EXPRESSION, PURIFICATION AND QUANTITATION OF HEMAGGLUTININ-NEURAMINIDASE FOR DOSE-RANGING ASSESSMENT OF A RECOMBINANT PROTEIN-BASED NEWCASTLE DISEASE SUBUNIT VACCINE

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LIST OF SYMBOLS

%	Percentage
˚C	Degree celcius
±	Plus or minus
×	Multiplication
>	Greater than
≥	Greater than or equal to
≤	Less than or equal to
V	Voltage

LIST OF ABBREVIATIONS

AF	Allantoic fluids
AMPV	Avian paramyxovirus
BCA	Bicinchoninic acid
BME	β-mercaptoethanol
BSL 2	Biosafety level 2
BSL-3-Ag	Biosafety level 3 agricultural
C	Cloacal
$CaCl_2$	Calcium chloride
CH_3COONa	Sodium acetate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	Critical micelle concentration
cRBCs	Chicken red blood cells
CT	Cytoplasmic
CV	Coefficient of variation
DDM	n-dodecyl-β-D-maltoside
DIVA	Differentiation of infected from vaccinated animal
Dpc	Day-post-challenge
Dpv	Day-post-vaccination
ECE	Embryonated Chicken Eggs
EDTA	Ethylenediaminetetraacetic acid
EID_{50}	50% Embryo infectious dose
ELISA	Enzyme-linked immunosorbent assay
F	Fusion
FBS	Fetal bovine serum
GOIs	Genes of interest
HA	Hemagglutination
HAC	Hydroxyapatite chromatography
HAU	Hemagglutination unit
HCl	Hydrochloric acid
HI	Hemagglutination-inhibition
HN	Hemagglutinin-neuraminidase

HPLC	High performance liquid chromatography
HyNPV	Hybrid baculovirus expression vector
IACUC	Institutional Animal Care and Use Committee
IC-BEVS	Insect cell-baculovirus expression vector system
ICPI	Intracerebral pathogenicity index
IEC	Ion-exchange chromatography
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
L	Large
LC/MS-MS	Liquid chromatography-mass spectrometry
M	Matrix
MOI	Multiplicity of infection
MUNANA	2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid
MWCO	Molecular weight cutoff
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Dibasic sodium phosphate
NaCl	Sodium chloride
NaH_2PO_4	Disodium phosphate
$NaH_2PO_4•H_2O$	Monobasic monohydrate sodium phosphate
ND	Newcastle disease
NDV	Newcastle disease virus
NP	Nucleoprotein
OTG	octyl-β-D-1-thioglucopyranoside
P	Phosphoprotein
P	Probability
PBS	Phosphate buffer saline
PMSF	Phenylmethylsulfonyl fluoride
Q	Quaternary ammonium
rHN	NDV HN with a hexa-histidine fusion tag
rpm	Revolutions per minute
SC	sodium cholate hydrate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
*Sf*21	*Spodoptera frugiperda* 21

SP	Sulfopropyl
SPF	Specific-pathogen-free
TM	Transmembrane
TMB	3,3′,5,5′-Tetramethylbenzidine
TX-100	Triton X-100
TX-114	Triton X-114
WB	Western blot

EXPRESSI, PENULENAN DAN KUANTIFIKASI HEMAGGLUTININ-NEURAMINIDASE UNTUK PENILAIAN PENJULAT DOS VAKSIN SUBUNIT PENYAKIT NEWCASTLE BERASASKAN PROTEIN REKOMBINAN

ABSTRAK

Vaksin subunit berasaskan hemagglutinin-neuraminidase rekombinan (HN) yang tidak patogenik dan dihasilkan menggunakan kultur sel serangga adalah merupakan alternatif yang berpotensi untuk menggantikan vaksin konvensional aktif and nyahaktif penyakit Newcastle. Namun begitu, pembangunan proses untuk penghasilan dan penilaian keberkesanan vaksin subunit HN dikekangi oleh ketiadaan piawai rujukan, yang merupakan asas kepada kaedah analisis kuantitatif yang mantap dan sensitif. Dalam kajian ini, strategi penulenan hiliran telah dibangunkan untuk memperoleh NDV HN yang telah diekspresi dengan tag gabungan hexa-histidine (rHN) untuk memudahkan pengesanan menggunakan antibodi generik. Seterusnya, rHN berketulenan tinggi (~95%) diperolehi dari pengekstrakan detergen dan penulenan dua peringkat yang melibatkan kromatografi kolom pertukaran ion-hidroksiapatit telah digunakan sebagai piawai rujukan bagi pembangunan ELISA kuantitatif. Lengkung kalibrasi untuk ELISA yang dibangunkan didapati linear di antara 15.6–1000 ng mL^{-1}. Ketepatan inter and intra asai yang dinyatakan sebagai pekali variasi (CV) didapati masing-masing kurang daripada 10% and 12%. Kuantiti pemulihan rHN pada daripada tahap penulenan yang berbeza kemudiannya dipantau. Kuantiti rHN dari lisat sel kasar juga ditentukan menggunakan kaedah ELISA bagi membolehkan kajian julat dos respon antibodi dan keberkesanan perlindungan terhadap penyakit. Dos rHN (1500 ng) yang lebih tinggi telah didapati berkolerasi dengan pengurangan ketara bagi peluhuran

virus dan pencapaian imuniti kelompok, seperti yang ditunjukan oleh nisbah ayam yang lebih tinggi dengan perlindungan titer antibodi HI $\geq \log_2 3$. Pada kesimpulannya, hasil kajian ini menekankan kepentingan pembangunan proses hiliran dalam membolehkan kuantifikasi yang mantap dan penilaian potensi vaksin subunit rekombinan.

EXPRESSION, PURIFICATION AND QUANTITATION OF HEMAGGLUTININ-NEURAMINIDASE FOR DOSE-RANGING ASSESSMENT OF A RECOMBINANT PROTEIN-BASED NEWCASTLE DISEASE SUBUNIT VACCINE

