Molecular identification of Newcastle disease viruses and their control through vaccination and enhanced biosecurity

ニューカッスル病ウイルスの分子的識別とワクチン接種およびバイオセキュリティ強化による防除に関する研究

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DISSERTATION CONTENTS

ABBREVIATIONS

AI	Avian influenza
AIV	Avian influenza virus
AFA	Anti-freeze agent
AGE	Adenoviral gizzard erosion
ANOVA	Analysis of variance
APEC	Avian pathogenic *E. coli*
APMV-1	Avian paramyxovirus type 1
ARV	Avian reovirus
bp	Base pair
BSB	Blocking solution for bacteria
BSV	Blocking solution for virus
°C	Degrees Celsius
$Ca(OH)_2$	Calcium hydroxide

CCP	Critical control points
CDC	Centers for disease control and prevention
CEF	Chicken embryo fibroblast
CFU	Colony forming unit
CK	Chicken kidney
CA	Citric acid
Cm	Centimeter
cDNA	Complementary deoxyribonucleic acid
CPE	Cytopathic effect
CS	Calf serum
dpv	Day post vaccination
dW_2	Redistilled water
DHL	Deoxycholate hydrogen sulfide lactose
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EMEM	Eagle's minimum essential medium
FAO	Food and agricultural organization
FAdV	Fowl adeno virus
FBS	Fetal bovine serum
$FdCa(OH)_2$	Food additive–grade calcium hydroxide
Fig	Figure
GM	Growth medium
h	Hour

HA	Hemagglutination
HACCP	Hazard analysis critical control point
HOCl	Hypochlorous acid
HPAI	Highly pathogenic avian influenza
HPAIV	Highly pathogenic avian influenza virus
HHS	Hepatitis hydropericardium syndrome
HSD	Honestly significant difference
FTA	Flinders technology associates
IBH	Inclusion body hepatitis
ICPI	Intracerebral pathogenicity index
IU	International unit
J-GAP	Japan good agricultural practices
LB	Luria-bertani
LPAIV	Low pathogenic avian influenza virus
M	Molar
MAFF	Ministry of agriculture, forestry and fisheries
Min	Minute
mL	Milliliter
mM	Millimolar
MM	Maintenance medium
MOI	Multiplicity of infection
NaOCl	Sodium hypochlorite
NBRC	National institute of technology and evaluation biological resource center

ND	Newcastle disease
NDV	Newcastle disease virus
NDVs	Newcastle disease viruses
NT	Not tested
OCl^-	Hypochlorite ion
OIE	Office international des epizooties
PBS	Phosphate buffered saline
PFU	Plaque forming unit
ppm	Parts per million
QAC	Quaternary ammonium compound
RBC	Red blood cell
RF	Reduction factor
RSS	Runting stunting syndrome
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
SE	*Salmonella* Enteritidis
SI	*Salmonella* Infantis
ST	*Salmonella* Tiphymorium
SL	Slaked Lime
SE	Standard Error
Sec	Second
SOPs	Standard Operation Procedures

$TCID_{50}$	Fifty percent tissue culture infective dose
USA	United states of America
vNDVs	Virulent strains (Velogenic and mesogenic) of Newcastle disease viruses
w/v	Weight per volume
WHO	World health organization
µL	Microliter

General introduction

1. General introduction

Poultry production systems comprise an important industry across the world and supply major protein sources for humans in the form of meat and eggs. Poultry infectious diseases, especially avian influenza (AI), Newcastle disease (ND), colibacillosis, salmonellosis, diseases from fowl adenovirus (FAdV), and avian reovirus (ARV) are highly contagious and detrimental to the poultry industry. Highly pathogenic avian influenza (HPAI) or ND have high morbidity and mortality rates of up to 100% due to their incidence and rapid transmission, resulting in severe economic losses for the poultry industry not only from animals losses due to diseases but also from trade restrictions and embargoes [Guan et al., 2013, Hofstad and Yoder, 1966, Li et al., 2009, Motin et al., 2005, OIE, 2009, Seo and Lee, 2013, Ssematimba et al., 2012, Yao et al., 2014].

ND virus (NDV) is one of the major poultry pathogens that seriously endangers the poultry industry, resulting in highly contagious septic, a fatal and destructive disease affecting a wide variety of poultry and wild birds worldwide [Ewies et al., 2017]. The economic impact of ND is severe, for example with estimated losses of 288.49 million US dollars annually in Bangladesh [Khatun et al., 2018], 23 million US dollars in Nigeria [Shittu et al., 2016], and 162 million US dollars in the USA [Cattoli et al., 2011]. In 2017-2019, over 20 Asian and 30 African countries reported over 5,400 ND outbreaks to the World Organization for Animal Health (OIE), a large fraction of which were from Iran (n = 492), Ghana (n = 454), Afghanistan (n = 442), and Zambia (n = 425); only 40 reported outbreaks were from Kenya [OIE, 2020]. The virus is shed directly from infected birds by droplets during sneezing and coughing and through feces indirectly, thus contaminating the air, objects, and floor of the farms [Seal et al., 2000, Spekreijse et al., 2013].

Large amounts of airborne pathogens on poultry farms are not only a potential threat to the poultry industry through high mortality and reduced production capacity but also a danger to

poultry farm personnel due to their zoonotic importance [Bródka et al., 2012, Gast et al., 2004b, Hao et al., 2013, Zhao et al., 2011, Zheng et al., 2013]. Prevention and control of diseases largely depend on biosecurity, and disinfectants are very important tools for biosecurity programs designed by the poultry industry [Stringfellow et al., 2009]. The devastating effect of NDV can be controlled with vaccines.

1.1. Background information of ND

ND, caused by virulent strains of NDVs, is a highly contagious, septicemic, destructive, and fatal disease affecting wide varieties of poultry and other avian species. NDV belongs to the genus *Avian orthoavulavirus 1* of the family *Paramyxoviridae* [Amarasinghe et al., 2018, Dimitrov et al., 2019, Ewies et al., 2017]. NDV is an enveloped virus with a non-segmented, single-stranded RNA genome of negative polarity of approximately 15.2 kb [Mayo, 2002], which encodes for six major proteins: nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein and large RNA-dependent RNA polymerase (L) shown in Fig 1 A and Fig 1 B. NDVs are classified into velogenic (highly virulent), mesogenic (moderately virulent), and lentogenic (low virulent or avirulent) strains [Alexander D.J., 2000]. The two surface glycoproteins, F and HN proteins, play a vital role in the virulence of NDVs [Huang et al., 2004; Panda et al., 2004]. The F protein, precisely the amino acid sequence of its cleavage site, is known to be the major virulence determinant [Chambers et al., 1986]. The thermostability of NDV is located within the HN protein [Wen et al., 2016]. All velogenic viruses and mesogenic strains possess multiple (more than two) basic amino acid residues between positions 113 and 116 ($_{113}$R-Q-K/R-R$_{116}$) and phenylalanine at position 117 of the F protein [Aldous et al., 2003; Panda et al., 2004]. In contrast, lentogenic strains possess few basic amino

acids 113 and 116 ($_{113}$K/R-Q-G/E-R$_{116}$) and leucine at position 117 of the F protein and can only be recognized by trypsin-like protease present in the respiratory and gastrointestinal tracts [Aldous et al., 2003, Panda et al., 2004].

ND was first described in 1926, in Indonesia, and one year later in Newcastle upon Tyne in England, and then gradually spreads worldwide [Ganar et al., 2014]. In Japan, the first outbreak of ND was recorded in 1930 [Nakamura et al., 1933] and large outbreaks continued until vaccination with ND live vaccine (Hitchner B1/47 strain) was administered in 1967. There have been fewer outbreaks of ND in small flocks that were not immunized against NDV or had been vaccinated improperly [Mase et al., 2002, Upadhyaya et al., 2013]. The virulent NDV strains are endemic in poultry in most of Asia (India, Pakistan, Vietnum, Combodia etc.) Africa (Ghana, Cameroon, Central African Republic, Gambia, Nigeria etc.) and some countries of North and South America (Maxico, Colombia, Venezuela etc.) [Ganar et al., 2014, Chang and Dutch, 2012].

To isolate NDVs from field samples, inoculation of the samples into the allantoic cavity of embryonated chicken eggs and inoculation to different cells and organ cultures are commonly used [Alders, R. G. and Spradbrow, 2001]. In addition to isolation or detection of the virus or serological evidence of infection, characterization of viral virulence at any level is essential for planning control procedures because NDVs differ between virulent and avirulent viruses [Aldous and Alexander, 2001].

Direct sequencing of NDV complementary deoxyribonucleic acid (cDNA) for NDV detection was reported by Stauber *et al*. (1995). Seal *et al*. (1995) used degenerate oligonucleotide primers for the amplifications of regions of the M and F genes, including the cleavage site encoding region. Following phylogenetic analysis of the aligned sequences, it was possible to classify the viruses for epidemiological studies and predict the pathotype of each virus reliably. Reverse

transcription-polymerase chain reaction (RT-PCR) and sequencing is widely used for the determination of the virulence of APMV-1 viruses or phylogenetic studies [Creelan et al., 2002].

NDV transmission occurs through ingestion of contaminated objects, inhalations, feces, nasal discharge, sick birds, contaminated feed, air, water, equipment, vehicles, contaminated poultry products, and human clothing [Abdisa and Tagesu, 2017]. Similar to the avian influenza virus, wild birds are also considered to play an essential role in the spread of NDV by the high nucleotide homology of viruses between wild birds and poultry [Ayala et al., 2016].

NDV is also recognized as a zoonotic agent. The clinical sign predominantly shown by those infected is conjunctivitis; however, there have also been occasional reports of ND causing flu-like symptoms [Alexander and Senne, 2008]. The majority of people reported to be infected with NDV are those with close contact with poultry (such as abattoir workers) or laboratory staff. As yet there have been no reports of human to human spread [Alexander and Senne, 2008].

Between 1926 and 1981, four ND panzootic occurred worldwide [Dzogbema et al., 2021] and outbreaks are still occurring. If a poultry is infected with virulent forms of NDVs, referred to mesogenic or velogenic (vNDVs) and an outbreak of ND occurs, the country is obligated to report it to the OIE, and trading partners may suspend imports of poultry or poultry products from that country [Alexander et al., 2004, Miller, 2013]. The World Livestock Disease Atlas surveyed 176 countries included in the OIE Animal Health Yearbooks from 2006 to 2009. It concluded that ND is the fourth most problematic poultry disease, behind HPAI, avian infectious bronchitis, and low pathogenic influenza [Anonymous, 2011].

1.2. Vaccine and vaccination for ND

Worldwide control of ND is often based on specific vaccination, and biosecurity with good management practice. ND vaccines induce immune responses that reduce or completely prevent clinical disease and mortality from ND, decrease the amount of vNDVs shed into the environment, and thus decrease the chance to infect the vaccinated animals [Miller et al., 2009]. Both live vaccines or inactivated vaccines containing apathogenic, lentogenic, or asymptomatic intestinal NDV strains or inactivated vaccines are used [Peeters and Koch, 2021]. These vaccines have been divided into mesogenic vaccine containing inactivated moderately virulent strains which requires intramuscular injection and lentogenic vaccine containing avirulent strains with their preferred mode of administration being eye drop, intranasal beak installation, drinking water, aerosol, or dipping from hatching till grow-outmode of administration being eye drop. [Alexander, 2003, Cho et al., 2008].

Currently available commercial vaccines prevent the morbidity and mortality of ND. However, they are unable to prevent infection with, replication, and shedding of vNDV strains [Bello et al., 2018, Igwe and Agbakwuru, 2019, Liu et al., 2018]. Although there are also thermostable vaccines that have been specifically developed to be used in village chickens [Spradbrow, 1992], most of the ND vaccine strains are thermolabile, such as Lasota and B1, and a few of them are thermostable such as V4, I2, and TS09-C [Bensink and Spradbrow, 1999, Lomniczi, 1975, Wen et al., 2016]. Furthermore, most live vaccines are sensitive to heat and require a cold chain to maintain the quality of vaccines during transport and storage most live vaccines are sensitive to heat and require a cold chain to maintain the quality of vaccines during transport and storage. It is expensive to keep vaccines at low temperatures, and the cold chain may consume up to ~80% of the total cost of vaccination programs [Das, 2004]. The cold chain failure may lead to the rapid loss of potency and inadequate protection against disease. It is estimated that

roughly 50% of vaccine products are discarded because of poor thermostability [Schlehuber et al., 2011]. Especially in developing and less-developed countries with poor cold chain, the thermostable vaccines have been needed to protect chickens from ND [Schat and Aini, 1991, Tu et al., 1998, Wambura et al., 2000].

Animal cell culture offers many advantages over the traditional chicken eggs method. The cell culture method is rapid, convenient, and less expensive than eggs, supports easy scale-up, and allows evidence of viral proliferation to be examined microscopically [Siddique et al., 2017, De Leeuw and Peeters, 1999, Ravindra et al., 2009].

Therefore, developing thermostable vaccines that could be partially or entirely independent of a cold chain is of great importance. However, live vaccines contribute a lot to preventing ND; there are still many ND outbreaks worldwide, suggesting that ND vaccination and vaccine efficacy still need improvement [Dimitrov et al., 2016].

1.3. Background information on other infectious diseases

Avian influenza viruses (AIVs) are highly contagious, multisystemic, extremely variable viruses widespread in birds caused by type A influenza viruses, belongs to the family Orthomyxoviridae [Swayne and Suarez, 2000]. Based on pathogenicity in birds, the influenza A virus has been divided into two groups: low pathogenic avian influenza (LPAI) and HPAI in chickens in a variety of other domestic and wild birds. HPAI virus (HPAIV) can cause mortality as high as 100% in birds and devastate the poultry industry as a result of severe trade restrictions [Alexander, 2000]. HPAIV (Asian lineage of H5) was reported firstly in domestic geese in southern China in 1996, had continued to circulate and spread among poultry and wild birds in Asia, Europe, the Middle East, North America, and Africa [Cattoli et al., 2009, Harfoot and

Webby, 2017, Lee et al., 2015, Su et al., 2015]. Outbreaks of HPAI, which originate in poultry upon transmission of certain LPAI viruses (LPAIVs) from wild birds, have occurred often in the last decade [Capua and Marangon, 2006, Munster et al., 2005]. Many potential pathways of the virus's introduction and spread have been proposed, such as the movement of humans, vectors, contaminated environment, respiratory droplet and feces of an infected bird and other fomites [Fusaro et al., 2016, Hernández-Jover et al., 2015, Ssematimba et al., 2013]. Several outbreaks of HPAI caused by H5N1, H5N6, and H5N8 strains were found in different parts of Japan from December 2003 to April 2022 [OIE, 2022, Okamatsu et al., 2017, Saito et al., 2015, Sakoda et al., 2012, Shivakoti et al., 2010]. The control measures were based on a stamping-out policy without vaccination but included movement control, cleaning, destruction, burial, and disinfection of the affected premises [Mase et al., 2005].

