantly higher levels of neutralizing antibodies than the levels induced by rNDV expressing HA protein. Furthermore, the rAPMV-3 vector vaccine can overcome pre-existing antibodies to

NDV. Taken together, these results suggest that under field conditions, mass-vaccination with a rAPMV-3 expressing HA protein might provide better protection against H5N1 HPAIV than a rNDV expressing HA protein.

In summary, our results showed that although individual globular head and stalk domains of the S protein of IBV or the HA protein of HPAIV contains major and minor neutralizing epitopes, respectively, a vaccine construct incorporating either globular head or stalk domains of S or HA protein would likely not provide protection against IBV or HPAIV challenge in chickens. To be effective the vaccine must incorporate the intact S or the intact HA protein. Furthermore, we showed that other than rNDV strain LaSota vector, which induced balanced immune responses, a novel chimeric rNDV/APMV-2 and rAPMV-3 are promising vaccine vectors against IBV for *in ovo* immunization and against HPAIV for mass immunization under the field conditions, respectively.

*Perspective and future directions*

1. To develop improved recombinant chimeric APMVs IBV vaccines.
2. To develop recombinant chimeric APMVs vectored IBV vaccines for *in ovo* vaccination.
3. To develop rAPMVs vectored the S proteins of two antigenically diverse IBV strains to provide a broad protection against IBV in chickens.
4. To develop rAPMVs vectored the HA proteins of two antigenically diverse HPAIV strains to provide a broad protection against HPAIV in chickens.

# Bibliography


1. OIE. 2013. Chapter 2.3.2. Avian Infectious Bronchitis. Version adopted by the World Assembly of Delegates of the OIE in May 2018. In OIE terrestrial manual.www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.02_AIB.pdf.

2. OIE (World Organization for Animal Health). Avian Influenza. In OIE TerrestrialManual,2015Chapter2.3.4,http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf.

3. OIE (World Organization for Animal Health). Newcastle disease, In OIE TerrestrialManual,2012,Chapter2.3.14,http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14_NEWCASTLE_DIS.pdf

4. Cook, J. K. A., Jackwood M. & Jones R. C. The long view: 40 years of infectious bronchitis research. Avian Pathology. 41, 239-250 (2014).

5. OIE-Listed disease, infectious and infestations in force in 2019, http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019/.

6. Cavanagh, D. & Gelb, J. Jr. Infectious bronchitis. In Diseases of Poultry (ed. Saif, Y.M et al.) 117-135 (Ames, IA: Blackwell Publishing, 2008).

7. International Committee on Taxonomy of Viruses (ICTV) 2018, https://talk.ictvonline.org/taxonomy.

8. Masters, P. S. & Perlmon S. Coronaviruses. In Fields Virology (ed. Knipe, D. M. et al.) 825-858 (Lippincott Williams & Wilkins, 629 Philadelphia, 2013).

9. Cavanagh, D, Coronavirus avian infectious bronchitis virus. Veterinary Research. 38, 281–297 (2007).

10. Lai, M.M.C. & Cavanagh D. The molecular biology of coronaviruses. Adv Virus Res. 48:1â€“100 (1997).

11. Belouzard, S., Millet, J.K., Licitra, B.N. & Whittaker, G.R. Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses. 4, 1011-1033(2012).

12. Promkuntod, N., Van Eijndhoven, R. E., De Vrieze, G., Grone, A. & Verheije, M. H. Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus. Virology. 448:26–32(2014).

13. Cavanagh, D., Davis, P.J. & Mockett, A.P.A. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein

are associated with neutralization epitopes. Virus Research, 11, 141-150 (1988).

14. Ignjatovic, J. & Sapats, S. Identification of previously unknown antigenic epitopes on the S and N proteins of avian infectious bronchitis virus. Arch Virol. 150, 1813-1831(2005).

15. Collisson, E. W., Pei, J., Dzielawa, J. & Seo, S. H. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Developmental & Comparative Immunology. 24, 187–200 (2000).

16. Ladman, B. S., Pope, C. R. & Ziegler, A. F. Protection of chickens after live and inactivated virus vaccination against challenge with nephropathogenic infectious bronchitis virus PA/Wolgemuth/98. Avian Diseases. 46, 938–944(2002).

17. Meeusen, E. N. T., Walker, J., Peters, A., Pastoret, P. P. & Jungersen G. Current status of veterinary vaccines. Clinical Microbiology Reviews. 20, 489–510(2007).