ABSTRACT

Recombinant hemagglutinin-neuraminidase (HN) based subunit vaccine, which is non-infectious and can be produced using insect cell-culture systems, is a potential alternative to conventional live and inactivated Newcastle disease (ND) vaccines. However, process development for manufacture and efficacy assessment of HN based subunit vaccines has been hampered by the absence of reference standards, a cornerstone for robust and sensitive quantitative analytical methods. In this work, a downstream purification strategy was developed to obtain NDV HN which was expressed with a hexa-histidine fusion tag (rHN) to facilitate detection using generic antibodies. Highly purified rHN (~95%) attained after detergent extraction and two-stage ion-exchange-hydroxyapatite column chromatography was subsequently utilized as reference standards for quantitative ELISA development. The calibration curve for the developed ELISA was found to be linear between the range of 15.6–1000 ng mL^{-1}. The intra- and inter-assay precision expressed as a coefficient of variation (CV) were less than 10% and 12% respectively. Recovery of rHN at different stages of purification was monitored. Quantitation of rHN from crude cell lysates was subsequently performed for dose-ranging antibody response and protective efficacy studies. A higher dose (1500 ng) of rHN was correlated to a significant reduction in virus shedding and attainment of herd immunity, as indicated by a higher proportion of chickens (92%) with hemagglutination inhibition (HI) antibody titers $\geq \log_2 3$. The outcome of this study,

shows the importance of downstream process development in enabling robust quantitation and efficacy assessment of a recombinant subunit vaccine.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Newcastle disease (ND) is a highly contagious avian disease with high morbidity and mortality rates. In response to significant economic losses caused by ND outbreaks, strict biosecurity practices and routine administration of vaccines have been implemented for disease control and prevention. In spite of these measures, ND outbreaks continue to occur (Choi et al., 2013b; Shohaimi et al., 2015) leading to questions about current vaccine efficacy (Cornax et al., 2012; Kapczynski & King, 2005) and vaccination practices (Dortmans et al., 2012; van Boven et al., 2008). Suboptimal immunity can be attributed to antigenic divergence between vaccination and challenge strains (Miller et al., 2007; Yang et al., 2017), poor vaccine quality (Dortmans et al., 2012) and inadequate antigen content (Cornax et al., 2012; Maas et al., 2003). Recent advances in recombinant technology have allowed the generation of genotype matched virus-vectored (Cho et al., 2008; Miller et al., 2009a) and protein-based subunit vaccines (Lee et al., 2008) to overcome the issue of antigenic divergence. Recombinant ND subunit vaccines have been shown to be protective against virus challenge and could possibly facilitate serological differentiation between infected and vaccinated animals (DIVA) (Lee et al., 2008; Zoth et al., 2009).

The Newcastle disease virus (NDV), the etiological agent of ND, fusion (F) and hemagglutinin-neuraminidase (HN) integral membrane proteins are responsible for virus attachment and membrane fusion during infection (Nagy et al., 1990; Yuan et al., 2012). They are host immune response targets and have been extensively evaluated for subunit vaccines development (Lee et al., 2008; Mori et al., 1994). HN have been

expressed using bacteria (Jiang et al., 2015), yeast (Kang et al., 2016), plant (Gomez et al., 2009) and insect cell (Choi et al., 2013a; Lee et al., 2008) hosts. The insect cell-baculovirus expression vector system (IC-BEVS) is suitable for high-level expression of biologically active recombinant proteins and have been utilized for commercial production of vaccines against porcine circovirus (Chae, 2012) and swine fever virus (Depner et al., 2001). In comparison to conventional egg-based manufacture of live and inactivated ND vaccines, IC-BEVS overcomes the need for a continuous supply of specific-pathogen-free (SPF) embryonated eggs (Cox, 2012; Wang et al., 2006) and require shorter production times (Lin et al., 2018). In addition, with the utilization of a hybrid baculovirus expression vector (HyNPV) (Mori et al., 1992), production of a recombinant target protein can also be achieved with silkworm larvae and pupae hosts to attain further cost-efficiency (Fukanoki et al., 2001; Mori et al., 1994).

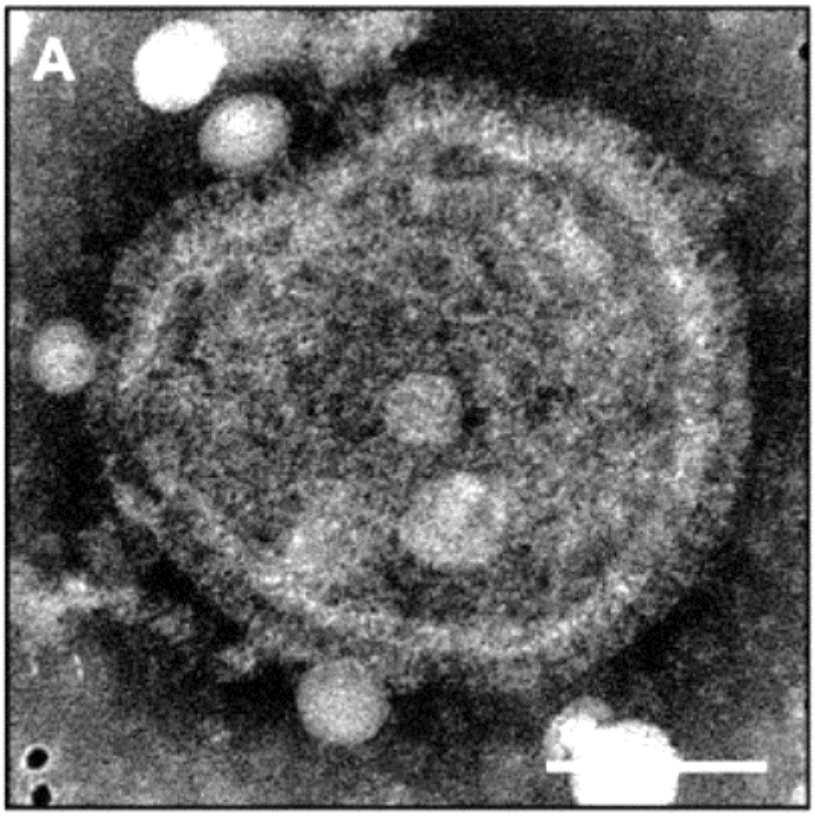


Figure 1.1 Electron microscopy of Newcastle disease virus (NDV) stained with phosphotungstic acid (PTA) (Scale bar: 100 nm). The figure was adapted from Mast & Demeestere (2009).

Despite the advance in recombinant subunit production, the correlation between HN based subunit vaccine antigen content to antibody response and protective efficacy have been poorly understood due to a lack of robust and sensitive quantitative analytical methods (Kamiya et al., 1994; Lee et al., 2008). Dose ranging studies are important as inactivated ND vaccines with suboptimal antigen content has been previously found to result in lower protection from mortality and a higher incidence of virus shedding (Cornax et al., 2012; Liljebjelke et al., 2008). Based on current guidelines from the World Organization for Animal Health (OIE), a ND vaccine is considered efficacious if greater than 90% protection from morbidity and mortality is attained after challenge

(Miller et al., 2009a; OIE, 2018). A reduction in virus shedding after NDV challenge has not been included in current ND vaccine efficacy evaluation guidelines (Cardenas-Garcia et al., 2015). However, there are views that virus shedding is also a critical parameter in evaluation of ND vaccines, as high incidences of virus shedding increases the risk of infection and transmission post-challenge (Miller et al., 2009a; Miller et al., 2007; van Boven et al., 2008). The effect of an optimal antigen content was found to be most pronounced in terms of virus shedding (Cornax et al., 2012; Liljebjelke et al., 2008). Determination of an optimal vaccine content is also important from a cost-perspective as real world utility of veterinary vaccines demand that vaccination costs be lower than cost of livestock.