FAdV infections have been confirmed in various avian species other than chickens, such as turkeys, geese, ducks, guinea fowl, pigeons, ostriches, and quails [Hess, 2020].Vertical and horizontal transmissions are known to be the primary routes of FAdV infection, and the virus is non-enveloped highly resistant to disinfectants [Grgić et al., 2006, McFerran and Smyth, 2000]. FAdVs cause inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), and adenoviral gizzard erosion (AGE) [Lin et al., 2020, Nakamura et al., 2011, Schachner et al., 2017].

Avian reoviruses (ARVs) are a significant cause of diseases in poultry. Since 2011, the poultry industry worldwide has been facing the consequences of the emergence of ARV variants. ARVs cause viral arthritis or tenosynovitis, chronic respiratory diseases, malabsorption syndrome, runting-stunting syndrome (RSS) provoke considerable economic losses [Hieronymus et al., 1983, Jones, 2000, Liu et al., 2003, Lu et al., 2015]. ARVs are present in poultry farms worldwide

because they are transmitted vertically and horizontally and are highly resistant to disinfectants [Jones, 2000].

Avian colibacillosis caused by avian pathogenic *E. coli* (APEC) is an infectious disease of birds and is considered one of the most important causes of morbidity and mortality, associated with heavy economic losses to the poultry industry that is communicable to humans [Lutful Kabir, 2010, Oliveira et al., 2010]. Clinically and sub-clinically affected animals shed *E. coli* through their feces in the environment [Ta Kagambè et al., 2012]. Besides, *E. coli* can survive a long time in the water, lettuce, soil, feces, manure, and porous and non-porous surfaces such as steel, rubber, plastic, concrete, etc., which may increase its enhancement for transmission to other hosts, flocks, and farms [Avery et al., 2005, Jiang et al., 2002].

Salmonella infection is one of the most important bacterial diseases in poultry caused by various *Salmonella* species, liable for heavy economic losses through mortality and reduced production [Haider et al., 2004]. Salmonella's foodborne illness caused by *Salmonella* is the leading causative agent with an estimated annual financial loss of 3.7 billion dollars and has severe public health impacts [Nidaullah et al., 2017]. The bacteria are passed out through the feces of infected birds, and lateral spread occurs through feces contaminated feeds, water, and litter [Shivaprasad, 2000]. Contaminated eggs and chicken meat are identified as the primary vehicle of *Salmonella* infection in humans, but anyhow, holding farms-based programs to prevent eggs contamination and education of consumers and food workers about the consumption of raw or undercooked eggs resulted in a great reduction of the human cases [Braden, 2006, Gast et al., 2004a].

1.4. Hazard Analysis Critical Control Point

The Hazard Analysis Critical Control Point (HACCP) system, which is scientific and systematic, identifying hazards and measures for their control to ensure food safety and monitors critical control points (CCP) all through the food chain to reduce or eliminate hazards [MHLW, 2003]. HACCP is a tool to assess risks and establish control systems that focus on prevention rather than relying mainly on end-product testing. Any HACCP system can accommodate change, such as advances in equipment design, processing procedures, or technological developments [MHLW, 2003]. With the rise of consumers concern about food safety, an approach to mitigating risks at every stage of food production from farm to table is becoming indispensable. Certification criteria for livestock farms that conduct hygiene management based on the HACCP approach (Farm HACCP) were issued in 2009 by the Ministry of Agriculture, Forestry, and Fisheries of Japan (MAFF) [Miyajima S., 2013].

AI, ND, salmonellosis, and colibacillosis are the threats associated with animal and human health, welfare, or food safety. Pathogen reduction plans include implementing specific HACCP plans, immunizing the birds or animals, standard operation procedures (SOPs) for sanitation, and testing programs for significant pathogens. Biosecurity enhancement materials and processes to maximize food safety can be carried out through the implementation of HACCP principles. HACCP strategies identify the areas where pathogens may enter and the ways to eradicate them. Measures aimed at preserving cleanliness, preventing pathogen build-up, and breaking possible transmission pathways are essential in the management of any modern farming enterprise, regardless of the animal species, including poultry. Contaminated people, equipment, and animals are the major risk factors for introducing pathogens into the farm.

1.5. Biosecurity

Biosecurity involves all measures to control the spread of disease-causing organisms such as AIV, NDV, FAdV, ARV, *Salmonella spp.,* and pathogenic *E. coli*. These measures comprise controlling human movement, isolating poultry from contaminated equipment and animals, controlling insects and rodents, vaccination, disinfection, and good housekeeping. The most significant risk factors for introducing pathogenic organisms are contaminated people, equipment, and animals. The prime target of biosecurity is to protect against the risk posed by disease and organisms. Therefore, biosecurity is the risk management practice in defense against biological threats [Meyerson and Reaser, 2002]. The objective of a quality biosecurity practice in livestock farms includes the prevention of the introduction of infectious agents into the farms, the prevention of disease agents spread from one infected area or flock to another, and the control of the incidence of diseases and their public health concerns.

For establishing good biosecurity practice, it is essential to explore and recognize the risk, find out the critical points in terms of pathogen introduction and spread, and apply suitable control measures by the level of risk. Cleaning and disinfection of all surfaces in poultry farms to ensure biosecurity should be performed regularly to prevent diseases, including bird flu [Martin et al., 2009]. Biosecurity and an effective disinfectant program will reduce foodborne pathogens, immunosuppressive viruses, reportable diseases, and opportunistic infections. Biosecurity measures have become a great strategy to minimize pathogen outbreaks in animal farms and disinfectants are a potent tool in a good biosecurity program [Stringfellow et al., 2009].

1.6. Biosecurity materials

1.6.1. Quaternary ammonium compounds (QACs)

Quaternary ammonium compounds (QACs) disinfectants are cationic compounds (-$NH4^{+}$) and are the most commonly used disinfectants in livestock farms and food processing industries [Gerba, 2015, Stringfellow et al., 2009]. They are efficacious against all enveloped viruses. They are usually odorless, non-staining, non-corrosive, safe for personal use, and inexpensive. They do not lose their efficacy when mixed with an anti-freeze solution (ethylene glycol or propylene glycol) [Davison et al., 1999]. QACs are membrane-active agents interacting with the cytoplasmic membrane (lipid or protein) of bacteria and intracellular targets (lipids) of viruses followed by disorganization of membrane; leakage of intracellular material; degradation of proteins and nucleic acids; stop metabolic activities and lysis of cell wall caused by autolytic enzymes [Gerba, 2015]. In the present studies, two types of QACs were used, one is Rontect® containing didecyl dimethyl ammonium chloride and another is Pacoma® containing trimethyl ammonium chloride.

1.6.2. Food additive-grade calcium hydroxide (FdCa(OH)$_2$

Food additive-grade calcium hydroxide (FdCa(OH)$_2$ is a colorless, white dry alkali powder, which is made from natural calcium carbonates through the calcinations process, with an average diameter of the powder particle 10 μm, by Fine Co., Ltd. (Tokyo, Japan). Generally, Ca(OH)$_2$ has been used as biosecurity material widely to control specific pathogens because of cost-effectiveness [Alphin et al., 2009]. In addition, Ca(OH)$_2$ is listed as an effective disinfectant material for epidemic outbreaks of AI and Aujeszky's disease and is suggested as in situ disinfectant regularly [Alphin et al., 2009, Koch and Euler, 1984]. FdCa(OH)$_2$ is relatively novel among disinfectant materials that can inactivate pathogens even in the presence of organic loads [Hakim et al., 2017a, 2017b, Toyofuku et al., 2017] and is, therefore, attractive as a potential biocidal agent.

The high concentrations of hydroxyl ions from calcium hydroxide modify the pH gradient of the cytoplasmic membrane, damaging its protein [Mohammadi et al., 2012]. Thus, the integrity of the cytoplasmic membrane is altered because of high alkalinity and by acting on the organic components and transporting the nutrients or by a saponification reaction in which the phospholipids or unsaturated fatty acids of the cytoplasmic membrane are destructed in the peroxidation process [Mohammadi et al., 2012].

1.6.3. Slaked lime or calcium hydroxide

Slaked lime (SL), an inorganic compound containing 65-70% $Ca(OH)_2$ whose particle size is less than 150 μm and a highly alkaline substance, was also used to inactivate pathogens [Thammakarn et al., 2015b]. It has been used as a trapping agent for enhancing livestock farm biosecurity in Japan. In Japan, MAFF recommends that livestock farmers spread SL powder for standby sterilization [Takehara, 2021]. Livestock farmers tend to use SL powder as a disinfectant for foot baths to inactivate viruses and bacteria on their boots during the freezing season or try to use it in combination with QACs in the hope of replacing FdCa(OH)2 [Takehara, personal communication]. However, it takes more time, and its synergistic effects with QACs at low temperatures have not been demonstrated in saturated conditions.

1.7. Aim of the present studies

Highly effective vaccination, including biosecurity, is the first defense line and the essential instrument to reduce the microbial load generally and the level of pathogens in poultry farms [Gehan et al., 2009] to protect the poultry industry from some infectious diseases including NDV. Furthermore, it is important to evaluate the efficacy of some biosecurity materials against

some critical viral and bacterial pathogens with adequate methods to find out the effective working concentration, contact time, and environmental conditions like temperature for its virucidal and bactericidal activity, alongside its durability in contaminated carrier surfaces, as to help farmers to promote their strategy for enhancing biosecurity in their farms and food processing industries.

Therefore, the following objectives of the present studies were to ensure immunization and biosecurity-

a) To isolate, identify, and molecular characterize avian NDV from birds in Afghanistan.

b) To develop a Vero cell adapted, thermostable ND vaccine (Vero-20 strain) as an alternative to the traditional embryonated chicken eggs-based vaccine.

c) The synergistic microbicidal efficacies of QACs with FdCa$(OH)_2$ and SL at low temperatures.

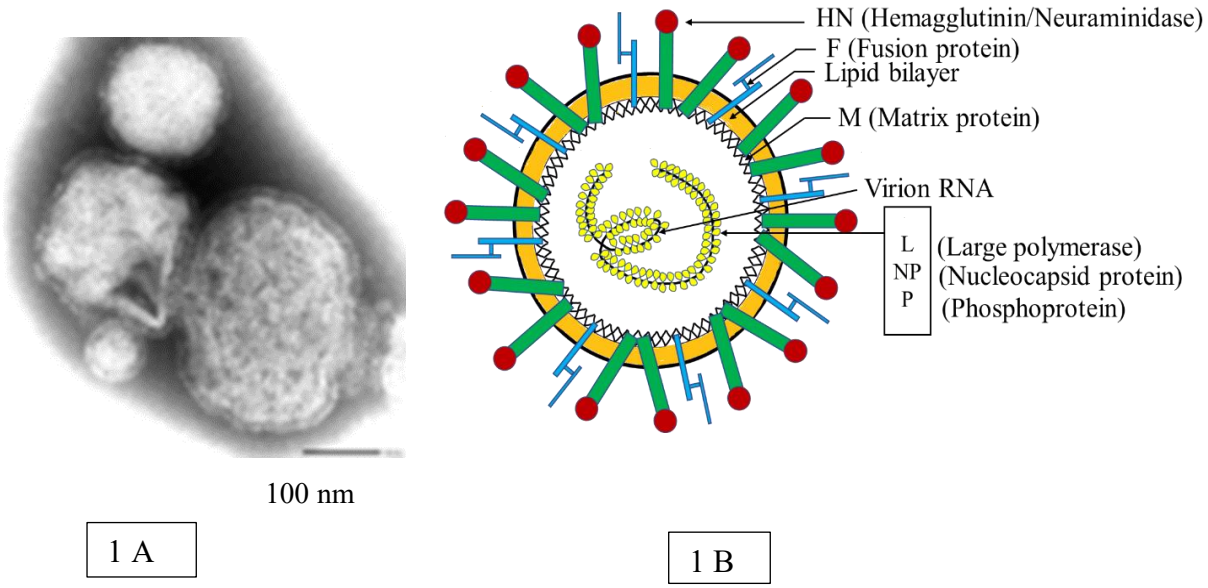

Fig 1 A and 1 B. Schematic view of NDV: Electron microscopy of NDV and molecular structure of NDV.

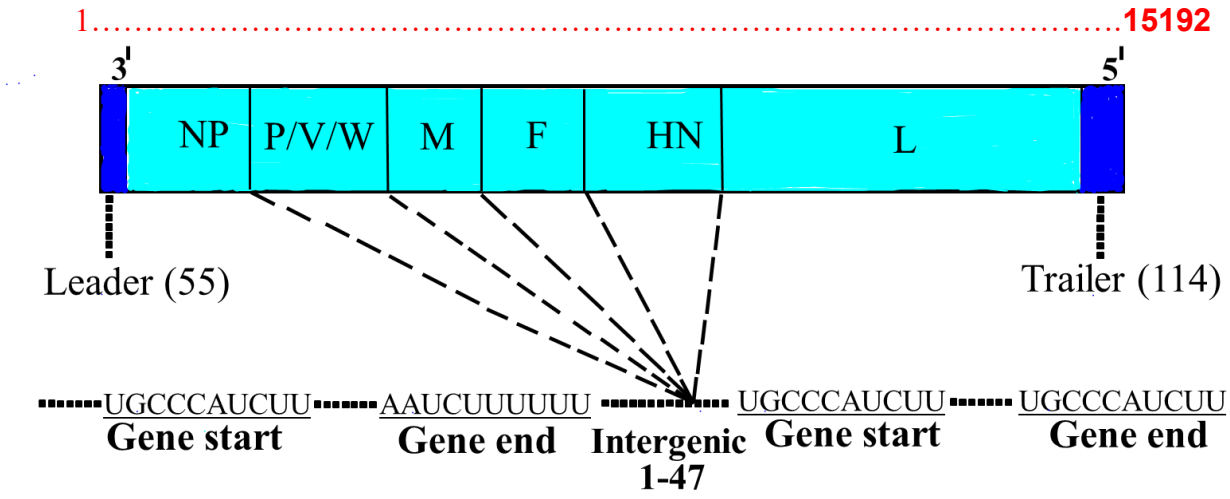

Fig 2. Genetic map of genome RNA of NDV

Chapter I

Isolation, identification and molecular characterization of Newcastle disease virus from field outbreaks in chickens in Afghanistan

I-1. Introduction

Newcastle disease virus (NDV) is one of the leading poultry pathogens severely endangering the poultry industry, causing a highly contagious septicemic, fatal, and destructive disease that attacks chicken and turkeys. Since its first appearance in Indonesia in 1926 [Alexander, 2000] and the Newcastle upon Tyne in England in 1927 [Ganar et al., 2014], four worldwide Newcastle disease (ND) panzootic have occurred [Miller and Koch, 2013].