18. Bande, F., Arshad, S. S., Bejo, M. H., Moeini, H. & Omar AR. Progress and challenges toward the development of vaccines against avian infectious bronchitis. Journal of Immunology Research.ID 424860(2015).

19. Palese, P. & Shaw, M.L. Orthomyxoviridae: The Viruses and Their Replication. In Fields Virology (ed. Knipe D.M, Howley P.M). 1151-1186 (Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2013).

20. Suarez, D.L. Influenza A virus. In Animal Influenza (ed. Swayne, DE) 3-30 (Animal Influenza, Wiley- Blackwell, Ames, IA, 2006).

21. Sutton, T.C. The Pandemic Threat of Emerging H5 and H7 Avian Influenza Viruses, Viruses.10, 461(2018).

22. Harfoot, R., Webby, R.J., H5 influenza, a global update, Journal of Microbiology. 55, 196–203(2017).

23. Swayne, D.E. Impact of vaccines and vaccination on global control of avian influenza. Avian Disease. 56, 818–828 (2012).

24. Swayne, D.E., Pavade, G., Hamilton, K., Vallat, B., Miyagishima, K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. Rev. Sci. Tech. 30, 839–870(2011).

25. Swayne, D. E., Principles for Vaccine Protection in Chickens and Domestic Waterfowl against Avian Influenza Emphasis on Asian H5N1 High Pathogenicity Avian Influenza, Ann. N.Y. Acad. Sci. 1081, 174–181(2006).

26. Veits, J., Wiesner, D., Fuchs, W., Hoffmann, B., Granzow, H., Starick, E., Mundt, E., Schirrmeier, H., Mebatsion, T., Mettenleiter, T.C., Römer-Oberdörfer, A. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. Proc. Natl. Acad. Sci., 103, 8197–8202(2006).

27. Balzli, C.L., Bertran, K., Lee, D.H., Killmaster, L., Pritchard, N., Linz, P., Mebatsion, T., & Swayne DE. The efficacy of recombinant turkey herpesvirus vaccines targeting the H5 of highly pathogenic avian influenza virus from the 2014–2015 North American outbreak. Vaccine 36: 84–90 (2018).

28. Bublot, M., Pritchard, N., Cruz JS, Mickle, T.R., Selleck, P., Swayne, D.E. Efficacy of a fowl pox-vectored avian influenza H5 vaccine against Asian H5N1 highly pathogenic avian influenza virus challenge. Avian Dis. 51, 498–500 (2007).

29. Toro, H. & Tang, D.C. Protection of chickens against avian influenza with non-replicating adenovirus-vectored vaccine. Poul. Sci. 88, 867–871 (2009).

30. Pavlova, S.P., Veits, J., Mettenleiter, T.C. & Fuchs, W. Live vaccination with an H5-hemagglutinin-expressing infectious laryngotracheitis virus recombinant

protects chickens against different highly pathogenic avian influenza viruses of the H5 subtype. Vaccine 27, 5085–5090(2009).

31. Cui, H. et. al. Avirulent Marek's disease virus type 1 strain 814 vectored vaccine expressing avian influenza (AI) virus H5 haemagglutinin induced better protection than turkey herpesvirus vectored AI vaccine. PLoS One, 8.e53340(2013).

32. Nayak, B., Kumar, S., DiNapoli, J.M., Paldurai, A., Perez, D.R., Collins, P.L. & Samal S.K. Contributions of the avian influenza virus HA, NA, and M2 surface proteins to the induction of neutralizing antibodies and protective immunity. J. Virol. 84, 2408–2420(2010).

33. Suarez, D.L., Pantin-Jackwood, M.P. Recombinant viral-vectored vaccines for the control of avian influenza in poultry. Veterinary Microbiology 206, 144–151(2017).

34. Klenk, H.D., Rott, R., Orlich, M., Blo¨dorn, J. Activation of influenza A viruses by trypsin treatment. Virology, 68, 426–439 (1975).

35. Lazarowitz, S. G., Choppin, W. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology, 68, 23, 440–454(1975).

36. Kirkpatrick, E., Qiu, X., Wilson, P.C., Bahl, J., Krammer, F. The influenza virus hemagglutinin head evolves faster than the stalk Domain, Scientific Reports. 8, 10432 (2018).

37. Koutsakos, M.; Kedzierska, K.; Subbarao, K. Immune Responses to Avian Influenza Viruses, Journal of Immunology 2019, 202, 382-391.