For conventional live and inactivated ND vaccines generated by egg-based manufacturing, antigen content is usually estimated via the determination of 50% embryo infectious dose (EID50) or hemagglutination (HA) assay (Cornax et al., 2012; Liljebjelke et al., 2008). Although HA assay can be applied to preparations without live viruses as starting material, the assay which relies on HN agglutination capabilities requires fresh red blood cells and has insufficient quantitation sensitivity (Carvalho et al., 2017) as required for vaccine formulation and dose-range studies. Since both EID50 and HA assays are impractical for HN based subunit vaccines, previous dose-ranging studies for these subunit vaccines have been performed by varying the number of insect cells which was used as expression hosts (Kamiya et al., 1994; Lee et al., 2008). Nevertheless, replication of this cell number approach was difficult as variation in antigen expression levels can occur within a study or between studies (Imasaki et al., 2018). Evaluation on the effect of HN content on subunit vaccine efficacy which was previously conducted using the cell number approach also does not allow for direct correlation between HN content to antibody response, protective efficacy (morbidity

and mortality) and viral shedding as the actual amount of HN could not be determined. Therefore, alternative quantitative analytical methods that allow for absolute antigen quantitation are necessary to facilitate dose-ranging studies with HN.

A reference standard is critical to the development of immunochemical (Western blot (WB) and ELISA) and chromatographic (RP-HPLC and LC/MS-MS) based quantitative analytical methods. Although successful expression of HN using insect cell expression hosts have been demonstrated (Lee et al., 2008), to the best of our knowledge, a suitable method to obtain HN of high purity has not been reported. The availability of highly purified HN would allow for total protein quantitation via protein assays such as micro bicinchoninic acid (BCA) without interference from other proteins. Accordingly, reference standards can then be prepared and utilized for the development of widely applicable quantitation methods such as enzyme-linked immunosorbent assay (ELISA). To facilitate the development of an ELISA that is less subject to antigenic variability, generic antibodies targeting fusion tags (Li et al., 2009) which can be added to the HN can be also utilized.

In this study, the expression of properly folded recombinant HN with a hexa-histidine tag (rHN) at the N-terminal was first inferred from biological activity assays. Subsequently, a downstream purification strategy involving detergent extraction and two-stage ion-exchange-hydroxyapatite column chromatography was developed to obtain highly purified rHN. Purified rHN was then utilized as reference standards for development of an ELISA based analytical method. The method was applied for quantitation of rHN from crude cell lysates prior to formulation of subunit vaccines for dose-ranging antibody response and dose-ranging protective efficacy and viral shedding studies.

1.2 Research objectives

The specific objectives for the present study are:

1. To express and characterize expressed rHN for use as a reference standard and evaluation as a protein-based subunit vaccine candidate.
2. To develop a detergent extraction and purification strategy to obtain rHN of high purity.
3. To develop a quantitative ELISA utilizing high purity rHN as reference standards and measure the intra- and inter-assay precision of the method.
4. To determine the optimal rHN subunit vaccine dose required to induce protective HI antibody titer, protect against morbidity and mortality, reduce virus shedding and achieve herd immunity.

CHAPTER 2

LITERATURE REVIEW

2.1 Newcastle disease

Newcastle disease (ND) is a highly contagious avian disease that causes devastating economic losses to commercial producers and small scale poultry farmers. In 2012, a total of 1,211 cases of ND outbreaks was reported in Vietnam. In the same year, numerous outbreaks of ND in Punjab, Parkistan led to depopulation of approximately 45 million birds at an approximate cost of USD 39 million (Rehan et al., 2019). In 2013, a total of 32,094 chickens was destroyed following several outbreaks of virulent ND in Malaysia (OIE, 2018). Based on the potential to spread rapidly and cause severe economic impact on the poultry industry, ND has been listed as a notifiable disease by the World Organization of Animal Health (OIE).

2.2 Classification of Newcastle disease virus

Newcastle disease virus (NDV), also known as avian paramyxovirus serotype 1 (AMPV-1), is the etiological agent of ND (Doyle, 1935). NDV is a member of the genus *Orthoavulavirus* which is classified under the subfamily *Avulavirinae* of the family *Paramyxoviridae* (Mayo, 2002). The genus *Orthoavulavirus* also contains other avian paramyxoviruses (AMPV 2 – AMPV 9) that are contagious to avian species.

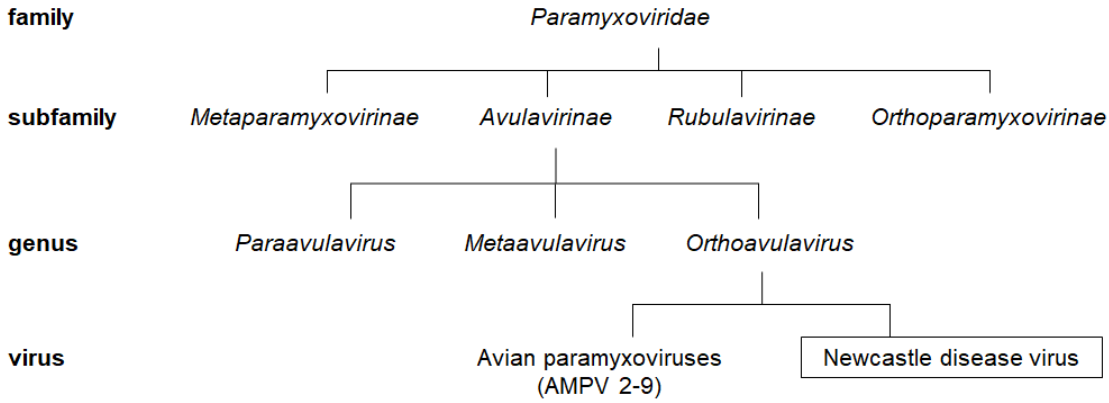


Figure 2.1 Taxanomic organization of *Paramyxoviridae*.

2.3 Clinical signs and pathotypes

Clinical signs observed in birds with ND include lack of appetite, lethargy, fluffed up feathers, greenish diarrhea, swelling, ulceration, respiratory distress (gasping, coughing, sneezing and rattling) and neurological symptoms (depression, torticollis, paralysis and head twisting) (Figure 2.2). However, in certain circumstances, rapid death of chickens can occur without apparent clinical signs. Other clinical signs that are observed upon NDV infections include a drop in egg production, mis-shapened and thin-shelled eggs with watery albumin, enlarged internal organs and hemorrhagic lesions.