The host range of NDV is wide, with about 241 species from 27 of the 50 orders of birds [Carrasco et al., 2013, Madadgar et al., 2013]. ND is endemic in various developing countries, including Afghanistan, and has a high economic impact. Due to this disease, the poultry industry is facing losses of millions of dollars worldwide [Susta et al., 2011].

NDVs are classified into velogenic (highly virulent), mesogenic (moderately virulent), and lentogenic (of low virulence or avirulent) strains based on their pathogenicity to chickens [Dortmans et al., 2011]. Among NDVs, velogenic and mesogenic strains both have the amino acid sequence ^{112}R/K-R-Q-R/K-R-F^{117} in the F cleavage site, whereas the lentogenic viruses carry ^{112}G/E-K/R-Q-G/E-R-L^{117} motif [Aldous et al., 2003].

NDVs are divided into two distinct genetic clades, class I and class II, based on their complete sequences of full F genes [Kim et al., 2007]. Class II NDVs have been classified into at least twenty-one (I-XXI) genotypes and multiple sub-genotypes [Dimitrov et al., 2019]. All NDV strains in class I and most of the genotypes I and II viruses within class II are avirulent strains, while virulent NDV strains belong to genotypes III to IX and XI to XVI of class II [Courtney et al., 2013]. Notably, genotype VII of class II NDV has become significant, due to strains associated with the most recent outbreaks in Asia, Europe, the Middle East, Africa, and South America [Yang et al., 2017]. Genotype VII has been further classified into nine sub-genotypes (VII a-k) [Molini

et al., 2017]. Although in a new classification system VII has three sub-genotypes, VII 1.1, VII 1.2, and VII 2, till now [Dimitrov et al., 2019a]. However, there are no published reports on the isolation and molecular characterization of NDVs in Afghanistan.

Isolation and identification of NDV are usually done by propagating viruses in embryonated chicken eggs, followed by hemagglutination (HA) and hemagglutination inhibition (HI) tests using NDV-monospecific antiserum [OIE, 2012a]. Recently, reverse transcription-polymerase chain reaction (RT-PCR) has been applied to the identification of NDV [Zhang et al., 2010].

In the present study, the viruses were isolated from infected or dead chicks in Kabul, Afghanistan, and HA-positive samples suspected of NDV were transported to Japan using Whatman Flinders Technology Associates (FTA®) cards (Whatman International Ltd., United Kingdom), while the molecular characteristics of NDV were examined by RT-PCR assays and sequencing.

I-2. Materials and methods

I-2.1. Outbreak histories in broiler farms in Kabul, Afghanistan

Farm A: The farm had 3,000 chicks that were 31 days old. Clinical signs of disease and mortality in the birds began at 27 days of age. The mortality rate on the day of specimen collection increased to 40%. The birds had been vaccinated twice for NDV (strain LaSota) at 6 and 20 days of age.

Farm B: Farm B also housed 3,000 birds that were 27 days old on the sampling date. Clinical signs of disease and bird mortality began at 25 days old. Mortality on the sampling day increased to 50%. The vaccination history of the flock was unknown.

Farm C: Farm C has housed 6,000 chicks that were 35 days old at the time of sampling. Clinical signs of disease and bird mortality began at 29 days of age. The mortality rate on the sampling day increased to 25%. The chicks were vaccinated twice with strain LaSota at 6 and 20 days of age.

I-2.2. Samples collection

Between August and October 2020, samples including fresh feces, oropharyngeal swabs, and parts of the trachea, lungs, and small intestine were collected from three of the above small scale broiler farms suspected of NDV infection in Kabul, Afghanistan. Oropharyngeal swabs were collected from moribund or dead birds using rayon cotton bulb swabs from Eiken Chemical Co., Ltd. (Tochigi, Japan). Tissue samples were aseptically collected from dead or dying birds and placed in plastic bags. Fresh feces were collected from the floors and placed in tubes. The swabs were placed in vials containing transport medium (brain heart infusion broth 3.7%) with an antibiotic-fungicide cocktail (Penicillin 1000 IU/mL, streptomycin 1 mg/mL, Amphotericin B 5 µg/mL) and kept at 4˚C. The samples were transferred to the laboratory within 3 h. All samples were stored at -20˚C until the day of inoculation.

I-2.3. Virus isolation by chicken embryo inoculation and confirmation by HA test

Only oropharyngeal swabs and fecal samples were used for attempting to isolate the virus from the collected samples. Oropharyngeal swabs were thawed, vortexed, and kept for about 1 h at room temperature before inoculation. The fecal samples were thawed, and their 20% suspensions were prepared in phosphate-buffered saline (PBS: 0.14 M NaCl, 2 mM KCl, 3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4), containing antibiotics (penicillin: 10,000 units/mL;

streptomycin:10,000 µg/mL; and amphotericin B: 50µg/mL) [Jahangir et al., 2008], then incubated for 1.5 h at room temperature. The suspensions of swabs and fecal samples were then centrifuged at 10,000 × *g* for 5 min. Aliquots of 200 µL of the supernatants were inoculated into the allantoic cavity of each two 10-day-old chicken embryos/sample. The eggs were incubated at 37˚C for five days and candled twice daily to check the viability of the embryos. Upon the death of embryos or at the end of the incubation period, the allantoic fluid was harvested and centrifuged at 10,000 × *g* for 5 min. The supernatant was then subjected to HA test using 0.5% chicken red blood cells [Sever, 1962].

I-2.4. Flinders technology associates (FTA®) card preparation

One hundred microliters of the HA positive samples - four confirmed isolates from each farm - were inoculated to Whatman (Whatman International Ltd., United Kingdom) FTA® Card and transferred to the laboratory of animal health, department of veterinary medicine, faculty of agriculture, National University Corporation Tokyo University of Agriculture and Technology (TUAT) under a Material Transfer Agreement with Cartagena for their molecular characterization and classifications.

I-2.5. RNA extraction from FTA® card

Each FTA® card with 100 µL allantoic fluid sample was cut into small pieces under an aseptic condition using autoclaved scissures and forceps and kept in microtubes inside a biosafety cabinet. Viral RNA was extracted from the FTA® card using ISOGEN-II (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions and stored at -80°C until use.

I-2.6. Reverse transcription PCR assay

RT-PCR was performed using PrimeScript One-Step RT-PCR Kit ver.2 (Takara Bio Inc.) with NDV-F2 (5′-TGGAGCCAAACCGCGCACCTGCGG-3′) and NDV-R2 (5′-GGAGGATGTTGGCAGCAT-3′) primers [Mase et al., 2009]. Briefly, 2 µL of viral RNA was mixed with 1 µL of PrimeScript 1 step Enzyme Mix, 12.5 µL of 2 × 1-Step Buffer, 0.1 µL each of primer, and 9.3 µL of RNase-Free dH_2O for a total of 25 µL, and incubated at 50°C for 30 min, to make cDNA. After stopping the RT reaction – run at 95°C for 15 min – the PCR reaction was performed with 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and final incubation at 72°C for 10 min was conducted. The first quarter of the coding region of the F gene (from nucleotide (nt) 47 to 420), which includes an essential structure such as the cleavage site, was selected for this analysis. The region comprising the 3′ of the M gene and the 5′ of the F gene was amplified for sequencing.

I-2.7. Agarose Gel Electrophoresis

The amplified RT-PCR products were subjected to agarose gel electrophoresis. The predicted size of the PCR products was approximately 766 base pairs (bp).

I-2.8. Nucleotide sequencing of the partial F-gene, collection of sequences, and phylogenetic analysis

According to the manufacturer's instructions, the obtained PCR products were purified with QIAquick Gel Extraction Kit (Qiagen Inc., Tokyo, Japan). The purified PCR products were sequenced from both directions using NDV-F2 and NDV-R2 primers [Mase et al., 2009].

Class II complete F gene dataset provided by the international consortium that published the current NDV classification system [Dimitrov et al., 2019a] and deposited in GitHub (https://github.com/NDVconsortium/NDV_Sequence_Datasets) was used (1901 sequences as of 30 October 2020). Also, BLASTN searches were employed to detect the sequence similarities between the isolated strains and reference strains in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Using MEGA-X software version 10.1.8, a phylogenetic tree was constructed using the maximum likelihood method with 1,000 bootstrap replicates.

I-3. Results

I-3.1. Clinical signs and symptoms

The infected broilers manifested dizziness, loss of appetite, depression, gasping, and paralysis of the neck, legs, or wings. The clinical symptoms, as well as the pathological lesions detailed ahead, suggested the occurrence of ND in the three farms mentioned above [Terregino and Capua, 2009].

I-3.2. Postmortem lesions

It was observed that necrotic lesions were present in the intestine's mucosa, gizzard, and proventriculus during a postmortem examination of dead birds. Hemorrhagic lesions were very

prominent in the mucosa of the proventriculus. The air sacs contained whitish translucent material, the lungs were somewhat swollen, and the liver and spleen were enlarged.

I-3.3. Virus isolation

Allantoic fluid inoculated with samples from diseased chickens showed HA activity at a titer of around 1:128.

I-3.4. Confirmation of NDV by RT-PCR from FTA® cards samples

Results from one-step RT-PCR confirmed the presence of the NDV M and F genes by using FTA® cards samples (Fig I-1). As shown in Fig I-1, a band of the expected size (766 bp) was amplified in all samples except No. 11.

I-3.5. Nucleotide sequencing and phylogenetic analysis

From each farm sample, Nos. 1, 8, and 10 of farms A, B, and C, respectively, were sequenced. The amino acid sequence of the F-protein cleavage site of 3 samples was ^{112}R-R-Q-K-R-F^{117}. The NDV isolates obtained were designated Ck/AF/AH01/2020, Ck/AF/AH08/2020, and Ck/AF/AH10/2020, respectively.

NDV partial F gene nucleotide sequencing data of these three samples were deposited in the DNA Data Bank of Japan (DDBJ) of the National Institute of Genetics. The accession numbers for the F nucleotide sequences are LC656353, LC656354, and LC656355.

A phylogenetic analysis was performed to study the evolutionary relationship between the NDVs isolated in Afghanistan in 2020 and viruses typical of other geographical regions (Fig I-2).

A complete tree was constructed for classification purposes based on partial F gene sequencing and phylogenetic relationship compared with deposited F gene sequences of class II NDV (n=1901) in GitHub, which was the available one (https://github.com/NDVconsortium/NDV_Sequence_Datasets) [Dimitrov et al., 2019a]. This phylogenetic analysis confirmed the nucleotide distance results and classified all three isolates studied here as members of class II genotype VII and sub-genotype VII1.1. A phylogenetic tree representing all available viruses of genotype VII was constructed for imaging purposes. As expected, the isolates studied here clustered together with the viruses that showed the highest nucleotide identity.

I-4. Discussion

NDV is one of the most devastating diseases in the poultry industry in many Asian countries. The clinical symptoms and the pathological lesions suggested indeed the occurrence of ND in the three farms as mentioned above [Terregino and Capua, 2009], and were confirmed virologically for the first time in Afghanistan. Although hundreds of NDV gene sequences are currently available, not a single data is accessible regarding the genomic characteristics of NDVs isolated from the poultry industry in Afghanistan.

In this study, Ck/AF/AH01/2020, Ck/AF/AH08/2020, and Ck/AF/AH10/2020, these three isolates were sequenced and found to cluster into genotype VII, sub-genotype VII1.1 in class II NDV. The position of multiple basic amino acids (at least three arginines (R) or lysine (K)) within the cleavage site of the F protein is determined as a marker of virulent strains [OIE, 2012a]. Furthermore, the cleavage site of the F protein has the motif ^{112}R-R-Q-K-R-F^{117}, which was previously reported as a typical motif for virulent NDV strains [Shabbir et al., 2013]. Hence, in

the present study, three isolates, namely Ck/AF/AH01/2020, Ck/AF/AH08/2020, and Ck/AF/AH10/2020, were designated virulent strains by all means. It is worth noting that genotype VII was responsible for the fourth panzootic outbreak, which spread in Asia, Europe, the Far East, and South Africa in 1980. Forty years ahead, it is still an active virulent strain worldwide [Lomniczi et al., 1998, Perozo et al., 2012], with a significant, now orderly probed outcome in Afghanistan.

Based on phylogenetic analyses, four main ND panzootic have occurred in poultry since 1926. For identifying the sub-genotype, the F gene nucleotide sequence, with particular emphasis given on the variable region (47-420 nucleotide position), is considered a standard criterion for genotyping [Qin et al., 2008]. The phylogenetic analysis of the partial F gene in this study indicated that the three isolates, as mentioned earlier, belong to the sub-genotype VII1.1, the predominant genotype causing NDV outbreaks across many Asian countries, including nearby Kyrgyzstan [Bogoyavlenskiy et al., 2009, Liu et al., 2015]. Moreover, our results of the phylogenetic analysis suggest that the isolates are closely related to the Chinese NDV isolate from 2013 (KF208469) and point to 94.58% nucleotide similarities with it. This is the first NDV genomic characteristics report of the poultry industry in Afghanistan. The current NDV strains we characterized were clustered with the Chinese NDV isolates in sub-genotype VII1.1, suggesting that the present isolates could have been introduced from China to Afghanistan. Geographically, Afghanistan does share a 74 km border with China. In addition, informal trade of live poultry has been reported between China and neighboring countries [FAO, 2019, Zhou et al., 2015]. Another factor could be that migratory birds play a role in the circulation of NDV [Umali et al., 2014]. To investigate the issue, surveillance of wild birds for NDV is a fundamental demand for establishing the epidemiology of the virus.

In summary, phylogenetic analysis of the F gene of the Ck/AF/AH01/2020, Ck/AF/AH08/2020, and Ck/AF/AH10/2020 isolates showed that sub-genotype VII1.1 viruses of class II continue to circulate in the poultry industry of Afghanistan. The current study also provides the first report on the molecular characterization of the F gene of NDV strains isolated from Afghanistan. In addition, the present NDV strains were isolated from vaccinated flocks which may be the result of immune failure; therefore, this study has provided essential information regarding the genetic nature of circulating NDV, which may help for a further and complete genome study, diagnosis, and control of the disease in Afghanistan.

I-5. Conclusion

The multiple sequence alignment (MSA) exhibited that the isolates have high homology (94.8%) with other reported NDV isolates. The phylogenetic analysis revealed that the isolates obtained in this study from fulminant diseased chickens in Afghanistan were closely related to velogenic or highly pathogenic types of NDV.