38. Kim, S.H. & Samal, S.K. Newcastle disease virus as a vaccine vector for development of human and veterinary vaccines. Viruses. 8, 183 (2016).

39. Kim, S.H. & Samal, S.K. Innovation in Newcastle Disease Virus Vectored Avian Influenza Vaccines, Viruses. 11, 3, 300 (2019).

40. Sutton, T.C., Chakraborty, S., Mallajosyula, V.V.A., Lamirande, E.W., Ganti, K., Bock, K.B., Moore, I.N., Varadarajan, R., Subbarao, K. Protective efficacy of influenza group 2 hemagglutinin stem-fragment immunogen vaccines, npj Vaccines, 2, 35 (2017).

41. Krammer, F. & Palese, P. Universal influenza virus vaccines that target the conserved hemagglutinin stalk and conserved sites in head domain. The Journal of Infectious Diseases, 219 (2019).

42. Yassine, H.M., Boyington, J. F., McTamney, P.M., Wei, C.J., Kanekiyo, M., Kong, W.P., Gallagher, J.R., Wang, L., Zhang, Y., Joice, M.G. Hemagglutinin-

stem nanoparticles generate heterosubtypic influenza protection. Nature Medicine, 21 (2015).

43. Mallajosyula, V.V.A. et. al. Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. PNAS, E2515(2014).

44. Thangavel, R.R. & Bouvier, N.M. Animal models for influenza virus pathogenesis, transmission, and immunology. J Immunol Methods. 0, 60-79 (2014).

45. Zeng, H. et. al. Tropism and infectivity of a seasonal A(H1N1) and a highly pathogenic avian A(H5N1) influenza virus in primary differentiated ferret nasal epithelial cell cultures. Journal of Virology, 23, 2019.

46. Samal, S.K. Newcastle disease and related avian paramyxoviruses. pp.69-114. In The Biology of Paramyxoviruses (ed. Samal S.K.), 69-114 (Caister Academic Press, Norfolk, UK, 2013).

47. Alexander, D.J. Avian paramyxoviruses. Veterinary Bulletin 50, 737–752 (1980).

48. Conzelmann, K.K. NONSEGMENTED NEGATIVE-STRAND RNA VIRUSES: Genetics and Manipulation of Viral Genomes Annu. Rev. Genet. 32, 123–62(1998).

49. Kim, S.H., Xiao, S., Shive, H., Collins, P.L., Samal, S.K. Replication, neurotropism, and pathogenicity of avian paramyxovirus serotypes 1-9 in chickens and ducks. PloS one. 7, e34927(2012).

50. Kumar, S., Militino, D.F., Nayak, B., Collins, P.L. & Samal S.K. Experimental avian paramyxovirus serotype-3 infection in chickens and turkeys. Veterinary research 41, 2010042 (2010).

51. Kim, S.H., Paldurai, A. & Samal S. K. A novel chimeric Newcastle disease virus vectored vaccine against highly pathogenic avian influenza virus. Virology, 503, 31-36 (2017).

52. Manoharan V. K., Khattar S. K., Paldurai A., Kim S. H., Samal S. K. A Y527A mutation in the fusion protein of Newcastle disease virus strain LaSota leads to a hyperfusogenic virus with increased replication and immunogenicity. J Gen Virol. 97, 287-92 (2016).

53. Liu, D. & Inglis, S. Identification of two new polypeptides encoded by mRNA5 of the coronavirus infectious bronchitis virus. Virology, 186, 342–347 (1992).

54. Liu, D. & Inglis, S. Internal entry of ribosomes on a tricistronic mRNA encoded by infectious bronchitis virus. J Virol. 66, 6143–6154 (1992).

55. Liu, D., Cavanagh, D., Green, P., Inglis, S. A polycistronic mRNA specified by the coronavirus infectious bronchitis virus. Virology. 184, 531–544 (1991).

56. Heald-Sargent, T., Gallagher, T., Ready, F, The coronavirus spike protein and acquisition of fusion competence. Viruses. 4, 557–80 (2012).

57. Shang, J. et al. Cryo-EM structure of infectious bronchitis coronavirus spike protein reveals structural and functional evolution of coronavirus spike proteins. PLoS Pathog, 14(2018).

58. Neuman, B.W. et. al. A structural analysis of M protein in coronavirus assembly and morphology, J Structure Biol, 174, 11-22 (2011).

59. Vennema, H.. et al. Nucleocapsid-indipendent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. EMBO J, 15, 2020-2028(1996).