Severity of clinical signs and course of the disease are influenced by host-related factors (bird species, age and immune status) and other factors such as pathotypes of virus strains and environmental stress (Alexander, 2000; Cattoli et al., 2011; OIE, 2018). For instance, young birds are more susceptible to infection and tend to develop more severe clinical signs than in older birds. Among the members of Gallidae family, chickens are more likely to develop ND than others including goose, turkeys,

junglefowl, patridges and quail upon infection (Spickler, 2016). As severity of ND induced in birds is a function of the virulence of virus strains, NDV has been grouped into five pathotypes. The five pathotypes are viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric (OIE, 2018). Viscerotropic velogenic is characterized by a rapid course of disease with high mortality and hemorrhagic lesions in the intestinal tract. On the other hand, neurotropic velogenic is typically associated with severe neurological and respiratory signs in the absence of gut lesions. Neurotropic velogenic strains also have relatively lower mortality than viscerotropic velogenic strains. Mesogenic, albeit less virulent, may cause acute disease and mortality in young birds. Asymptomatic enteric and lentogenic often refer to subclinical disease with mild clinical signs, in which the mortality is negligible. (Alexander, 2000; OIE, 2018).

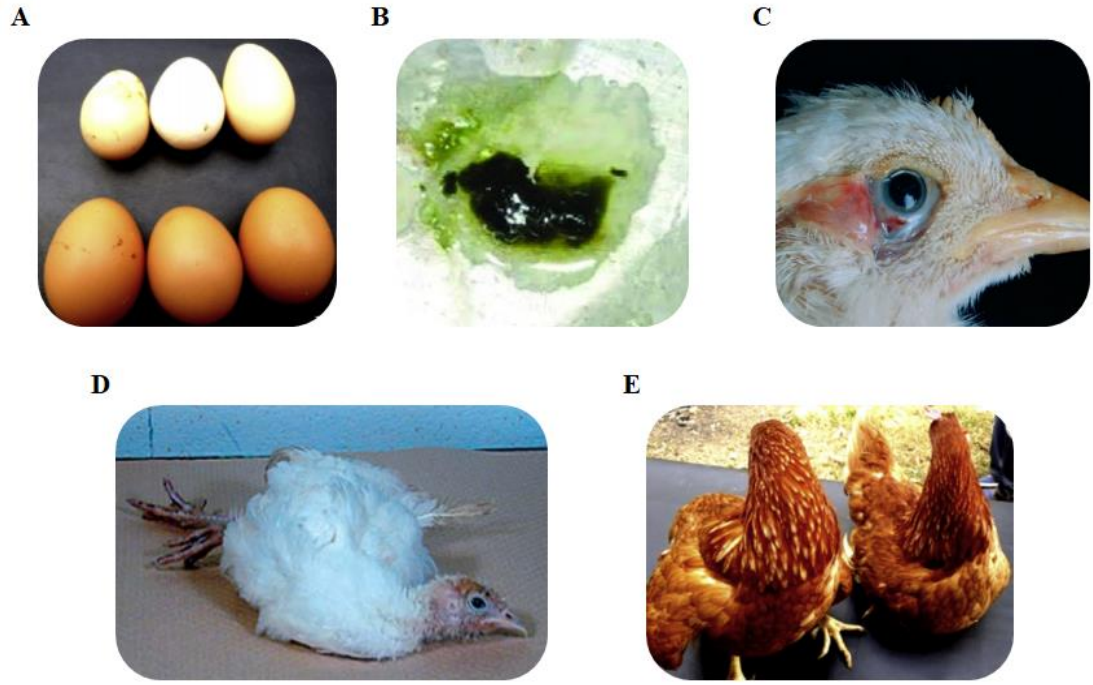


Figure 2.2 Clinical signs of Newcastle disease. (A) Mis-shapened or thin-shelled eggs (Igwe et al., 2018); (B) Greenish, watery faeces (Ashraf et al., 2018); (C) Swelling and ulceration of tissues around eyes (Kommers et al., 2003); (D) Paralysis (Piacenti et al., 2006); (E) Torticollis (Igwe et al., 2018).

2.4 Phylogenetic classification of Newcastle disease virus

Although all NDV strains are serologically classified under a single AMPV serotype, they can be antigenically and genetically diverse (Miller et al., 2009a). Several classification systems based on the partial F gene sequence or complete genome sequence of NDV have been developed to facilitate the study of NDV evolution and genetic diversity (Ballagi-Pordany et al., 1996; Czegledi et al., 2006). However, simultaneous use of different classification methods led to confusion in nomenclature and discrepancies in genetic groups assignment. In 2012, a unified classification system

based on complete F gene sequences and a number of objective criteria such as phylogenetic tree topology, evolutionary nucleotide distances, branch support and epidemiological independence of at least four isolates per genotype or sub-genotype NDV was proposed by Diel et al. (2012). This system classified NDV into two classes, with class I which consist of a single genotype and class II which consist of 18 genotypes (I - XVIII) (Courtney et al., 2013; Czegledi et al., 2006; Miller et al., 2010; Snoeck et al., 2013). Nevertheless, the utilization of non-curated complete F sequence datasets and only some of the objective criteria in the classification of NDV continued to result in inaccurate naming of new genotypes and assigning of sub-genotypes (Dimitrov et al., 2019).

To overcome the deficiencies of the classification system developed by Diel et al. (2012), an updated phylogenetic classification system with new nomenclature criteria was recently developed (Dimitrov et al., 2019). For the new classification system, complete fusion (F) gene sequence datasets were curated for NDV phylogenetic analyses. For analyses, the cut-off value for evolutionary nucleotide distance between sub-genotypes was raised from 3% to 5% to avoid excessive delineation. On the other hand, the bootstrap support value at the genotypes and sub-genotypes defining nodes were increased to 70% and above to reduce the risk of misclassification. While the system proposed by Dimitrov et al. (2019) maintains the existing two NDV classes and genotypes, the number of sub-genotypes was reduced and three new class II genotypes (XIX, XX and XXI) were identified. In this system, the sub-genotypes of both class I and II were named using Arabic numerals separated by periods instead of lowercase Latin letter.