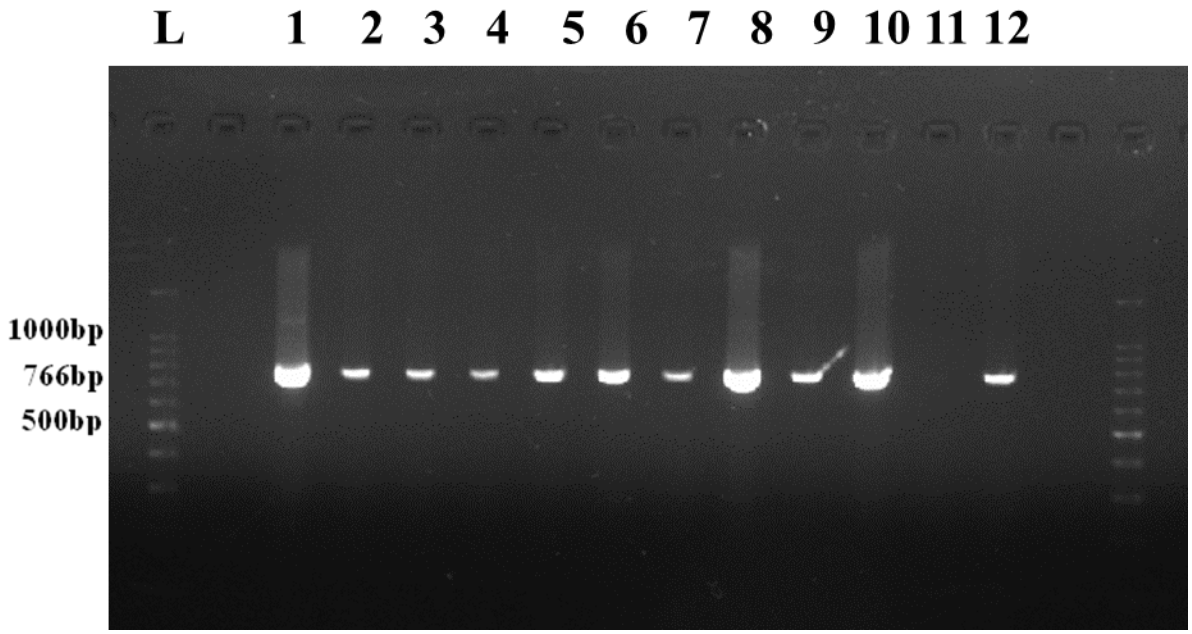

Fig I-1. Agarose gel electrophoresis of RT-PCR products. Lane L: ladder, Lane 1, 2, 3, and 4 were from farm A, 5, 6, 7, and 8 were from farm B, 9, 10, 11, and 12 were from farm C. Positive results correspond to 766 bp RT-PCR products.

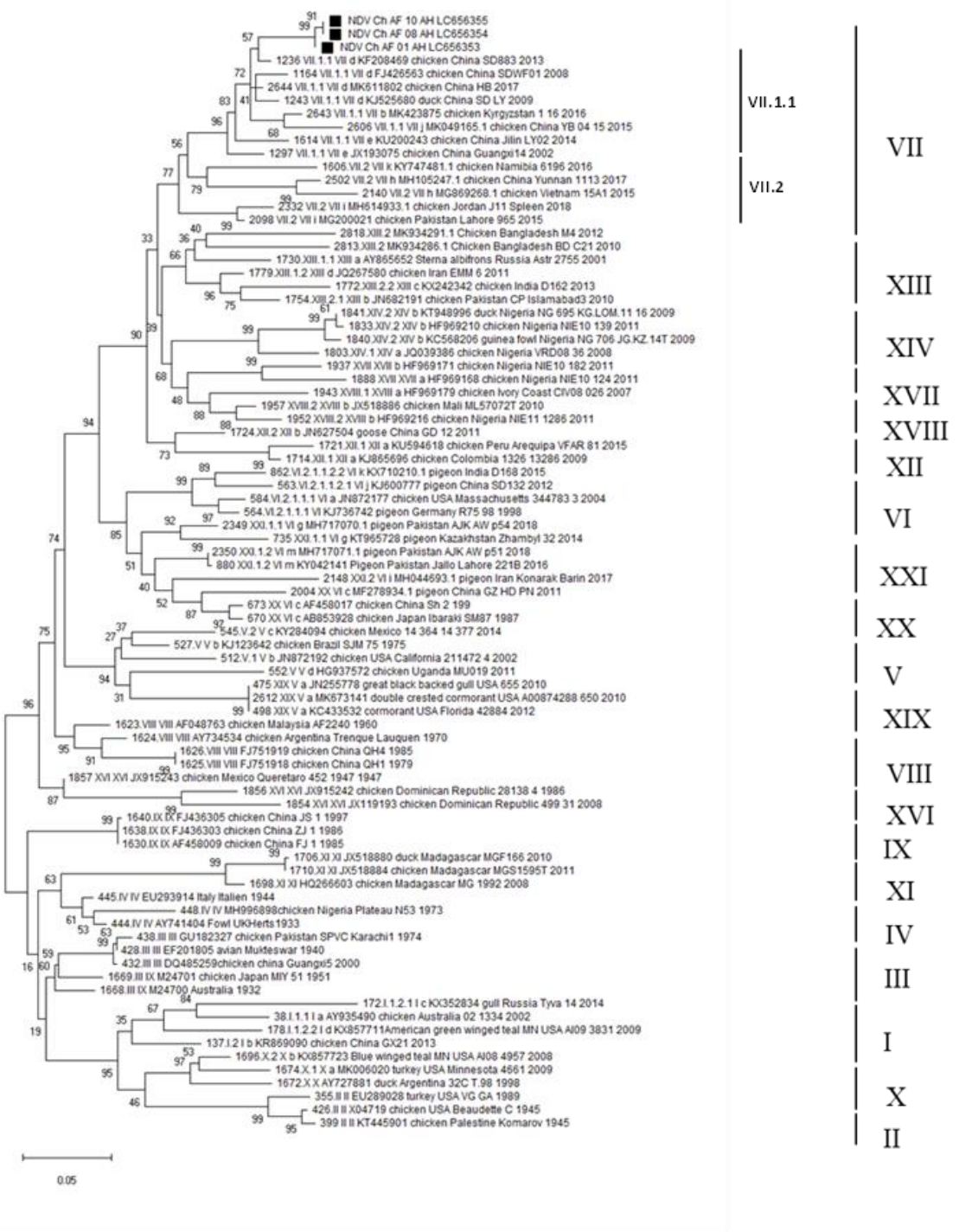

Fig I-2. Phylogenetic tree of Newcastle disease virus based on partial F gene nucleotide sequence (47-420) data of our isolates, together with class II different genotypes/sub-genotypes from current classification system by Dimitrov et al., 2019 (10). The tree was constructed using the maximum likelihood method (1,000 bootstrap replicates) in MEGA-X software. Bootstrap values are shown at the nodes. The sequences determined in this study are marked with square.

Chapter II

Establishment of a thermostable candidate Newcastle disease vaccine strain and its adaptation to Vero cells

II-1. Introduction

Newcastle disease (ND) is a highly contagious, fatal, septicemic avian disease and poses a considerable threat to the poultry industry worldwide [Pedersen et al., 2004]. Molecular methods based on the L and F-protein sequences have shown two categories of NDV of classes I and II, in reference to one of the antigenic determinants in one serotype [Czeglédi et al., 2006].

Genotype I in class II such as V4, Ulster, and others, commonly isolated from poultry as well as some class I lentogenic viruses isolated from wild birds and live bird markets (LBM), are vaccine strain candidates, since they induce minimal adverse immune reactions [Ballagi et al., 1996, Chansiripornchai and Sasipreeyajan, 2006]. Strains of low virulence originating from waterfowl and producing no apparent signs of disease are frequently used as potential live vaccines [Alexander and Senne, 2008, Shim et al., 2011]. Antibodies to HN and F proteins are neutralizing and are the major protective components induced by ND vaccines [Xiao et al., 2012]. The effective prevention and control of avian infectious diseases usually depend on vaccination in many countries. However, most live vaccines are sensitive to heat, and subsequently require a cold chain to maintain the quality of vaccines during transport and storage. It is expensive to keep vaccines at a low temperature, and the cold chain may consume up to ~80% of the total cost of vaccination programs [Das, 2004]. Moreover, the cold chain is not always reliable. Temperature excursions outside the optimal temperature range are frequently observed during transport and storage [Matthias et al., 2007, Nelson et al., 2007], due to inappropriate cold chain equipment, human error, and power shortages [Nelson et al., 2004. Setia et al., 2002, Techathawat et al., 2007]. The situation is even worse in developing and less-developed countries. Hemagglutination (HA) and neuraminidase (NA) activities are functions solely of the HN protein [Hsu et al., 1979, Scheid and

Choppin, 1973]. HN protein is shown to be a crucial determinant of NDV thermostability [Wen et al., 2016].

ND vaccines have traditionally been produced by growing virus strains in embryonated chicken eggs. NDV is harvested from the allantoic fluid and processed to create a vaccine [Gallili and Ben-Nathan, 1998]. However, this traditional method poses some drawbacks, such as poor-quality control, high labor intensity, time-consuming, and need for a high amount of specific pathogens-free (SPF) eggs; also, unavailability of SPF eggs with causes the spread of some diseases, and requires large area for the incubation of eggs [Souza et al., 2009]. Animal cell culture offers many advantages over the traditional embryonated chicken eggs method [Arifin et al., 2011]. The method is rapid, convenient, and less expensive than eggs, supports easy scale-up, and allows evidence of viral proliferation to be examined microscopically [Abera, 2018, Souza et al., 2009]. Cell-substrate systems in which NDVs have been propagated include Vero cells from African green monkey kidney, chicken embryo fibroblasts (CEF), and Douglas Foster one (DF-1) cells [De Leeuw and Peeters, 1999, Ravindra et al., 2009].

Biosecurity and immunization are the best ways by which to combat ND. In developing countries like Bangladesh and Afghanistan, these policies are not strictly applied. Several reports showed vaccines cannot protect chickens completely even after ND vaccinations due to emerging of NDV variants with genetic distances [Dimitrov et al., 2017, Miller et al., 2013, 2007]. However, it has been reported that B1 vaccine can protect chickens from these new variants [Mase, 2022]. Some countries may not store vaccines at the proper temperature. Temperature directly affects the potency of vaccines. The cold chain for storage and transportation could not be effectively maintained due to interruption in the electrical supply needed for this system. In Afghanistan, virulent NDVs were isolated from vaccinated chicken farms, and the problems with proper vaccine storage were speculated.

Thermostable vaccines that are widely used are based on vaccine strains from class II genotype I (i.e I2, V4, and PHY-LMV42), which are avirulent and safely used in chickens of all ages [Garcia et al., 2013]. Strains of NDV that have increased stability to heat are especially advantageous in rural areas of the world with limited refrigeration capacities [Alders, 2014]. Although the thermostability of NDV may appreciably vary between strains, there are very little data on this attribute in reference to various virus strains.

The present study aims to find a thermostable ND vaccine candidate strain isolated from a duck, adapt the strain to Vero cells.

II-2. Materials and methods

II-2.1. Viruses and cells

NDV velogenic strain Sato [Takehara et al., 1987], mesogenic strain TCND [Bankowski, 1958] and lentogenic strains B1, Ishii [Mase et al. 2002], and APMV/northern pintail/Japan/Aomori/2003 (dk-Aomori/03, thereafter referred as NDV261) [Sakai et al., 2007] were used. Strains Sato, Ishii, and TCND were kindly supplied by Kitasato Institute (Tokyo, Japan), and the vaccine strain B1 was purchased from Nisseiken Co., Ltd. (Tokyo, Japan).

Vero cells were cultured at a 1.5 x 10^5 cells/mL concentration. Additionally, primary chicken kidney (CK) cells were prepared according to the methods described [Takehara et al., 1991].

II-2.2. Screening of thermostable strains

Vials containing 0.5 mL aliquots of NDV strains Sato, TCND, B1, Ishii, and NDV261 were taken from a deep freezer at -80°C, and the aliquots were thawed. They were kept on ice or in a

water bath at 56°C for 30, 60, and 120 min, then transferred quickly onto ice, so as to stop the inactivating heat. All aliquots were assessed for HA activity by standard methods [OIE, 2012a].

The lentogenic virus, NDV261 showed adequate HA titer after incubation for 2 h at 56°C and was selected for further studies.

II-2.3. Adaptation of thermostable NDV to Vero cell line

At each passage in Vero cells, NDV261 was heat-treated at 56°C for 30 min, inoculated onto Vero cells, incubated at 37°C for 60 min, then supported with a maintenance medium (MM) containing 1 µg/mL trypsin from bovine pancreas (Sigma Chemicals, MO, USA). The virus was passaged 20 times in Vero cells and the viruses at each passage were named NDV-Vero1 to NDV-Vero20.

II-2.4. Virus growth curve in Vero cells

To confirm the adaptation of the vaccine candidate to Vero cells, the growth curves of the original NDV261 and NDV-Vero20 were compared. A monolayer of Vero cells in 6-well plates was inoculated with the viruses at the multiplicity of infection MOI of 0.01 plaque-forming unit (PFU)/cell and incubated at 37°C for 60 min. The monolayer was washed thrice with phosphate-buffered saline (PBS) (pH 7.2), supported with the trypsin containing MM, thereby leading to 1 µg/mL as the final concentration, and incubated at 37°C. The culture supernatants were collected at 12, 24, 36, 48, 72, and 96 h post inoculation, centrifuged at 8,000 x g for 3 min, and stored at -80°C until tested.

These samples were titrated on Vero or CK cells for PFU/mL or fifty percent tissue culture infectious dose ($TCID_{50}$/mL), as described [Jahangir et al., 2010a]. For Vero cells, 0.5 µg/mL trypsin was included in the first overlay medium.

II-2.5. Thermal stability test

The thermal stability of NDV-Vero20 in liquid condition was evaluated at 37°C, 25°C, and 4°C storage temperatures for 0.5, 1, 1.5, 2, 5, 7, and 10 days. At each time point, the virus was picked up and titrated, as detailed above.

II-2.6. Reverse-transcription polymerase chain reaction (RT-PCR), and sequencing of F and HN genes

Specific primer sets used to amplify the part of F genes and HN genes of NDV261 and NDV-Vero20 are shown in Table II-1. RT-PCR and sequencing reactions were performed, and the sequence data were deposited to DDBJ, as described in chapter 1.

The first quarter of the coding region of the F gene (from nucleotide (nt) 47 to 420), includes essential structures such as the cleavage site, and the coding region of the HN gene from nt 45 to 1685 was selected for this analysis. Sequence analysis was performed using MEGA X software, version 10.1.8. Also, BLASTN searches were employed to detect the sequence similarities between the two strains NDV261 and NDV-Vero20, together with reference strains in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

To predict the HN relationship between experimental strains and vaccine strains, multiple sequence alignment of the HN gene of NDV261 and NDV-Vero20 was done together with other representative thermostable vaccines I2–AY935499, I2 progenitor–AY935500, TS09C-JX110635,

NDV4-C-JX443519, and thermolabile Lasota–JF950510, LaSota C5–KC844235, and D58 strain-EU305607. The parameters used for the sequence analysis were multiple alignments (Claustal W), sequence identity plotter, and sequence matrix at both amino acid and nucleotide levels. The transmembrane domains were predicted using Dense Alignment Surface (DAS) transmembrane domain prediction server- http:www.sbc.su.se/-miklos/DAS/maindashtml [Cserzö et al., 1997].