60. Sturman, L.S., Holmes, K.V., Behnke, J. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. Journal of virology. 33, 449–462 (1980).

61. Winter, C., Schwegmann-Weßels, C., Cavanagh, D., Neumann, U., and Herrler, G. Sialic acid is a receptor determinant for infection of cells by avian infectious bronchitis virus. Journal of General Virology, 87,1209–1216 (2006).

62. Shahwan, K., Hesse, M., Mork, A.K., Herrler, G. and Winter, C. Sialic acid binding properties of soluble coronavirus spike (S1) proteins: differences between infectious bronchitis virus and transmissible gastroenteritis virus, Viruses. 5, 1924–1933 (2013).

63. Madu, I.G., Chu, V.C., Lee, H., Regan, A.D., Bauman, B.E., and Whittaker, G.R. Heparan sulfate is aselective attachment factor for the avian coronavirus infectious bronchitis virus Beaudette. Avian Diseases, 51, 45–51 (2007).

64. Chu, V. C., McElroy, L.J., Chu, V., Bauman, B.E., and Whittaker, G.R. The avian coronavirus infectious bronchitis virus undergoes direct low-pH dependent fusion activation during entry into host cells. J. Virol. 80, 3180–3188(2006).

65. Brierley, I., Digard, P., Inglis, S.C. Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot. Cell, 57, 537–547 (1989).

66. Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J Gen Virol. 81, 853–879 (2000).

67. Mielech, A.M., Chen, Y., Mesecar, A.D., Baker, S.C. Nidovirus papain-like proteases: Multifunctional enzymes with protease, deubiquitinating and deISGylating activities. Virus research (2014).

68. Sethna, P.B., Hofmann, M.A., Brian, D.A., Minus-strand copies of replicating coronavirus mRNAs contain antileaders. Journal of virology. 65, 320–325 (1991).

69. Keck, J.G., Makino, S., Soe, L.H., Fleming, J.O., Stohlman, S.A., Lai, M.M. RNA recombination of coronavirus. Advances in experimental medicine and biology. 218:99–107 (1987).

70. de Haan, C.A, Rottier, P.J. Molecular interactions in the assembly of coronaviruses. Adv Virus Res. 64, 165–230 (2005).

71. Sisk, J.M., Frieman, M.B., and Machamer, C.E., Coronavirus S protein-induced fusion is blocked prior to hemi fusion by Abl kinase inhibitors. Journal of General Virology, 99, 619–630 (2018).

72. Cavanagh, D., Mawditt, K., Welchman, D.D.B., Britton, P. & Gough, R.E. Coronaviruses from pheasants (Phasianus colchicus) are genetically closely

related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys, Avian Pathology, 31, 81–93 (2002).

73. Dea, S. & Tijssen, P. “Detection of turkey enteric coronavirus by enzyme-linked immunosorbent assay and differentiation from other coronaviruses,” American Journal of Veterinary Research, 50, 226–231 (1989).

74. Jonassen, C.M. et al Molecular identification and characterization of novel coronaviruses infecting graylag geese (Anser anser), feral pigeons (Columbia livia) and mallards (Anas platyrhynchos). Journal of General Virology, 86, 1597–1607 (2005).

75. Cavanagh, D. Coronaviridae: a review of coronaviruses and toroviruses. In Coronaviruses with Special Emphasis on First Insights Concerning SARS. (ed Cavangah D.) 1–54, (Birkh¨auser, Basel, Switzerland, 2005).

76. Circella, E., Camarda, A., Martella, V., Bruni, G., Lavazza, A., & Buonavoglia, C. Coronavirus associated with an enteric Advances in Virology 9 syndrome on a quail farm. Avian Pathology, 36, 3, 251–258 (2007).

77. Bande, F., Arshad, S.S., Omar, A.R., Bejo, M.H., Abubakar, M.S., Yusuf, Abb., Pathogenesis and Diagnostic Approaches of Avian Infectious Bronchitis, Advances in Virology, 4621659(2016).

78. Chhabra, R., Chantrey, J., Ganapathy, K. Immune responses to virulent and vaccine strains of infectious bronchitis virus in chickens. Viral Immunology, 28, 475-488(2015).

79. King, D. & Cavanagh, D. Infectious bronchitis. Diseases of Poultry, 9,471–484 (1991).

80. Wit J. J. & Cook K. A. Factors influencing the outcome of infectious bronchitis vaccination and challenge experiments. Avian Pathology 43, 485-497(2014).