Based on the updated classification system by Dimitrov et al. (2019), class I viruses are classified into a single genotype with three sub-genotypes (1.1.1, 1.1.2 and

1.2), while class II viruses are divided into 21 genotypes (I – XXI) with multiple sub-genotypes (Dimitrov et al., 2019). Class I comprise of viruses primarily isolated from waterfowls and shorebirds, of which are avirulent in chickens. Class II viruses, which include both avirulent and virulent strains were recovered from domestic poultry and wild birds (Kim et al., 2007; Miller et al., 2009b). Class II genotype II viruses are primarily of low virulence except for one, a neurotropic velogenic strain isolated in 1948 namely TXGB (Miller et al., 2010). Specifically, LaSota, Hitchner B1 and VG/GA strains which have been extensively utilized as live NDV vaccines for ND control, belong to genotype II. Nevertheless, genotype V, VI, and VII, which contain only virulent strains, have recently become the predominant genotypes circulating worldwide (Figure 2.3). Among these, genotype VII viruses are particularly important given their association with many recent ND outbreaks in Asia, Africa and Middle East countries (Bogoyavlenskiy et al., 2009; Snoeck et al., 2009; Tan et al., 2010). In addition, genotype VII viruses possess the ability to infect not only wild birds but also vaccinated chickens with high morbidity and mortality rates (Qin et al., 2008; Yu et al., 2001).

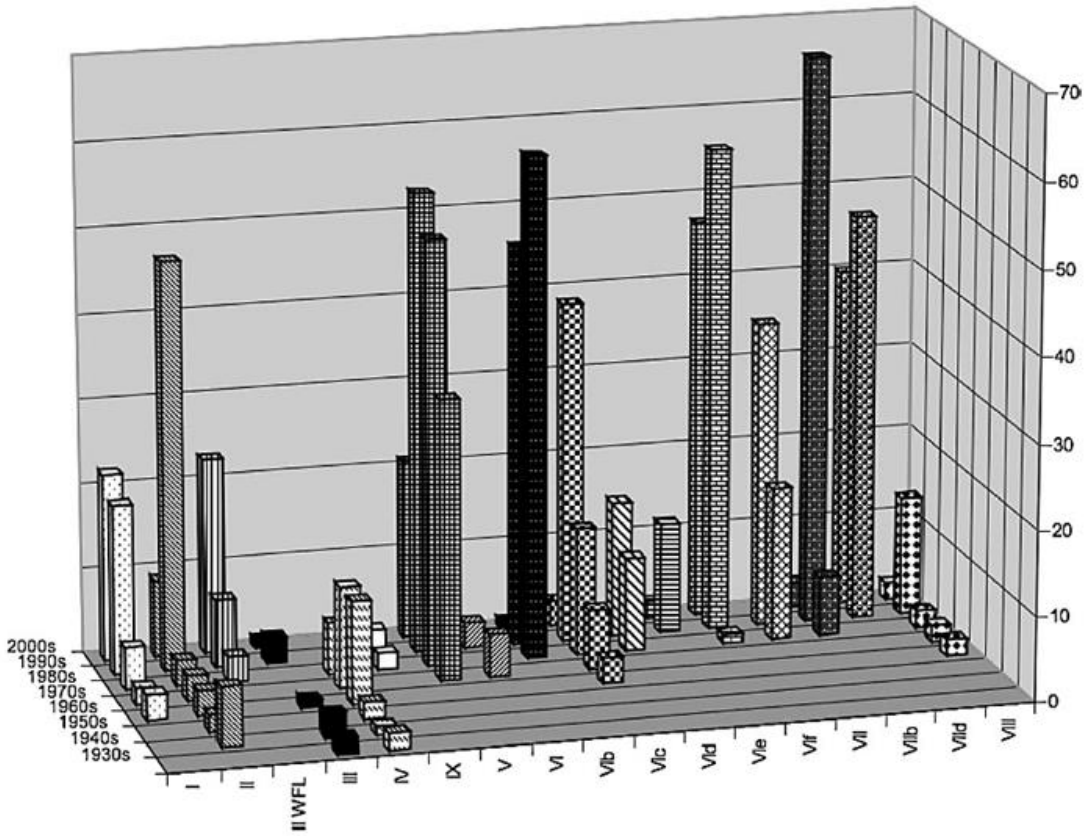


Figure 2.3 The number (*n*) of NDV isolates reported to GenBank from 1930s–2000s was graphically presented based on genotype (G). G I (1962-2004) *n* = 52, G II (1945-2001) *n* = 78, G III (1930-2002) *n* = 14, G IV (1933-1989) *n* = 37, G V (1970-2003) *n* = 158, G VI (1968-2002) *n* = 343, G VII (1981-2002) *n* = 250, G VIII (1965-2000) *n* = 24, G IX (1985-1997) *n* = 11. Figure adapted from Miller et al. (2010).

2.5 Control and prevention

Implementation of an intensive vaccination program along with stringent bio-security practices is critical for effective control and prevention of ND (Alexander, 2000; Fentie et al., 2014; Kapczynski et al., 2013; Miller et al., 2007). ND vaccination regimens for breeders and layers often involve priming with live vaccines and boosting with inactivated vaccines to produce a uniform and long-lasting antibody response. Conventional live and inactivated ND vaccines have been widely utilized for disease control worldwide (Meulemans, 1988; Senne et al., 2004), each having its advantages and disadvantages. Live vaccines are advantageous given the ease of administration, rapid onset of immunity and low production cost. However, live vaccines are susceptible to neutralization by maternal antibodies and can cause mild respiratory disease in vaccinated chickens (Dimitrov et al., 2017). A temperature fluctuation during storage of live vaccines may also lead to a loss in potency and reduced vaccine efficacy (Sieng et al., 2017; Williams & Paixão, 2018). More importantly, the high pathogenicity of virulent NDV in embryonated eggs and risk of virulent virus escaping from production facilities also restricted the generation of genotype-matched live vaccines (Dimitrov et al., 2017). Whilst inactivated vaccines are relatively safer and have been shown to induce high levels of protective antibody over a prolonged period. The preparation of inactivated vaccines involves chemical treatment by β-propiolactone and formaldehyde, which has been reported to be toxic and carcinogenic (Beard et al., 1975; Daszak et al., 2000). A withdrawal period is also often required before the vaccinated chickens are safe for consumption (Dimitrov et al., 2017).

2.6 Current situation and potential solutions

In spite of proper implementation of ND control strategies, outbreaks which continue to occur, suggests that there is room for improvement to current vaccines or vaccination regimens. There are views that recurrent outbreaks can be a result of antigenic and genetic divergence between vaccination and challenge strains (Miller et al., 2007; Yang et al., 2017), poor vaccine quality (Dortmans et al., 2012) and inadequate antigen content (Liljebjelke et al., 2008; Maas et al., 2002). In terms of antigenic or genetic differences, an average difference of 12% in HN amino acid sequence between current vaccination strains and circulating strains have been reported (Cornax et al., 2012; Miller et al., 2013; Miller et al., 2007). Variability in antigen content, with up to 100-fold differences in HN content between batches of inactivated ND vaccines produced by four different manufacturers have also been reported (Maas et al., 2003).