II-2.7. Immunogenicity of NDV-Vero20 to chicks

Immunogenicity of NDV-Vero20 toward chicks was evaluated after inoculating the virus to chicks. Animal experimental work was performed in strict accordance with Animal Care guidelines of the Tokyo University of Agriculture and Technology (Tokyo, Japan) with permit number (R03-28). Six 1-day-old unvaccinated commercial layer chicks, hereafter designated "conventional chicks," were purchased from Tomaru Co., Ltd. (Gunma, Japan), labeled, settled in plastic cages inside the isolators (CL-5443, Clea Japan, Tokyo, Japan), and kept until 4 day-old, then used for the experiments.

Blood samples were taken from chicks at 4, 14 and 30-day-old to detect maternal antibodies through a jugular vein, because their wing vessels was too narrow to collect blood from the wing vein. At 30 day-old, the birds were divided into two groups - the treatment group (#1-3) and the control group (#4-6) and housed in separate cages in separate isolators. At 30 day-old, the treatment group was inoculated with the virus of 10^6 PFU per chick through eye drop of 150 µL/bird. And the control group received 150 µL PBS/bird. All the experimental birds were bled through a wing vein at 37, 44, 51, and 60 day-old, namely at 7, 14, 21, 30 days post-vaccination (dpv).

Oropharyngeal swabs were collected from all chicks using a rayon cotton bulb swab (Japan Becton Dickinson, Tokyo, Japan) at 0, 2, 3, 5, 7, 14, and 21 dpv. The swabs were put in vials containing transport medium (brain heart infusion broth 3.7%, penicillin 100 IU/mL, streptomycin 1 mg/mL, Amphotericin B 5 mg/mL [Pedersen et al., 2014], vortexed, and kept for 1 h at 4 °C, then stored -30°C up to the day of inoculation. Swab samples were titrated on a monolayer of CK cells in 96-well microplates. Serial ten-fold dilution was prepared per swab sample in MM, and the samples were inoculated to CK cells seeded in 96-well plates of 100 µL/well and four wells per dilution. Cytopathic effects (CPE) were observed daily in the inoculated plate, and HA test was performed five days post-inoculation to confirm the result.

Virus neutralization (VN) test was performed in CK cells to calculate the chicken's maternal and immune antibody titers against NDV using the 50% plaque reduction method, with a constant amount of virus and varying serum dilution as described [Ruenphet et al., 2012]. Briefly, serum samples were collected from conventional chicks before vaccination at 4, 14, and 30 day-old, and post-vaccination at 37, 44, 51, and 60 day-old. The sera were then diluted serially four-fold in PBS, and mixed with an equal volume of NDV Sato [Takehara et al., 1987]. The neutralizing antibody titer at 50% plaque-reduction point was calculated by means of Behrens-Karber's method [Matumoto, 1949].

II-3. Results

II-3.1. Thermostability

The NDV261 strain survived with HA titer (64 $\log_2$) after exposure at 56°C for 120 min initially whereas NDV Sato, NDV TCND, NDV Ishii and NDV B1 showed <4 $\log_2$. NDV-Vero20 was exposed at 56°C for 30 min more than 20 times and showed HA activity higher than 8 $\log_2$ HA titer as indicated in Table II-2.

Virus titers ($log_{10}TCID_{50}$/mL) on Vero cells of NDV-Vero10, NDV-Vero15, and NDV-Vero20 after heat treatment at 56°C for 30 min were 5.25 ± 0.14, 6.83 ± 0.08, 8.00 ± 0.00, respectively.

II-3.2. Virus growth curve in Vero cells

Multicycle growth kinetics of NDV261 and NDV-Vero20 viruses in Vero cells at MOI 0.01 PFU/cell were titrated on Vero and CK cells (Fig II-1). NDV-Vero 20 grew faster and higher in Vero cells than the original virus, NDV261, and the virus titers measured on CK cells were higher than on Vero cells (Fig II-1). The peak titers were measured at 36 h post-inoculation.

II-3.3. Thermal stability test

The thermostability of NDV-Vero20 was evaluated after incubation at 37°C, 25°C, and 4°C for 0.5, 1, 1.5, 2, 5, 7, and 10 days, respectively. The infectious titer (log_{10}TCID50/mL) little decreases along time in all selected temperatures, as shown in Fig II-2.

II-3.4. F and HN gene sequence analysis

Accession number of the sequences of part of F gene and complete HN gene for NDV261 and NDV-Vero20 were LC709183, LC709184, LC709182 and LC709181, respectively. The same amino acid sequence was found in the F gene cleavage site of both NDV261 and NDV-Vero20. These strains possess ^{112}GKQGR*L^{117} at the cleavage site (Table II-3), which is of an avirulent type [Sakai et al., 2007].

The HN gene of both NDV-Vero20 and NDV261 strains contain coding sequence (cds) comprising 1850 nucleotides coding for 616 amino acids. The transmembrane domains at positions

23–44 and 25–42 of both NDV261 and NDV-Vero20 are similar to two predictable thermostable domains of the representative vaccine strains, but differ from thermolabile strains (Table II-4). The sequence identity matrix for HN gene of NDV-Vero20, NDV261, and other representatives thermostable and thermolabile vaccine strains of NDV in different genotypes (genotypes I, II, and VIII) were provided in Table II-5. Point variations between thermostable and thermolabile viruses are shown (Fig II-3).

II-3.5. Immunogenicity of the strain NDV-Vero20

The virus shedding was confirmed by isolating NDV in CK cells. The virus was recovered from all vaccinated chicks at 2, 3 and 5 dpv and from 2 chicks at 7 dpv, but not from mock infected control chicks (Table II-6). The result from VN test showed that all chicks had high maternal antibody titers, which were gradually decreased. After vaccination at the 30 day-old, the vaccinated group exhibited marked immunity titers and retained immunity up to 60 day-old, whereas the control group's passive immunity titer was diminishing to <160 as shown (Fig II-4) [Sakai et al., 2006].

II-4. Discussion

For NDVs, thermostability is measured by HA activity or infectivity after exposure to a constant temperature (56°C) for different time intervals [King, 2001]. The criteria of the NDV thermostable lentogenic strains is their HA persistence longer than 30 min at 56°C and at least 20 min for a 2 $\log_{10}$ reduction of infectivity titer at 56°C temperatures [Lomniczi, 1975]. The HA activity of thermostable strains remains at 56°C for 120 min, while for other lentogenic thermolabile strains (B1, LaSota, and F strains) persisted only for 5 minutes [Spalatin and Hanson,

1966]. Some thermostable strains like I2, HR-V4, Ulster, and V4, have been isolated, characterized, and found to furnish suitable protection efficacies [Bensink and Spradbrow, 1999, Ideris et al., 1990, Simmons, 1967, Spalatin and Hanson, 1976].

In the present study, NDV261 was a confirmed thermostable lentogenic strain and it was adapted to Vero cells. NDV-Vero20 exhibited a high HA titer (Table II-2). This finding was similar to that reported for the thermostable NDV I-2 strain (Bensink and Spradbrow, 1999) and another one [Omony et al., 2016].

NDV-Vero20 grew better than the original NDV261 in Vero cells (Fig II-1), and its high infectivity titer is similar to others' findings [Ahamed et al., 2004, Siddique et al., 2017], thus confirming its adaptation to Vero cells.

In the thermal stability test, NDV-Vero20 showed little decrease in infectious titer (log_{10}TCID50/mL) after incubation at 37°C, 25°C, and 4°C for 0.5, 1, 1.5, 2, 5, 7, and 10 days (Fig II-2). This finding is in similarity to the thermostable NDV I-2 strain [Boumart et al., 2016].

The intracerebral pathogenicity index (ICPI) of NDV261 was 0.0, indicating that it was an avirulent type [Sakai et al., 2007]. The partial F gene sequence of both NDV261 and NDV-Vero20 possess ^{112}GKQGR*L^{117} at their cleavage sites, which were denoted as an avirulent type (Table II-3).

The mutations of S315P and I369V in the HN protein could contribute to enhancing viral thermostability [Ruan et al., 2020]. The presence of cysteine amino acid residue at position 123 has been reported to be essential for intramolecular disulfide bonds that stabilize the oligomeric HN structure gene [Crennell et al., 2000]. The R416 is essential for receptor binding, while NA and HA activities were likewise conserved even after exposure to 56° C, as were the receptor binding sites involving E401, R416, and Y526 [Connaris et al., 2002]. Several amino acid residues

have been reported as functional amino acid residues responsible for receptor binding, while NA and HA activities were likewise conserved even after exposure to 56° C, F220, S222, L224, E401, R416, and Y526 in HN protein. Thirteen amino acid residues at positions 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531, and 542 in HN protein have been reported to be essential for intramolecular disulfide bonds that stabilize the oligomeric HN structure gene, increasing the hydrophobic properties of the entire HN molecule responsible for thermostability after exposure to 56 °C [Ponnusamy et al., 2009, Römer-Oberdörfer A et al., 2003]; in that connection, findings found in HN gene of NDV-Vero20 are presented (Fig II-3).

The predicted amino acids present in the transmembrane domain of NDV-Vero20 23LVFRIAILLLTVVTLAISAAL44 and 25FRIAILLLTVVTLAISAA42 are similar to representative thermostable strains and different from thermolabile strains (Table II-4) [Omony et al., 2016].

Protein stabilities have been predicted by sequence feature-based predictions upon amino acid substitutions, while using models [Teng et al., 2010]. Our observed amino acids substitutions at V24I, A43S, A45L, E49G, A62R, A73T, N95K, S120N, C123W, H203Y, I266V, S269R, V369I, V404I, V453I, S464P, V477I, D494G, E495V, and I509T in our thermostable NDV-Vero20 strain all decreased protein stability with prediction confidence of >80%, which in our case partly explains thermostability phenotype (as shown in Fig II-3) [Omony et al., 2016]. The sequence similarities for both amino acid and nucleotide of NDV-Vero20 with other thermostable and thermolabile strains showed that thermostability present in NDV-Vero20 strain irrespective of genotyping may be due to some similarities of amino acids in HN genes of other thermostable NDV vaccine strains (Table II-5) [Ruan et al., 2020].

When three 30-day-old chicks were inoculated with NDV-Vero20, the virus was recovered until 7 day-old (Table II-6), and the VN titer remained at protective levels for more than 30 days (Fig II-4). HI antibody titer of NDV 3 $\log_2$ (i.e 1:8) and above is consider as positive for specific immunity [Allan and Gough, 1974, Hossain et al., 2010, Numan et al., 2005] and sera with antibody titers 4 $\log_2$ reported [Ghaniei and Mohammadzadeh, 2012, OIE, 2012b]. There was no weight loss observed in the vaccinated birds (data not shown), and no clinical signs were observed in the vaccinated birds.

Today, thermostable strains like HR-V4 and I-2 are preferred due to their maintained potency without the need for a cold chain [Jeon et al., 2008]. Considering that ND vaccination partly failed in the field due to the cold chain inadequacy in hot climates, the thermostability of NDV vaccine strains has been improved and then used in licensed live vaccines [Lomniczi, 1975]. The I-2 strain derived from the parental V4 strain has a better thermostability and is now licensed in Australia [Alders, 2014, Nyaupane et al., 2016]. Our finding of the thermostability of NDV-Vero20 was mostly similar to findings by Omony et al. (2016), who identified thermostable NDV strains exhibiting persistence after heating at 56°C for one h, and also on the molecular basis of HN gene for thermostability. Furthermore, one thermostable strain has been proved to perfectly protect the chicken from the virulent challenge [Jeong et al., 2013]. Like in the Australian experience of enhancing thermostability of V4 vaccine strain from a subpopulation of the known heat-stable virus also adopted by Ideris et al. (1990), we subjected clean ND-virus isolates to the same temperature (56 °C), followed HA titers for 2 h, and selected the yield as thermostable isolates.

Based on these outcomes of the present study, it was concluded that the NDV-Vero20 strain is an avirulent, appropriately thermostable, Vero cell adapted, highly immunogenic, and low

cumulative titer drop candidate vaccine strain, which constitutes a proper alternative to traditional embryonated chicken egg-passaged based vaccine.

II-5. Conclusion

Our experimental strain NDV-Vero20 is a Vero cell adapted, most robust candidate vaccine strain that can resist high temperatures and thus be advantageously used in rural areas, and in tropical or subtropical countries. In the future, this thermostable vaccine candidate strain is expected to help control Newcastle disease in Afghanistan and tropical regions such as Bangladesh.

Table II-1. Specific primers used for RT-PCR

Gene	Primer	Primer sequence	Position	Product	Reference
M	NDV-F2	5′-TGGAGCCAAACCGCGCACCTGCGG-3′	4235-4258	766 bp	Mase et
F	NDV-R2	5′-GGAGGATGTTGGCAGCAT-3′	4983-5000		al., 2009
HN	NDVHN1F2	5′-GGCTTCMCAACATCCGTTCTAC-3′	6364-6385	652 bp	Byarugaba et al. 2014
	NDVHN1R2	5′-GAATGYGAGTGATCTCTGCA-3′	6997-7016		
	NDVHN2F2	5′-CATGAGYRCTACCCAYTACTG-3′	6948-6968	591 bp	
	NDVHN2R2	5′-GATAGATAAGATGGCYTGCTG-3′	7519-7539		
	NDVHN3F2	5′-GGGTGGCAAAYTACCCAGGAG-3′	7295-7315	768 bp	
	NDVHN3R2	5′-GTATTGGATATTTCRGCAATGC-3′	8042-8063		
	NDVHN4F2	5′-GCATACACGACATCGACATG-3′	7984-8003	513 bp	
	NDVHN4R2	5′-CGGTARCCCAGTYAATTTCCA-3′	8477-8497		

Reference - Primer sequence alignment with JX443519.1 NDV4-C strain collected from NCBI online databank link; https://www.ncbi.nlm.nih.gov/nucleotide/JX443519.1?report=genbank&log$=nuclalign&blast_rank=1&RID=80WME57B013&from=1&to=15186

Table II-2. Effect of heat treatment (56°C) on hemagglutination activity of the NDV-Vero20 after each passage on Vero cells from the original NDV261.