81. Nix, W. A., Trorber, D. S., Kingdom, B. F., Keeler, J. C. L. & Gelb, J. J. Emergence of subtype strains of the Arkansas serotype of infectious bronchitis virus in Delmarva broiler chickens. Avian Disease. 44, 568-581(2000).

82. McKinley, E. T., Hilt, D. A. & Jackwood, M. W. Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. Vaccine. 26, 1274–1284(2008).

83. Lee, S. W. et al. Attenuated vaccines can recombine to form virulent field viruses. Science. 337, 188(2012).

84. Wakenell, P. S., Sharma, J. M. & Slocombe, R. F. Embryo vaccination of chickens with infectious bronchitis virus: histologic and ultrastructural lesion

response and immunologic response to vaccination. Avian Diseases, 39, 752-765(1995).

85. Tarpey, I., Orbell, S. J. & Britton, P. Safety and efficacy of an infectious bronchitis virus used for chicken embryo vaccination. Vaccine, 24, 6830–6838(2006).

86. Shi, X. M., Zhao, Y. & Gaoetal H. B. Evaluation of recombinant fowl pox virus expressing infectious bronchitis virus S1 gene and chicken interferon-$\gamma$ gene for immune protection against heterologous strains. Vaccine. 29, 1576–1582(2011).

87. Johnson, M. A., Pooley, C., Ignjatovic, J. & Tyack, S. G. A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. Vaccine. 21, 2730–2736(2003).

88. Toro, H. et al. Infectious bronchitis virus S2 expressed from recombinant virus confers broad protection against challenge. Avian Disease. 58, 83-89(2014).

89. Zhao, R. et al. Recombinant Newcastle disease virus expressing the infectious bronchitis virus S1 gene protects chickens against Newcastle disease virus and infectious bronchitis virus challenge. Vaccine. 35, 2435-2442(2017).

90. Chen, H. Y., Yang, M. F. & Cui, B. A. Construction and immunogenicity of a recombinant fowl pox vaccine co-expressing S1 glycoprotein of infectious bronchitis virus and chicken IL-18. Vaccine. 28, 8112–8119(2010).

91. Parks, G. D. & Lamb, R. A. Paramyxoviridae. In Fields Virology (ed. Knipe, D. M.) 957-996 (Lippincott Williams & Wilkins, 629 Philadelphia, 2013).

92. Krishnamurthy, S. & Samal, S. K. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. J. Gen. Virol. 79:2419–2424 (1998).

93. de Leeuw, O. & Peeters, B. Complete nucleotide sequence of Newcastle disease virus: Evidence for the existence of a new genus within the subfamily Paramyxovirinae. J. Gen. Virol. 80:131–136(1999).

94. Huang, Y., Wan, H.Q., Liu, H.Q., Wu, Y.T., Liu & X.F. Genomic sequence of anisolate of Newcastle disease virus isolated from an outbreak in geese: a novelsix nucleotide insertion in the non-coding region of the nucleoprotein gene. Brief Report. Arch. Virol. 149 (7), 1445–145(2004).

95. Czegledi A, Ujvari D, Somogyi E, Wehmann E, Werner O, et al. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 120: 36–48 (2006).

96. Murphy, S.K. & Parks, G.D. Genome nucleotide lengths that are divisible by six are not essential but enhance replication of defective interfering RNAs of the paramyxovirus simian virus 5. Virology 232:145–57(1997).

97. Calain, P. & Roux, L. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. J. Virol. 67, 4822-4830(1993).

98. Huang, Z., Krishnamurthy, S., Panda, A, Samal, S.K. Newcastle Disease Virus V Protein Is Associated with Viral Pathogenesis and Functions as an Alpha Interferon Antagonist J Virol.16: 8676–8685(2003).

99. Park, M.S., García-Sastre, A., Cros, J.F., Basler, C.F., Palese, P. Newcastle Disease Virus V Protein Is a Determinant of Host Range Restriction. Journal of Virology, 9522–9532 (2003).

100. Kolakofsky, D., Boy de la Tour, E., Bruschi, A. Self-annealing of Sendai virus RNA. J. Virol. 14:33–39(1974).

101. Morrison, T.G. Structure and function of paramyxovirus fusion protein. Biochemica et Biophysica Acta 1614:73-84(2003).

102. Gravel, K.A. & Morrison T.G. Interacting Domains of the HN and F Proteins of Newcastle Disease Virus, J Virol. 77:11040–11049(2003).