The concerns above highlight the need for continuous research towards improved ND vaccines that can lead to better immunological outcomes. In this study, efforts were directed to generate recombinant genotype-matched NDV HN protein based subunit vaccine (Section 2.6.1) and development of robust quantitation methods to facilitate formulation of the recombinant subunit protein vaccine for dose-ranging immunological studies (Section 2.6.2).

2.6.1 Generation of genotype-matched protein-based subunit vaccines for ND

There are views that increased antigenic and genetic relatedness of vaccination strains to circulating field strains would improve ND control from the perspective of

antibody response, protection from morbidity and mortality and reduction of virus shedding (Miller et al., 2013; Miller et al., 2009a; Miller et al., 2007).

A study by Miller et al. (2009a) reported that inactivated ND vaccine genotype-matched to challenge virus was able to induce a higher HI antibody level and significantly reduce virus shed. Similarly, a study by Yang et al. (2017) has also showed that a higher protection rate against genotype VII NDV challenge as measured by high HI antibody titers and marked reduction in virus shedding, was achieved with antigenically-matched inactivated ND vaccines. However, the high pathogenicity of genotype VII NDV in embryonated eggs and requirement for high-level bio-containment facilities have hampered production of genotype-matched live or inactivated ND vaccines (Dimitrov et al., 2017). To overcome the limitations above, recombinant DNA technology has been utilized to enable the generation of virus-vectored or protein-based subunit vaccines genotype-matched to prevalent circulating NDV strains. NDV HN glycoprotein is one of the major targets of host immune response. Studies have previously shown that replacement of the HN gene of NDV genotype II with a gene coding for HN of virulent NDV genotype VII through the use of reverse genetics methods resulted in improved antibody response and a significant reduction in virus shedding amongst vaccinated subjects (Bu et al., 2019; Liu et al., 2017). On the other hand, HN has also been extensively evaluated for protein-based subunit vaccine development (Lee et al., 2008; Nagy et al., 1991). Recombinant HN based subunit vaccines have been shown to be protective against virus challenge and allow for differentiation of infected from vaccinated chickens (DIVA). HN based subunit vaccines which are non-replicating, also overcomes the issue of potential reversion to virulence-associated with live-attenuated vaccines (Meeusen et al., 2007). The utilization of recombinant subunit vaccines also circumvent the need for

carcinogenic inactivating agents as required for preparation of inactivated vaccines (Thomann, 2003).

NDV HN has been expressed using different expression hosts including bacteria (Jiang et al., 2015; Lee et al., 2010), yeast (Khulape et al., 2015), plant (Berinstein et al., 2005; Gomez et al., 2009) and insect cells (Kamiya et al., 1994; Lee et al., 2008; Nagy et al., 1991). In this study, the insect-cell baculovirus expression vector system was selected for expression of NDV HN. A detailed review on the properties of NDV HN protein is provided in Section 2.7.1. The advantages of the IC-BEVS system selected for NDV HN production is detailed in Section 2.7.2.

2.6.2 Development of robust quantitative methods for rHN

Recombinant HN produced using insect cells have been shown to be capable of conferring protection to immunized subjects. However, the correlation between antigen content and HN based subunit vaccine efficacy has to our best knowledge not been determined using robust quantitative analytical methods.

The antigen content of conventional live and inactivated ND vaccines generated by egg-based manufacture have been conventionally estimated via determination of 50% embryo infectious dose (EID_{50}) or hemagglutination (HA) assay (Cornax et al., 2012; Liljebjelke et al., 2008). However, the EID_{50} method can only be applied to vaccine preparation involving live viruses as starting material. On the other hand, the HA assay which relies on the agglutination capability of HN requires fresh red blood cells. HA assay has insufficient quantitation sensitivity as required for vaccine formulation and dose-ranging studies owing to the limited ability of the assay to detect small changes in HN concentration (Ryu, 2017; Thompson et al., 2013).

Previous dose-ranging studies of HN based subunit vaccines were conducted by varying cell number of the expression hosts (Kamiya et al., 1994; Lee et al., 2008). The cell number approach does not allow for a direct correlation between antigen content and subunit vaccine efficacy as the actual amount of HN cannot be determined. Replication of the cell number approach can also be difficult due to possible variation in antigen expression levels within a study or between studies (Imasaki et al., 2018). Therefore, alternative quantitative analytical methods that allow for absolute antigen quantitation are necessary to evaluate the effect of HN content on subunit vaccine efficacy via dose-ranging studies. In addition to vaccine efficacy, the determination of optimal vaccine content is also important from a cost-perspective as the real-world utility of veterinary vaccines demands that vaccination costs be lower than the cost of the livestock.

Possible alternatives to quantitate HN, such as immunochemical (Western blot and ELISA) and proteomic-based (HPLC and LC/MS-MS) quantitative analytical methods would require HN protein of high purity as reference standards. The availability of a high purity HN reference standard allows the generation of a standard curve for accurate quantitation of HN protein (Tschantz et al., 2008). Therefore in this study, efforts were directed to develop a successful downstream purification strategy to attain rHN of high purity as reference standards for the development of a quantitative ELISA. A detailed review on strategies in downstream processing development and quantitation method development is provided in Section 2.8 and Section 2.9.

2.7 Expression of rHN using IC-BEVS

2.7.1 NDV Hemagglutinin-neuraminidase

NDV is an enveloped single-stranded RNA virus with a genome size of 15186, 15192 or 15198 nucleotides. The RNA genome encodes six structural proteins, in the order from 3' to 5': nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large (L) protein (Figure 2.4) (Wakamatsu et al., 2006; Wilde et al., 1986). In addition to structural proteins, transcriptional editing of P gene mRNA can result in translation of two additional proteins (W and V), which have been identified as non-structural proteins (Peeters et al., 2004; Steward et al., 1993).