NDV strain	Time (min)	HA titer
NDV Sato	120	$<2^2$
NDV TCND	120	$<2^2$
NDV B1	120	$<2^2$
NDV Ishii	120	$<2^2$
NDV 261	120	2^6
NDV 261	Before heat therapy	2^7
NDV-Vero1	30	2^5
NDV-Vero2	30	2^7
NDV-Vero3	30	2^5
NDV-Vero4	30	2^6
NDV-Vero5	30	2^3
NDV-Vero6	30	2^3
NDV-Vero7	30	2^3
NDV-Vero8	30	2^3
NDV-Vero9	30	2^3
NDV-Vero10	30	2^3
NDV-Vero11	30	2^3
NDV-Vero12	30	2^4
NDV-Vero13	30	2^4
NDV-Vero14	30	2^4
NDV-Vero15	30	2^5
NDV-Vero16	30	2^5
NDV-Vero17	30	2^5
NDV-Vero18	30	2^5
NDV-Vero19	30	2^6
NDV-Vero20	30	2^7

Vero cells and the viruses at each passage were named NDV-Vero1 to NDV-Vero20.

Table II-3. Partial F gene sequencing of original and Vero cell adapted candidate vaccine strain

Type	Amino acid sequences with cleavage site of F gene	Reference
NDV261 F gene	^{112}GKQGR*L^{117}	(Sakai et al., 2007)
NDV-Vero20 F gene	^{112}GKQGR*L^{117}	In this experiment
NDV261 F gene	^{112}GKQGR*L^{117}	In this experiment

Table II-4. Predicted transmembrane amino acid sequences analysis of the HN gene of NDV-Vero20, NDV261, and commercially available thermostable and thermolabile vaccine strains.

NDV strain	Start	End	Length	Cutoff	Predicted amino acid sequence
NDV-Vero20	23	44	22	1.7	**LVFRIAILLLTVVTLAISAAAL**
	25	42	18	2.2	**FRIAILLLTVVTLAISAA**
	208	222	15	1.7	LGVLRTSAIGRVFFS
NDV261	23	44	22	1.7	**LVFRIAILLLTVVTLAISAAAL**
	25	42	18	2.2	**FRIAILLLTVVTLAISAA**
	208	222	15	1.7	LGVLRTSAIGRVFFS
I-2[A]	23	44	22	1.7	**LVFRIAILLLTVVTLAISAAAL**
	25	42	18	2.2	**FRIAILLLTVVTLAISAA**
	424	430	7	1.7	ALLYPMI
	557	563	7	1.7	RIVPLLV
I-2 progenitor[A]	23	44	22	1.7	**LVFRIAILLLTVVTLAISAAAL**
	25	42	18	2.2	**FRIAILLLTVVTLAISAA**
	424	430	7	1.7	ALLYPMI
	557	563	7	1.7	RIVPLLV
TS09-C[A]	24	44	21	1.7	**VFRIAILLSTVVTLAISAAAL**
	25	42	18	2.2	**FRIAILLSTVVTLAISAA**
	210	211	2	1.7	VL
	424	430	7	1.7	ALLYPMI
	557	563	7	1.7	RIVPLLV
NDV4-C[A]	24	44	21	1.7	**VFRIAILLSTVVTLAISAAAL**
	25	42	18	2.2	**FRIAILLSTVVTLAISAA**
	210	210	1	1.7	L
	424	430	7	1.7	ALLYPMI
	557	563	7	1.7	RIVPLLV
LaSota[B]	24	47	24	1.7	**IFRIAILFLTVVTLAISVASLLYS**
	25	45	21	2.2	**FRIAILFLTVVTLAISVASLL**
	557	563	7	1.7	RIVPLLV
LaSota C5[B]	24	47	24	1.7	**IFRIAILFLTVVTLAISVASLLYS**
	25	45	21	2.2	**FRIAILFLTVVTLAISVASLL**
	557	563	7	1.7	RIVPLLV
D58[B]	24	47	24	1.7	**IFRIAILFLTVVTLAISVASLLYS**
	25	45	21	2.2	**FRIAILFLTVVTLAISVASLL**
	557	563	7	1.7	RIVPLLV

The transmembrane domains marked with bold sequences showed similarities and variations between thermostable and thermolabile strains.
[A]Thermostable strains, [B]Thermolabile strains.

Table II-5. Sequence identity matrix (%) for CDS of HN gene.

Sequence	[a]NDV Vero20	[a]NDV 261	[a]I-2	[a]I-2 progenitor	[a]TS09 -C	[a]NDV4-C	[a]PHY-LMV42	[c]HR09	[b]LaSota	[b]LaSota C5	[a]D58	[a]Ishii
NDV-Vero20	**ID**	**99.82**	**95.59**	**95.77**	**97.43**	**97.98**	**98.34**	**92.29**	**94.30**	**94.67**	**94.12**	**98.63**
NDV261	99.72	**ID**	**95.40**	**95.59**	**97.24**	**97.79**	**98.16**	**92.29**	**94.12**	**94.49**	**93.93**	**98.90**
I-2	91.36	91.26	**ID**	**99.48**	**95.67**	**96.19**	**95.58**	**90.19**	**93.06**	**93.40**	**92.88**	**95.95**
I-2 progenitor	91.36	91.26	99.84	**ID**	**95.85**	**96.37**	**95.76**	**90.3**	**93.23**	**93.58**	**93.06**	**96.13**
TS09-C	93.15	93.06	95.47	95.50	**ID**	**99.03**	**97.28**	**90.89**	**93.75**	**94.10**	**93.58**	**97.79**
NDV4-C	93.39	93.29	95.55	95.59	99.81	**ID**	**98.16**	**91.42**	**94.27**	**94.62**	**94.10**	**98.34**
PHY-LMV42	93.30	93.25	92.98	92.98	95.30	95.57	**ID**	**91.59**	**94.29**	**94.66**	**94.11**	**98.46**
HR09	86.74	86.68	85.52	85.52	86.05	86.16	85.93	**ID**	**90.19**	**90.54**	**90.02**	**92.12**
LaSota	88.43	88.34	87.74	87.77	89.51	89.61	89.02	84.12	**ID**	**99.65**	**99.83**	**94.66**
LaSota C5	88.57	88.48	87.83	87.83	89.61	89.72	89.24	84.18	99.82	**ID**	**99.48**	**95.03**
D58	89.15	89.09	88.48	88.48	89.58	89.81	89.72	84.06	99.94	99.83	**ID**	**94.48**
Ishii	94.49	94.43	94.08	94.08	96.19	96.42	97.27	86.75	90.30	90.36	90.35	**ID**

[a]Genotype I, [b]Genotype II, [c]Genotype VIII. Amino acid differences are indicated in bold and nucleotide differences are in normal font. The difference from commonly used vaccine strains is high lightened.

Table II-6. Isolation of NDV after inoculation of layer chicks with NDV-Vero20 strain through eye drops

Chick number	Group	Days post-vaccination						
		0	2	3	5	7	14	21
1	Treatment	-	+	+	+	-	-	-
2	Treatment	-	+	+	+	+	-	-
3	Treatment	-	+	+	+	+	-	-
4	Control	-	-	-	-	-	-	-
5	Control	-	-	-	-	-	-	-
6	Control	-	-	-	-	-	-	-

[+]NDV was isolated from oropharyngeal swab.

[-]NDV was not isolated from oropharyngeal swab.

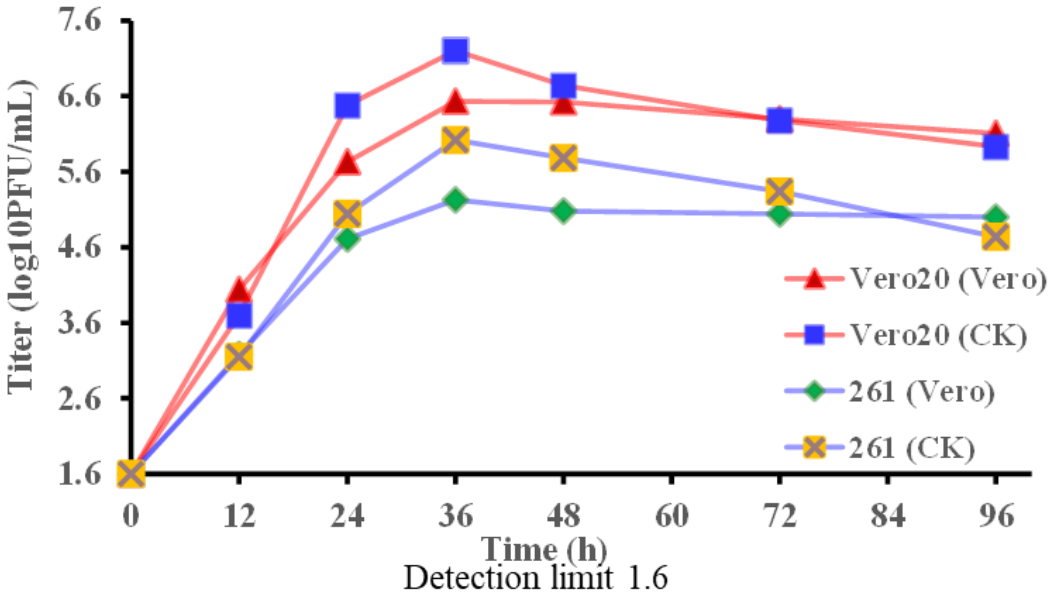

Fig II-1. Graphical representation of growth kinetics of NDV261 and NDV-Vero20 in Vero cell line and CK cells.

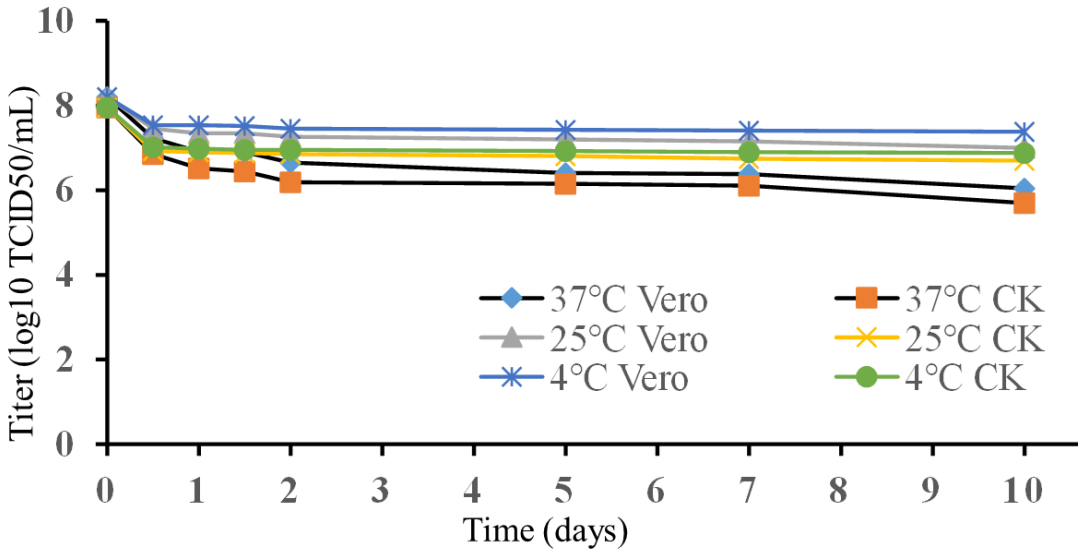

Fig II-2. Infectious titer drops (log_{10} $TCID_{50}$/mL) of the NDV-Vero20 vaccine candidate, as maintained at 37℃, 25℃, and 4℃ on CK and Vero cells.

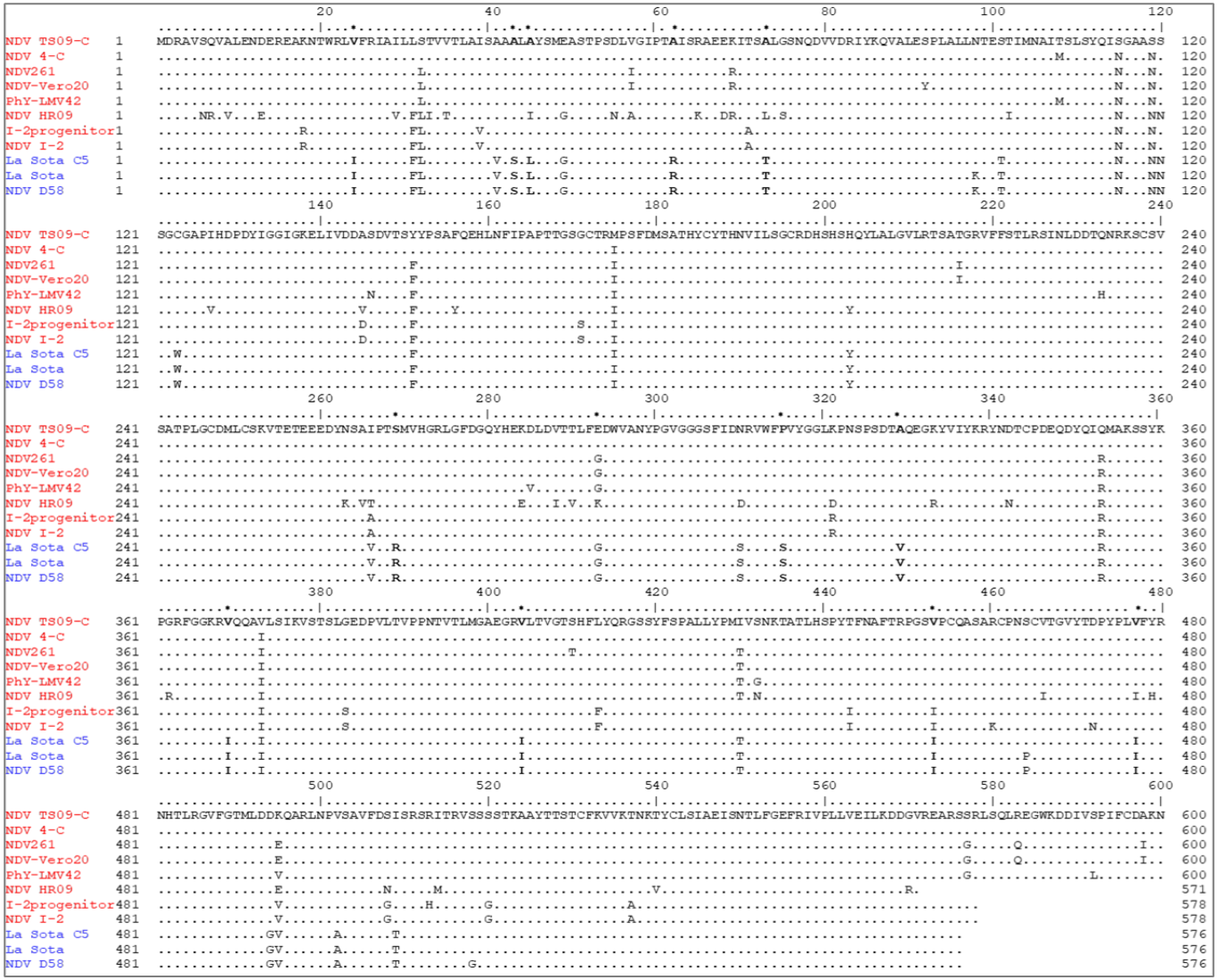

Fig II-3. Multiple amino acid sequence alignment of HN proteins. Red and blue colors indicate thermostable and thermolabile strains. Black bold fonts marked by asterik (*) denote point variation in identity between thermostable and thermolabile viruses.