103. Huang, Z., Panda, A., Elankumaran, S., Govindarajan, D., Rockemann, D.D, Samal S.K. The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. J Virol. 78:4176-84(2004).

104. Rout, S.N., Samal, S.K. The large polymerase protein is associated with the virulence of Newcastle disease virus. J Virol. 82:7828-36(2008).

105. Alexander, D.J. & Allan, W.H. Newcastle disease virus pathotypes. Avian Pathol. 3:269-78(1974).

106. Choi, K.S., Lee, E.K., Jeon, W.J., Kwon, J.H. Antigenic and immunogenic investigation of the virulence of the Newcastle disease virus fusion protein. J. Vet. Sci. 11,205-211(2010).

107. Kattenbelt, J.A., Stevens, M.P., Gould, A.R. Sequence variation in the Newcastle disease virus genome. Virus Res. 116:168-184(2006).

108. Collins, M.S., Strong, I, Alexander, D.J. Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses Arch Virol. 134:403-11(1994).

109. Huang, Z., Krishnamurthy, S., Panda, A., Samal, S.K. High-level expression of a foreign gene from the most 39-proximal locus of a recombinant Newcastle disease virus. J Gen Virol 82:1729–1736(2001).

110. Zhao, W., Zhang, Z., Zsak, L., and Yu, Q. P and M gene junction is the optimal insertion site in Newcastle disease virus vaccine vector for foreign gene expression. J. Gen. Virol. 96, 40–45.(2015).

111. Yoshida, A. & Samal, S.K. Avian Paramyxovirus type-3 as a vaccine vector: identification of a genome location for high level expression of a foreign gene. Frontiers in Microbiology journal. 8, 693(2017).

112. Carnero, E. Li, W. Borderia, A.V. Moltedo, B. Moran, T. & García-Sastre, A. Optimization of human immunodeficiency virus Gag expression by Newcastle disease virus vectors for the induction of potent immune responses. J. Virol. 83, 584–597(2009).

113. Zhao, H. & Peeters, B.P.H. Recombinant Newcastle disease virus as a viral vector: Effect of genomic location of foreign gene on gene expression and virus replication. J. Gen. Virol. 84, 781–788(2013).

114. Niesters, H.G. et al. The neutralization epitopes on the spike protein of infectious bronchitis virus and their antig enic variation. Adv Exp Med Biol. 218, 483–492(1987).

115. Promkuntod, N., Van Eijndhoven, R. E., De Vrieze, G., Grone, A. & Verheije, M. H. Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus. Virology. 448:26–32(2014).

116. Casais, R., Dove, B., Cavanagh, D. & Britton, P. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol. 77, 9084–9089(2003).

117. Nayak, B. et al. Immunization of chickens with Newcastle disease virus expressing H5 hemagglutinin protects against highly pathogenic H5N1 avian influenza viruses. PLoS One. 4, 6509 (2009).

118. Schroer, D. et al. Vaccination with Newcastle disease virus vectored vaccine protects chickens against highly pathogenic H7 avian influenza virus. Avian Disease. 53, 190–197(2009).

119. Nagy, A. et al. Recombinant Newcastle disease virus expressing H9 Ha protects chickens against heterologous avian influenza H9N2 virus challenge. Vaccine. 34, 2537-2545(2016).

120. Basavaajappa, M. K. et al. A recombinant Newcastle disease virus (NDV) expressing infectious laryngotracheitis virus (ILTV) surface glycoprotein D protects against highly virulent ILTV and NDV challenges in chickens. Vaccine. 32, 3555-3563(2014).

121. Reed, L.J. & Muench, H.A. A simple method of estimating fifty percent endpoints. Am J Hyg. 27, 493-497 (1983).

122. Yoshida, A. & Samal, S.K. Avian Paramyxovirus type-3 as a vaccine vector: identification of a genome location for high level expression of a foreign gene. Frontiers in Microbiology journal. 8, 693(2017).

123. U. S. Department of Agriculture (USDA). USDA Title 9. Code of federal regulations, standard requirements for IBV vaccines https://www.law.cornell.edu/cfr/text/9/113.327.

124. Eldemery, F., Kellye, S., Toro J.H. & Van Santen, V.L. Protection against infectious bronchitis virus by spike ectodomain subunit vaccine. Vaccine, 35, 5864 – 5871(2017).