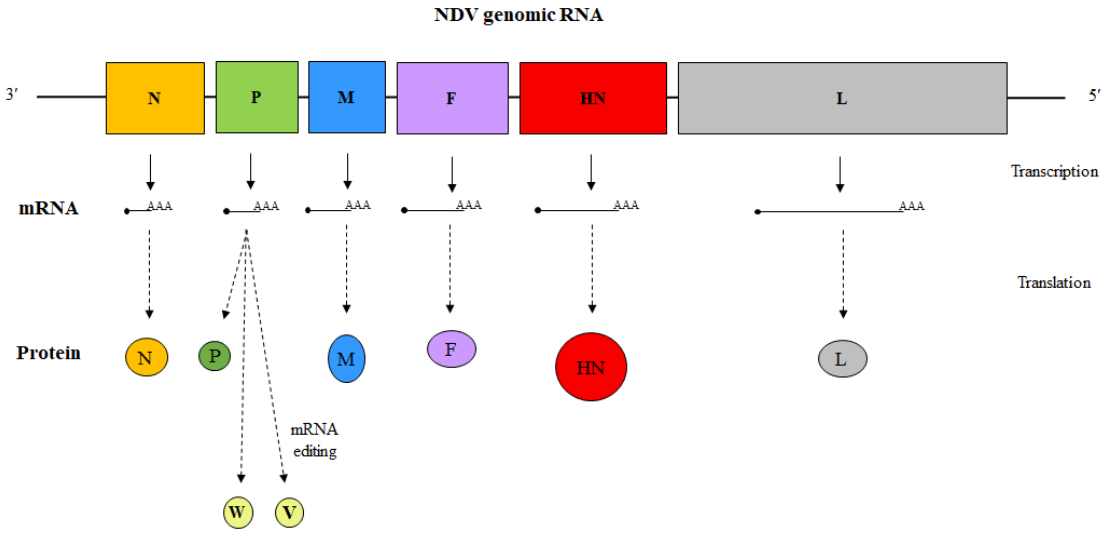


Figure 2.4 A schematic representation of NDV genomic organization and proteins encoded. N: nucleoprotein; P: phosphoproteins; M: matrix; F: fusion; HN: hemagglutinin-neuraminidase; L: large protein; V and W: non-structural proteins.

Of the six structural proteins, F and HN are two glycoproteins which present as spike-like structures on the virus surface (Figure 2.5). HN mediates the binding of virus to host cells and F protein promotes fusion between the virus envelope and host cell membrane (Lamb & Parks, 2007). As F and HN proteins play a crucial role in viral infection, both proteins are major targets of host immune responses (Yusoff & Tan, 2001).

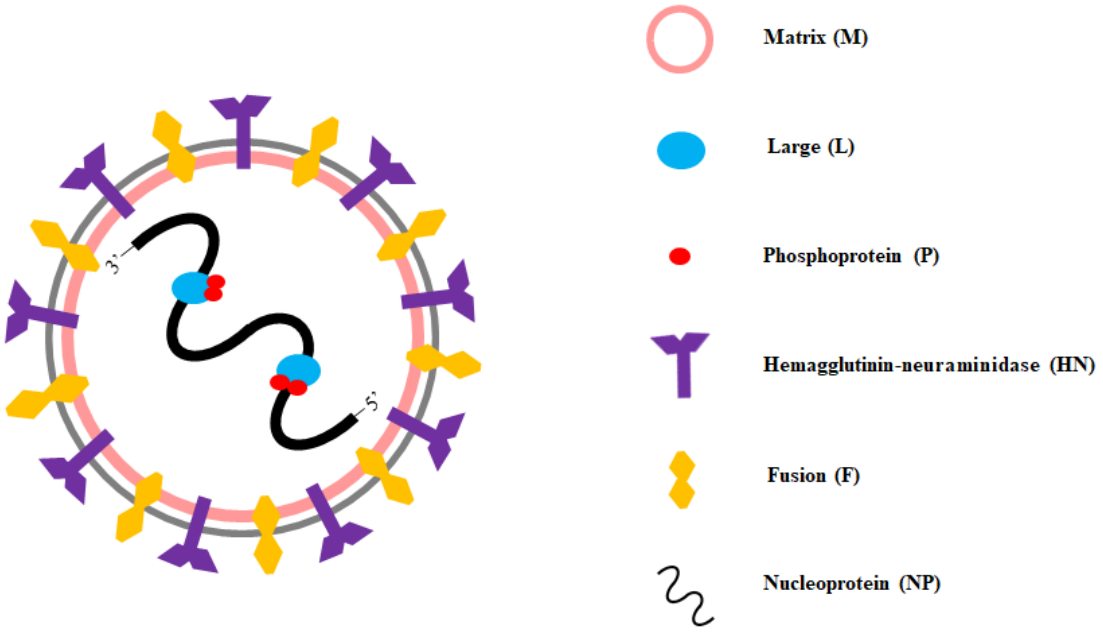


Figure 2.5 A schematic diagram of NDV virion structure. The NDV F and HN glycoproteins are located on the surface of the virion.

Hemagglutinin-neuraminidase (HN) is a multifunctional type II integral membrane protein, which plays a vital role in entry and egress of NDV (Yuan et al., 2011). HN exists on the virion surface and virus-infected cells as a tetramer (Li et al., 2004). The tetrameric structure of HN is formed by non-covalent interaction of two disulfide-linked dimers (Figure 2.6B) (Iorio et al., 2001).

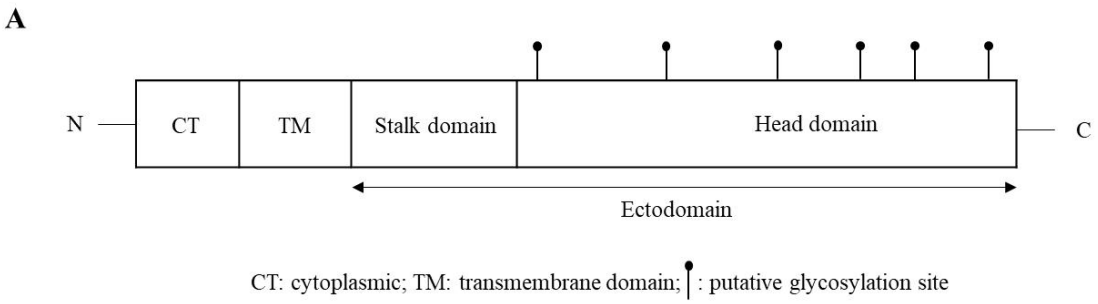
A HN monomer is composed of a cytoplasmic (CT) domain, a transmembrane (TM) domain and a large ectodomain (Figure 2.6). Truncation of the CT or TM domain

has been previously reported to affect the stability of the tetrameric structure and functional activities of HN (McGinnes et al., 1993; Parks & Lamb, 1990; Thompson et al., 1988). The HN ectodomain consists of a stalk domain that supports the head domain (Figure 2.6B). The head domain contains active sites for receptor binding and neuraminidase activities. Neutralizing antibodies against NDV have also been generated from seven antigenic sites of the HN monomer head domain (Mirza et al., 1993; Yuan et al., 2011).