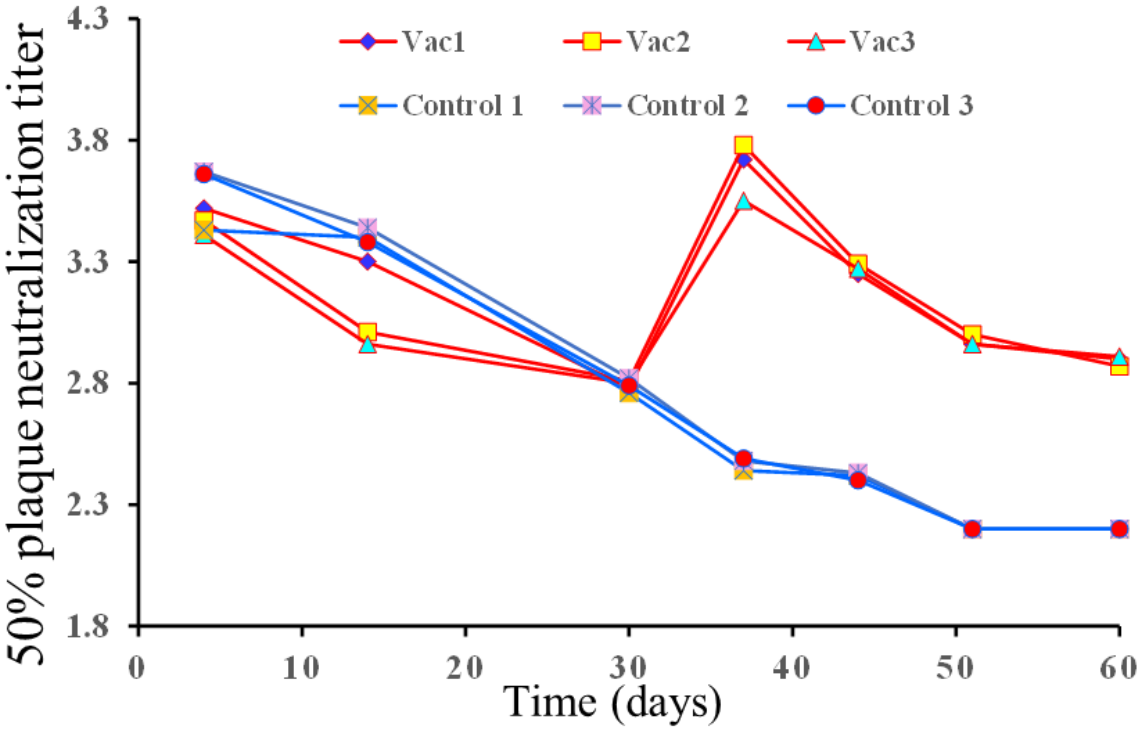

Fig II-4. 50% plaque reduction titer in $\log_{10}$ of NDV-specific antibodies of layer chickens following vaccination with NDV-Vero20 candidate vaccine.

Chapter III

Evaluation of disinfection materials in different environments to enhance farm biosecurity

III-1. Introduction

Enhancement of farm biosecurity through the use of appropriate disinfectants is one of the most important means of reducing the number of pathogens [Meroz and Samberg, 1995, Velkers et al., 2017]. Prevention and control of diseases largely depend on biosecurity, and disinfectants are essential tools for biosecurity programs [Alam et al., 2018a, Hakim et al., 2015, Thammakarn et al., 2015a]. Various environmental factors such as temperature, organic load, and short contact time can reduce disinfection abilities Alam et al., 2018c, Gerba, 2015, Stringfellow et al., 2009, Thammakarn et al., 2016]. The efficacies of some disinfectants will be lost at low temperatures [Alam et al., 2018b, Dee et al., 2005, Ito et al., 2018].

Poultry infectious diseases, especially avian influenza (AI), Newcastle disease (ND), infectious bursal disease (IBD), the diseases due to fowl adenovirus (FAdV), and avian reovirus (ARV), colibacillosis and salmonellosis are highly contagious and detrimental for the poultry industry. Since 2003, especially during the winter season, highly pathogenic avian influenza (HPAI) has been widespread and persistent in Asia and Africa, and discontinuously in Europe [Sakuma et al., 2021, Sims, 2007]. NDV is one of the notorious poultry pathogens severely endangering the poultry industry and resulting in a highly contagious septicemic, fatal, and destructive disease that affects a wide variety of domestic and wild birds worldwide [Diel et al., 2012, Dimitrov et al., 2019b]. FAdV and ARV are ubiquitous in poultry facilities because of their resistance to a wide range of disinfectants [Inoue et al., 2020, Mustaffa and Spradbrow, 1975, OIE, 2001, Savage and Jones, 2003].

Quaternary ammonium compounds (QACs) are common disinfectants widely used at livestock farms and food processing industries, owing to their relatively low toxicity and broad antimicrobial spectrum, alongside chemical stability. However, their inactivation efficacies are

diminished by organic material contamination or at low temperatures [Gerba, 2015, Stringfellow et al., 2009] and QACs are not able to inactivate non-enveloped viruses [Ito et al., 2018, OIE, 2001].

The synergistic virucidal activity of Food additive-grade calcium hydroxide $FdCa(OH)_2$ and QAC was shown in the suspension test at 2°C, and the spectrum of virucidal activity of QAC with the addition of $FdCa(OH)_2$ was also shown to be broadened against a non-enveloped virus [Ito et al., 2018]. The synergistic virucidal and bactericidal activities at 2°C were also demonstrated in the suspension and carrier tests [Alam et al., 2018b, 2018a]. It was also shown that FAdV and ARV were inactivated within 5 sec by $FdCa(OH)_2$ regardless of the presence or absence of organic matter and temperature conditions (25°C and 2°C) [Daio et al., 2020]. Guan *et al.* showed that preparations of disinfectant supplemented with methanol (MeOH) as an anti-freeze agent could be effectively applied at temperatures as low as -20°C against AIV for disinfection [Guan et al., 2015].

Quite recently, in the evaluation of disinfectants, it has become clear that some disinfectants could inactivate pathogens in suspension tests, but not in carrier tests [Miyaoka et al., 2021a, 2021b]. For the evaluation of disinfectants, the suspension test is the first screening and the carrier test is the second screening for the field usage [Miyaoka et al., 2021b, Sattar and Maillard, 2013].

Slaked lime (SL), an inorganic compound containing > 70% $Ca(OH)_2$ and a strong alkaline substance was also used to inactivate pathogens [Thammakarn et al., 2015c]. In Japan, the Ministry of Agriculture, Forestry, and Fisheries (MAFF) recommends to livestock farmers spread slaked lime powder for standby sterilization [Takehara, 2021]. Livestock farmers tend to use SL powder as a disinfectant for foot baths to inactivate viruses and bacteria on their boots during the freezing

season or try to use it in combination with QACs in the hope of replacing $FdCa(OH)_2$ (Takehara, personal communication). However, it takes 3 to 6 h for the SL powder to inactivate pathogens [Hakim et al., 2017c], and its synergistic effects with QACs have not been demonstrated under saturated conditions.

It is essential to keep disinfectants efficacy even in cold conditions. In the present study, the synergistic efficacies of QACs with alkaline agents such as $FdCa(OH)_2$ and SL were examined at low temperatures. It was also important to perform the experiments at the indicated temperature, for example, using aluminum racks to maintain the exact temperature as shown in Fig 3-1. Otherwise, the temperature will become high, and will not get the correct data.

III-2. Materials and methods

III-2.1. Experimental design

The current study was to evaluate the synergistic microbicidal efficacies of the mixtures of $FdCa(OH)_2$ and QACs or SL and QAC toward several viruses and bacteria, especially at low temperatures during the winter season for proper selection and dose maintenance of the disinfectants. Suspension tests were conducted with these disinfectants and evaluated for several contact times for both viruses and bacteria. For the experiments at -20°C, the anti-freezing agent was mixed with an equal volume of disinfectant solutions to avoid freezing. After disinfectant treatments, the remained viruses or bacteria were titrated.

III-2.2. Anti-freeze agent

A commercially available window washer, Kaihyo Super washer solution® made by KOGA Chemical Mfg. Co., Ltd. (Saitama, Japan), which contains 51-53 wt.% methanol along

with surfactants, chelator, and antiseptic agents was purchased at a local market and used as the anti-freeze agent (AFA) that lowers the freezing point of a water-based liquid. In this study, for the experiments at -20°C, QACs were diluted at 1:250 with 0.34 % $FdCa(OH)_2$ and mixed with AFA in equal amounts.

III-2.3. Chemical disinfectants and neutralizers

QACs, Rontect® containing didecyl dimethyl ammonium chloride and Pacoma® containing trimethyl ammonium chloride were purchased from Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) and Meiji Seika Pharma Co. (Tokyo, Japan), respectively. $FdCa(OH)_2$ powder with an average particle diameter of 10 µm was kindly supplied by Fine Co., Ltd. (Tokyo, Japan). Rontect® and Pacoma® were diluted 500 times with 0.17% $FdCa(OH)_2$ (thereafter, Rox500 and Pax500, respectively). For -20°C experiments, these QACs were diluted 1: 250 with 0.34% $FdCa(OH)_2$ and mixed equally with AFA (finally 1: 500 dilution for QACs and 0.17% $FdCa(OH)_2$: thereafter, Rox500-20 or Pax500-20, respectively). As a negative control, AFA mixed with phosphate buffer saline (PBS) in equal amounts was prepared and designated as AFA-PBS-20. SL of 70% calcium hydroxide, 70.0 Slaked lime® (made in China, imported by Kumamoto Bussan Co., Kumamoto, Japan), with particle diameter less than 150 µm was purchased. Virucidal efficacies of QAC with 0.24% SL (solid SL 0.24 g and 100 µL Rontect® added in 100 mL dW_2 defined as Ron + SL) and single 0.24% SL (solid SL 0.24 g added in 100 mL dW_2 designated as saturated SL) were also evaluated. For the experiments at 1°C, all solutions and viruses were kept on ice for around 1 h until the solution's temperature became 1°C before starting the experiments as shown in Fig III-1. For experiments at -20°C, all solutions containing 50% AFA were kept at -20°C freezers for at least 1 h until the temperature became -20°C before starting the experiments.

These solution's temperatures were confirmed with a bar thermometer and during the experiment, all microtubes were placed in aluminum racks and the temperature was strictly controlled (Fig III-1).

A chemical neutralizer, namely a blocking solution for the virus (thereafter BSV) containing 30% fetal bovine serum (FBS) in 0.7 M 4-(2-hydroxyl)-1-piperazineethanesulfonic acid (pH 7.2), was prepared for neutralizing the virus inactivation efficacy of solutions right after the given disinfectant contact time [Alam et al., 2018a]. Another blocking solution for bacteria (thereafter BSB) containing 30% FBS in 0.7 M Tris–HCl (pH 7.2) to stop the bacterial inactivation reaction was prepared and used right after the selected contact time of disinfectant application [Alam et al., 2018b].

III-2.4. Viruses, bacteria, and cells

A low pathogenic AIV, A/duck/Aomori/395/04 (H7N1) (Jahangir et al., 2010b) and virulent NDV strain Sato as enveloped viruses, and FAdV strain Ote and ARV strain Uchida [Daio et al., 2020] as non-enveloped viruses were prepared and titrated in the form of 50% tissue culture infective dose ($TCID_{50}$)/mL [Jahangir et al., 2010a] in primary chicken kidney (CK) cells. *Salmonella* Infantis (SI), *Escherichia coli* (EC) were grown in Luria Bertani (LB) medium and titrated on DHL agar as colony forming units (CFU)/mL as described [Alam et al., 2018b].

III-2.5. Evaluation of the virucidal and bactericidal activities using the suspension test

In the present study, two reaction temperatures (-20°C and 1°C) and several contact time points were used for investigating the inactivation process of the above-mentioned viruses and

bacteria in the aqueous phase using the suspension test. For the -20°C experiment with viruses and bacteria, all microtubes containing 400 µL of disinfectants were kept at -20°C freezer for 1 h. And for the 1°C investigation, all microtubes containing 400 µL of disinfectants were kept on ice for around 1 h.

Four hundred microliters of Rox500-20, Pax500-20 or AFA-PBS-20 were mixed separately with 100 µL of each virus, then incubated for indicated times at -20°C. In parallel, 400 µL of Rox500-20 or Pax500-20 were mixed with 500 µL of BSV in a microtube and then was added 100 µL of each viral solution in the microtube, to evaluate the blocking solution's effect (shown as 0 sec treatment and contact time).

At 1°C, microtubes containing 400µL Rox500, Pax500, Ron+SL, or saturated SL were mixed with 100 µL of each virus, and then incubated for the indicated times, in parallel with 0 sec.

After the process of incubation, the virus inactivation efficacy of each solution was stopped by adding 500 µL of BSV. The remaining viable virus titer in each sample, including the positive control that 100 µL of each virus was mixed with 200 µL of PBS, 200 µL of AFA, and 500 µL of BSV and then kept at 1°C for 1 h, was titrated (log_{10} $TCID_{50}$/mL) by inoculating it on CK cells in 96-well tissue culture plates (four wells per dilution, 200 µL final volume in each well) after making serial 10-fold dilutions.

For bactericidal evaluation, 400 µL of Rox500-20 or AFA-PBS-20 was mixed with 100 µL of each bacterial solution, and then incubated at -20°C for 3 or 5 min for Rox500-20 and 5 or 10 min for AFA-PBS-20. After incubation, the bactericidal efficacy of the tested solution was blocked by adding 500 µL of BSB. At last, bacterial viable counting in each sample was calculated (log_{10} CFU/mL) by plating 25 µL portions on DHL agar plates after making serial ten-fold

dilutions in PBS, followed by 24 h incubation at 37°C. In the case of positive control, 100 µL of bacteria were separately mixed with 200 µL of PBS, 200 µL of AFA, and 500 µL of BSB kept at 1°C for 1h, and then ten-fold serial dilutions were made.

The tested solution was evaluated in triplicate, and the titers were shown as mean ± SE. Inactivation was considered to be effective if ≥ 3 $\log_{10}$ reduced organism titers were obtained, indicating a more than 1,000 times viral or bacterial titer reduction [Lombardi et al., 2008, Takehara et al., 2009, Thammakarn et al., 2015a].