125. Kim, S.H., Subbiah, M., Samuel, A.S., Collins, P.L., Samal, S.K. Roles of the fusion and hemagglutinin-neuraminidase proteins in replication, tropism, and pathogenicity of avian paramyxoviruses J. Virol., 85:8582-8596(2011).

126. Shirvani, E., Paldurai, A., Manoharan, V.K., Varghese, B.P., Samal, S.K., A Recombinant Newcastle Disease Virus (NDV) Expressing S Protein of Infectious Bronchitis Virus (IBV) Protects Chickens against IBV and NDV. Sci Rep. 10(8)(2018).

127. Jordan, B. Vaccination against infectious bronchitis virus: A continuous challenge, Veterinary Microbiology 206:137–143(2017).

128. Hohdatsu, T., Yamada, M., Tominaga, R., Makino, K., Kida, K., Koyama, H. Antibody-dependent enhancement of feline infectious peritonitis virus infection in feline alveolar macrophages and human monocyte cell line U937 by serum of cats experimentally or naturally infected with feline coronavirus. J Vet Med Sci. 60:49-55(1998).

129. Wang, S.F., Tseng, S.P., Chia-Hung, Y., Yang, J.Y., Tsao, C.H., Shen, C.W., Chen, K.F., Liu, F.T., Liu, W.T., Chen, Y.M.A., Huang, J.C. Antibody-dependent SARS coronavirus infection is mediated by antibodies against spike

proteins, Biochemical and Biophysical Research Communications 451:208–214(2014).

130. Krammer, F. et al. Assessment of Influenza Virus Hemagglutinin Stalk-Based Immunity in Ferrets. Journal of Virology, 88, 6 3432–3442(2014).

131. Steel, J., Lowen, A.C., Wang, T.T., Yondola, M., Gao, Q., Haye, K., García-Sastre, A., Palesea, P. Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain, mBio, 1,1(2010)

132. Gao, W. et al. Protection of Mice and Poultry from Lethal H5N1 Avian Influenza Virus through Adenovirus-Based Immunization, Journal of Virology, 80, 959–1964(2006).

133. Sutton, T.C., Chakraborty, S., Mallajosyula, V.V.A., Lamirande, E.W., Ganti, K., Bock, K.B., Moore, I.N., Varadarajan, R., Subbarao, K. Protective efficacy of influenza group 2 hemagglutinin stem-fragment immunogen. vaccines, npj Vaccines, 2, 35(2017).

134. Ramakrishnan, M.A.; Determination of 50% endpoint titer using a simple formula, World J Virol. 5, 2, 85–86(2016).

135. Kim, S.H., Paldurai, A., Xiao, S., Collins, P.L., Samal, S.K. Modified Newcastle disease virus vectors expressing the H5 hemagglutinin induce

enhanced protection against highly pathogenic H5N1 avian influenza virus in chickens. Vaccine, 32, 4428-35(2014).

136. Silva, M.D., Labas, V., Nys, Y., Rehault-Godbert, S. Investigating proteins and proteases composing amniotic and allantoic fluids during chicken embryonic development. Poultry Science , 96,2931-2941(2014).

137. Krammer, F., Palese, P. Influenza virus hemagglutinin stalk-based antibodies and vaccines Curr Opin Virol. 3,521–530(2013).

138. Wang, T.T., Tan, G.S., Hai, R., Pica, N., Petersen, E., Moran, T.M., Palese, P. Broadly Protective Monoclonal Antibodies against H3 Influenza Viruses following Sequential Immunization with Different Hemagglutinins. PLoS Pathogen, 6,2 (2010)

139. Laursen, N.S. & Wilson, I.A. Broadly neutralizing antibodies against influenza viruses Antiviral Res. 98,3, 476–483(2013).

140. Nayak, B. Rout, S.N., Kumar, S., Khalil, M.S., Fouda, M.M., Ahmed, L.E., Kenneth, C. E., Perez, D.R., Collins, P.L., Samal, S.K. Immunization of chickens with Newcastle disease virus expressing H5 hemagglutinin protects against highly pathogenic H5N1 avian influenza viruses. PLoS One , 4, 6509(2009).

141. Wang, W., Christopher, J.D.F., Alvarado-Facundo, E., Vassell, R., Weiss, C.D. Intermonomer Interactions in Hemagglutinin Subunits HA1 and HA2Affecting Hemagglutinin Stability and Influenza Virus Infectivity, Journal of Virology, 89(2015).