HN has six potential glycosylation sites (Figure 2.6A) (Yusoff & Tan, 2001) and formation of oligosaccharides at each site contribute around 2 to 3 kDa to the existing molecular weight (Panda et al., 2004). This observation is consistent with previously reported results showing that unglycosylated and glycosylated HN had molecular weights of 63 kDa and 75 kDa respectively (Nagy et al., 1990; Ong et al., 2000). Loss of N-linked glycans has been reported to have a negative impact on protein folding, stability, solubility and transport of HN to the virus-infected cells surface (Olden et al., 1982). The loss of N-linked glycans on HN can also alter the virulence of NDV as elimination of the glycosylations sites by site-directed mutagenesis has been thought to reduce the virulence of NDV (Panda et al., 2004; Segawa et al., 2003). However, the effect of glycosylation on NDV HN immunogenicity remains to be elucidated. Nevertheless, the ratio of glycosylated and non-glycosylated forms of rHN is expected to be remain highly similar across different batches as long as expression conditions upstream are kept similar.

HN is capable of binding to red blood cell receptors and causes agglutination. Hemagglutination (HA) activity results from the ability of HN to recognize sialic-acid containing receptors and mediate virus attachment to host cells. HN also exhibit neuraminidase (NA) activity, which prevents self-agglutination of progeny NDV

virions during virus budding by cleaving sialic acid residues (Takimoto et al., 2002). HA and NA activities can be assayed to indirectly determine the conformation of recombinant NDV HN (Nagy et al., 1990; Ong et al., 2000). In addition to HA and NA activity, HN exhibits fusion promoting activity, which facilitate entry of NDV into host cells via interaction with NDV F protein (Morrison, 2003).



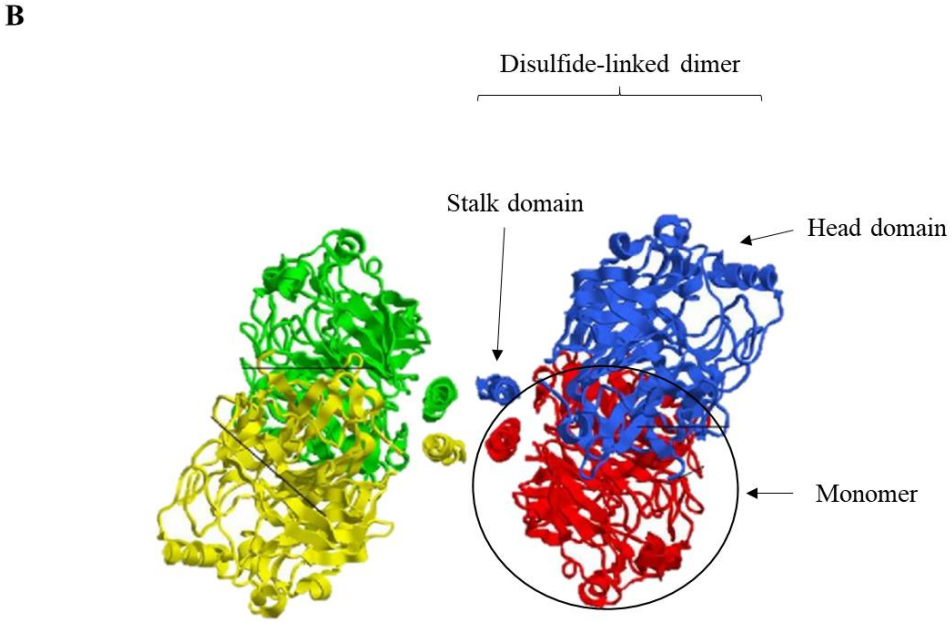


Figure 2.6 (A) Schematic diagram of NDV HN domain structure with six potential glycosylation sites. (B) Ribbon diagram of tetrameric HN ectodomain. A tetramer is formed from two disulfide-linked dimers and each monomer is shown in different colors (green, yellow, blue and red). The ectodomain of a HN monomer consists of a stalk domain supporting a head domain. Figure adapted from Palgen et al. (2015).

2.7.2 Insect cell-baculovirus expression vector system

The insect cell-baculovirus expression vector system (IC-BEVS) is becoming an increasingly popular platform for recombinant protein production (Felberbaum, 2015; Mena & Kamen, 2011; van Oers et al., 2015). A multitude of recombinant protein-based viral vaccines produced using IC-BEVS have been successfully licensed for veterinary use (Table 2.1). A number of other recombinant protein based vaccine candidates that have been produced using IC-BEVS are currently under field evaluation (Table 2.1).

Production of recombinant proteins using IC-BEVS involves infection of insect cells with recombinant baculoviruses carrying expression constructs containing a gene or genes of interest (GOIs) (Figure 2.7). Recombinant baculoviruses can be generated by homologous recombination between transfer vectors carrying the GOI and linearized baculovirus DNA (Hitchman et al., 2009; Shi & Jarvis, 2007). Linearized baculoviruses with a lethal deletion are replication-deficient and homologous recombination with complimentary transfer plasmids restores their viability (Kitts et al., 1990; Kitts & Possee, 1993). For expression of target recombinant protein, generated recombinant baculoviruses are added at a suitable multiplicity of infection (MOI) to insect host cells. Expression of recombinant protein in insect cells is mostly driven by a strong polh or p10 promoter located adjacent to the gene of interest (Belyaev et al., 1995; Smith et al., 1983).

Table 2.1 Baculovirus-expressed recombinant protein-based subunit vaccines for veterinary use.

Commercialized vaccines				
Virus	Protective agent	Target species	Product name	Reference
Swine fever virus	Envelope glycoprotein E2	Pig	Porcilis Pesti™	(Ahrens et al., 2000; Lipowski et al., 2000)
Swine fever virus	Envelope glycoprotein E2	Pig	Bayovac™ CSF E2	(Bouma et al., 1999; Depner et al., 2001; Dong & Chen, 2007)
Porcine circovirus type 2	ORF2 capsid protein	Pig	Ingelvac® CircoFLEX™	(Fachinger et al., 2008; Opriessnig et al., 2009)
Avian influenza virus	H5 protein	Chicken	Volvac® B.E.S.T. AI+ND	(Oliveira Cavalcanti et al., 2017)
Vaccines candidates in field trials				
Bovine diarrhea virus	Envelope glycoprotein E2	Cattle		(Thomas et al., 2009)
Japanese encephalitis virus	prME, E and NS1 proteins	Mouse		(Yang et al., 2005)
Newcastle disease virus	HN and F proteins	Chicken		(Lee et al., 2008)
East coast fever virus	p67 protein	Cattle		(Kaba et al., 2005)
Canine visceral leishmaniasis	Q-like protein	Dog		(Poot et al., 2009)
African horse sickness virus	VP2 protein	Horse		(Aksular et al., 2018)