III-3. Results

III-3.1. Evaluation of the virucidal activities of the solutions at -20°C

Table III-1 shows the virucidal activities of Rox500-20, Pax500-20, and AFA-PBS-20 toward AIV, NDV, FAdV, and ARV at -20°C. Rox500-20 was able to reduce the titers of enveloped viruses, namely AIV and NDV within 30 min and of non-enveloped viruses, namely FAdV and ARV, within 30 sec. Pax500-20 was almost as effective as Rox500-20, but slightly inferior. No viral inactivation was detected at 0 sec, where the neutralizing solution was added before virus addition, compared to the control viruses. AFA-PBS-20 had no virucidal effectiveness toward AIV, NDV, FAdV, and ARV within 30 min at -20°C.

III-3.2. Evaluation of the virucidal activities of the solutions at 1°C

Table III-2 shows the virucidal activities of Rox500, Pax500, Ron+SL, and saturated SL towards AIV, NDV, FAdV, and ARV at 1°C. Rox500 and Pax500 were able to inactivate FAdV, ARV within 30 sec, AIV within 10 min, and NDV within 30 min, respectively at an effective level (≥ 3 $\log_{10}$ $TCID_{50}$/mL). Ron+SL and saturated SL could not

inactivate AIV and NDV even after 60 min. However, Ron+SL could inactivate FAdV and ARV within 30 sec.

III-3.3. Evaluation of the bactericidal activities of Rox500-20 and AFA-PBS-20 solution at -20°C

Table III-3 shows the bactericidal activities of Rox500-20 and AFA-PBS-20 towards SI and EC at -20°C. Rox500-20 was able to inactivate EC to undetectable level (< 2.6 $\log_{10}$ CFU/mL) within 3 min and SI in effective level (≥ 3 $\log_{10}$ CFU/mL) within 3 min and to undetectable level within 5 min. AFA-PBS-20 had no significant bactericidal effectiveness toward SI and EC even after 10 min.

III-4. Discussion

Disinfection is essential for breaking down the infection chain of pathogens by reducing the risk of cross-contamination. Disinfectants are used for disease prevention and control [Meroz and Samberg, 1995]. The efficacies of most chemical disinfectants are affected by the presence of organic materials, low temperatures, and time of contact with pathogens [Gerba, 2015].

QACs are common disinfectants widely used in livestock farms and food processing industries, and their weakness, the decrease in inactivation activity at low temperature, has been overcome by the synergistic virucidal and bactericidal activities generated through the addition of FdCa$(OH)_2$ at 2°C [Alam et al., 2018b, 2018a, Ito et al., 2018].

Thus, evaluated changes in the efficacies of combined disinfectants used in this study under different contact times, at very low temperatures (-20°C), and low temperatures (1°C).

In the present study, the QACs and $FdCa(OH)_2$ mixtures with the methanol-based AFA namely Rox500-20 and Pax500-20, showed synergistic activity even at -20°C, as described previously for 2°C [Alam et al., 2018b, 2018a, Ito et al., 2018]. Pacoma® is the 2nd and Rontect® is the 3rd generation of QACs [Gerba, 2015], however as shown in Table III-1, their virucidal activities with $FdCa(OH)_2$ were not much different (Pacoma® was slightly inferior).

When the slaked lime powder was added to QAC to reach a saturated (0.17%) solution; so while the original slaked lime has 70% $Ca(OH)_2$ (and not 0.17g), 0.24g of slaked lime powder was added to 100 mL of 1: 500 diluted Rontect® to make 0.17% saturated solution - instead of $FdCa(OH)_2$ at 1°C - the synergistic effects were not demonstrated with QAC (Table III-2). The mixture with Rontect® and Pacoma® with $FdCa(OH)_2$, namely Rox500 and Pax500 inactivated AIV in 10 min and NDV in 30 min, respectively (Table III-2). The differences between $FdCa(OH)_2$ and the slaked lime were purity of $Ca(OH)_2$ and particle size; the $Ca(OH)_2$ content of $FdCa(OH)_2$ was 98% and that of the slaked lime was about 70%, and the particle size of the powder was 10 µm for $FdCa(OH)_2$ and less than 150 µm for the slaked lime as shown in MATERIALS AND MATHODS. Particle size seems to be important for inactivation activity as previously described [Thammakarn et al., 2014].

The mixture with Rontect®, $FdCa(OH)_2$, namely Rox500-20 could inactivate EC and SI at -20°C as shown in Table III-3. AFA itself as AFA-PBS-20 could not be able to decrease titers in 30 minutes and 10 minutes toward the above viruses and bacteria respectively as shown in Table III-1 and 4-3. Guan *et al*, (2015) showed that methanol as the anti-freeze agent had no adverse effect and the AIV was resistant to treatment with 20% MeOH alone for up to 30 min and it did not kill AIV.

III-5. Conclusion

It is recommended for enhancing farm biosecurity to use FdCa$(OH)_2$ for synergistic and broaden the spectrum of QACs in all seasons, as the mixtures can inactivate not only bacteria but also enveloped and non-enveloped viruses. Besides, it is not recommended to use QACs together with slaked lime.

Table III-1. Virucidal efficacies of Rox500-20, Pax500-20, and AFA-PBS-20 at -20°C

Solution type	Virus	Control	Viral titer ($log_{10}TCID_{50}$/mL) at different contact times				
			0 sec	5 sec	1 min	10 min	30 min
Rox500-20[a]	AIV	NT	8.66 ± 0.07	7.50 ± 0.00	7.33 ± 0.35	7.00 ± 0.00	3.50 ± 0.00[d]
Pax500-20[b]		NT	8.25 ± 0.11	7.00 ± 0.20	6.91 ± 0.07	5.50 ± 0.18	4.50 ± 0.00[d]
Rox500-20	NDV	NT	9.33 ± 0.07	8.50 ± 0.00	8.00 ± 0.17	6.67 ± 0.47	6.16 ± 0.59[d]
Pax500-20		NT	9.08 ± 0.18	7.41 ± 0.20	7.58 ± 0.24	6.67 ± 0.18	5.50 ± 0.12[d]
Rox500-20	FAdV	NT	8.50 ± 0.28	4.25 ± 0.38[d]	3.50 ± 0.00[d]	NT	NT
Pax500-20		NT	8.58 ± 0.30	3.50 ± 0.17[d]	3.17 ± 0.33[d]	NT	NT
Rox500-20	ARV	NT	8.91 ± 0.08	3.58 ± 0.65[d]	3.75 ± 0.25[d]	NT	NT
Pax500-20		NT	8.91 ± 0.08	4.58 ± 0.50[d]	3.42 ± 0.30[d]	NT	NT
AFA-PBS-20[c]	AIV	8.50 ± 0.28	NT	NT	NT	NT	7.58 ± 0.13
	NDV	9.25 ± 0.32	NT	NT	NT	NT	8.50 ± 0.20
	FAdV	8.25 ± 0.00	NT	NT	NT	NT	8.00 ± 0.00
	ARV	8.67 ± 0.07	NT	NT	NT	NT	8.25 ± 0.11

[a] Quaternary ammonium compounds (QAC), Rontect® from Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) was diluted 1:250 with 0.34 % $FdCa(OH)_2$ and mixed equally with the anti-freeze agent (AFA).

[b] QAC-Pacoma®, Meiji Seika Pharma Co. (Tokyo, Japan) was diluted 1:250 with 0.34 % $FdCa(OH)_2$ and mixed equally with the AFA.

[c] AFA mixed with phosphate buffer saline (PBS) in equal amount as AFA-PBS-20.

[d] Effective viral inactivation if ≥ 3 log_{10} tissue culture infective dose $_{50}$/ml ($TCID_{50}$/mL).
Data represent means ± SE of 3 different experiments. 'NT' denoted as not tested.

Table III-2. Virucidal efficacies of Rox500, Pax500, Ron + SL, and saturated SL at 1°C.

Solution type	Virus	Viral titer ($log_{10}TCID_{50}$/mL) at different contact times							
		0 sec	5 sec	30 sec	1 min	3 min	10 min	30 min	60 min
Rox500[a]	AIV	8.91 ± 0.24	NT	NT	6.08 ± 0.07	NT	4.41 ± 0.37[e]	4.16 ± 0.07[e]	3.75 ± 0.11[e]
Pax500[b]	AIV	8.58 ± 0.16	NT	NT	6.41 ± 0.13	NT	4.67 ± 0.07[e]	4.33 ± 0.13[e]	3.58 ± 0.07[e]
Rox500	NDV	9.25 ± 0.00	NT	NT	7.08 ± 0.07	NT	7.00 ± 0.23	4.67 ± 0.36[e]	4.58 ± 0.49[e]
Pax500	NDV	9.62 ± 0.15	NT	NT	8.00 ± 0.50	NT	7.50 ± 0.35	4.83 ± 0.36[e]	4.25 ± 0.50[e]
Ron+SL[c]	AIV	7.83 ± 0.068	NT	NT	6.17 ± 0.29	6.08 ± 0.136	NT	NT	5.67 ±0.07
	NDV	9.08 ± 0.068	NT	7.50 ± 0.00	7.50 ± 0.00	7.50 ± 0.00	NT	NT	6.33 ± 0.07
	FAdV	7.07 ± 0.068	5.25 ± 0.00	3.75 ± 0.71[e]	NT	NT	NT	NT	NT
	ARV	8.41 ± 0.068	5.16 ± 0.07[e]	3.83 ± 0.27[e]	NT	NT	NT	NT	NT
Saturated SL[d]	AIV	7.83 ± 0.068	NT	NT	7.50 ± 0.00	NT	7.50 ± 0.00	7.50 ± 0.00	7.25 ± 0.07
	NDV	9.08 ± 0.068	NT	NT	7.50 ± 0.00	NT	7.50 ± 0.00	7.50 ± 0.00	6.91 ± 0.13

[a] Quaternary ammonium compounds (QAC), Rontect® from Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) was diluted 1:500 with 0.17 % $FdCa(OH)_2$.

[b] QAC (Pacoma®), Meiji Seika Pharma Co. (Tokyo, Japan) was diluted 1:500 with 0.17 % $FdCa(OH)_2$.

[c] Solid slaked lime 0.24 g and 100 µL QAC-Rontect® added in 100 mL dW_2.

[d] Solid slaked lime 0.24 g added in 100 mL dW_2.

[e] Effective viral inactivation if ≥ 3 log_{10} tissue culture infective dose $_{50}$/mL ($TCID_{50}$/mL).

Data represent means ± SE of 3 different experiments. 'NT' denoted as not tested.

Table III-3. Bactericidal efficacies of Rox500-20 and AFA-PBS-20 at -20°C.

Solution Type	**Bacteria**	**Bacterial titer log_{10}CFU/mL at different contact times**			
		Control	3 min	5 min	10 min
Rox500-20[a]	*E. coli*	8.60 ± 0.00	<2.6 ± 0.00[c]	<2.6 ± 0.00[c]	NT
	Salmonella Infantis	9.00 ± 0.00	5.57 ± 0.09[d]	<2.6 ± 0.00[c]	NT
AFA-PBS-20[b]	*E. coli*	8.09 ± 0.40	NT	8.09 ± 0.36	7.91 ± 0.08
	Salmonella Infantis	8.24 ± 0.39	NT	8.24 ± 0.05	8.18 ± 0.28

[a] QAC-Rontect® was diluted 1: 250 with 0.34 % $FdCa(OH)_2$ and mixed equally with the anti-freeze agent.
[b] The anti-freeze agent (AFA) mixed with PBS in equal amount as AFA-PBS-20.
[c] Undetectable level, when < 2.6 log_{10} colony forming unit (CFU/mL). [d] Effective bacterial inactivation (≥ 3 log_{10} (CFU/mL). Data represent means ± SE of 3 different experiments.

Fig III-1. An aluminum rack was kept on ice. To ensure temperatures at 1°C or at -20°C, all reactions were performed in aluminum racks. For the 1°C experiments, the racks were placed on ice; for the -20°C experiments, the racks were kept in a -20°C freezer and removed from the freezer to a safety cabinet on ice. After solutions were added, the racks were immediately returned to the freezer.

General conclusion

General conclusion

Newcastle disease (ND) presents a permanent threat to global poultry production, with a significant economic impact. The health of animals is pivotal for successful poultry production; thus, biosecurity and specific immunoprophylaxis are essential parts of poultry management programs. Vaccination is routinely used in poultry flocks of all ages and production categories. Vaccine-induced immunity successfully protects animals from clinical disease and death but is unable to prevent infection with, or replication and shedding of, virulent field strains or challenge viruses; therefore, biosecurity is also a very important tool for controlling the infectious agents.

In my dissertation, isolation, identification and molecular characterization with a phylogenetic analysis of the F gene of Newcastle disease virus (NDV) isolates showed that sub-genotype VII1.1. viruses of class II continue to circulate in the poultry industry of Afghanistan in chapter I. Isolation and molecular characterization will be assisted to control ND. In this study, it was screened for thermostable NDV strain for making Vero cell line adapted candidate vaccine. In chapter-II, selected one NDV-Vero20 strain which was an avirulent, thermostable, Vero cell adapted, highly immunogenic, low cumulative titer drops and thermal stable candidate vaccine and alternative to traditional embryonated chicken egg-passaged based vaccine. The candidate vaccine besides being used for commercial flocks, as with commercially available V4, and I-2 thermostable vaccines, would be a suitable use for family or village flocks in rural areas due to its very low price, local production, and small quantities delivering a system with wide varieties of application routes in tropical or subtropical countries including Bangladesh. In the future, we hope that the thermostable candidate vaccine will find greater use and application to help control ND potentially in tropical worlds like Bangladesh and Afghanistan.

It has evaluated the synergistic efficacies of quaternary ammonium compounds (QACs) with alkaline agents such as food additive-grade calcium hydroxide ($FdCa(OH)_2$) and slaked lime (SL) at low temperatures toward avian potential pathogens in chapter-III. It is suggested for enhancing farm biosecurity to use $FdCa(OH)_2$ for synergistic and broadening the spectrum of QACs in all seasons, as the mixtures can inactivate not only bacteria but also enveloped and non-enveloped viruses. Besides, it is not recommended to use QACs together with SL. The present study aimed to evaluate the efficacy of some biosecurity materials against some critical viral and bacterial pathogens with adequate methods to find out the effective working concentration, contact time, and environmental conditions like low temperatures for its virucidal and bactericidal activity to help farmers to promote their strategy for enhancing biosecurity in their farms and food processing industries, especially in the winter season.

From the findings of the present studies, it can be concluded that surveillance, monitoring with the characterization of NDV, proper vaccination with avirulent thermostable strain, and enhanced biosecurity practices can prevent the ND successfully in the poultry industry.

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