142. Swayne, D.E. et al. Antibody Titer Has Positive Predictive Value for Vaccine Protection against Challenge with Natural Antigenic-Drift Variants of H5N1 High-Pathogenicity Avian Influenza Viruses from Indonesia, Journal of Virology, 89,7 (2015).

143. Jang, H., Elaish, M., Mahesh, K.C., Abundo1, M.C., Ghorbani, A., Ngunjiri, J.M., Lee, C.W. Efficacy and synergy of live-attenuated and inactivated influenza vaccines in young chickens, Plos one, 13, 4(2018).

144. Li, Z., Gabbard, J.D., Mooney, A., Chen, Z., Tompkins, S.M., He, B. Efficacy of Parainfluenza virus 5 mutants expressing hemagglutinin from H5N1 influenza A virus in mice. Journal of Virology 87,9604-9609(2013).

145. Römer-Oberdörfer, A., Veits, J., Helferich, D., Mettenleiter, T.C. Level of protection of chickens against highly pathogenic H5 avian influenza virus with Newcastle disease virus based live attenuated vector vaccine depends on

homology of H5 sequence between vaccine and challenge virus. Vaccine, 26, 2307–2313(2008).

146. Lardinois, A., Steensels, M., Lambrecht, B., Desloges, N., Rahaus, M., Rebeski, D., van den Berg, T. Potency of a recombinant NDV-H5 vaccine against various HPAI H5N1 virus challenges in SPF chickens. Avian Dis. 56,928–936(2012).

147. Cornelissen, L.A.H.M., de Leeuw, O.S., Tacken, M.G., Klos, H.C., de Vries, R.P., de Boer-Luijtze, E.A., van Zoelen-Bos, D.J., Rigter, A., Rottier, P.J., Moormann, R.J., de Haan, C.A. Protective efficacy of Newcastle disease virus expressing soluble trimeric hemagglutinin against highly pathogenic H5N1 influenza in chickens and mice. PLoS ONE 7:e4444(2012).

148. Nagai, Y., Klenk, H.D., Rott, R. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology, 72,496-508(1976).

149. Bukreyev, A., Huang, Z., Yang, L., Elankumaran, S., Claire, M., Murphy, B.R., Samal, S.K., Collins, P.L. Recombinant Newcastle disease virus expressing a foreign viral antigen is attenuated and highly immunogenic in primates. J. Virol. 79,13275–13284(2005).

150. Yoshida, A. & Samal, S.K. Avian Paramyxovirus Type-3 as a Vaccine Vector: Identification of a Genome Location for High Level Expression of a Foreign. Gene. Frontiers in microbiology 8:00693(2017).

151. Kumar, S., Nayak, B., Collins, P.L., Samal, S.K. Evaluation of the Newcastle disease virus F and HN proteins in protective immunity by using a recombinant avian paramyxovirus type 3 vector in chickens. J. Virol. 85, 6521–6534(2011).

152. Bukreyev, A., Skiadopoulos, M.H., Murphy, B.R., Collins, P.L. Nonsegmented negative-strand viruses as vaccine vectors. J Virol 80, 10293-10306(2006).

153. Nayak, B., Dias, F.M., Kumar, S., Paldurai, A., Collins, P.L., Samal, S.K. Avian paramyxovirus serotypes 2-9 (APMV-2-9) vary in the ability to induce protective immunity in chickens against challenge with virulent Newcastle disease virus (APMV-1). Vaccine ;30:2220-7 (2012).

154. Kim, S.H. & Samal, S.K. Heterologous prime-boost immunization of Newcastle disease virus vectored vaccines protected broiler chickens against highly pathogenic avian influenza and Newcastle disease viruses. Vaccine, 35,4133-4139(2017).

155. Chen, Z., Xu, P., Salyards, G.W., Harvey, S.B., Rada, B., Fu Z.F., He, B. Evaluating a parainfluenza virus 5-based vaccine in a host with pre-existing immunity against parainfluenza virus 5. Plose one 7(2012)

156. Yang, L., Sanchez, A., Ward, J.M., Murphy, B.R., Collins, P.L., Bukreyev, A. A paramyxovirus-vectored internasal vaccine against Ebola virus is immunogenic in vector-immune animals. virology 377:225-264(2008).

157. Alexander, D.J., Chettle, N.J., Parsons, G. Resistance of chickens to challenge with the virulent Herts 33 strain of Newcastle disease virus induced by prior infection with serologically distinct avian paramyxoviruses. Res Vet Sci. 26, 198–201(1979).