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**MOLECULAR EPIDEMIOLOGY
OF AVIAN INFLUENZA AND NEWCASTLE DISEASE USING
SAMPLES TRANSPORTED WITHOUT A COLD CHAIN**

Thesis submitted by

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Master of Tropical Veterinary Science (by research)

In May 2011

For the degree of Master of Tropical Veterinary Science
at the School of Veterinary and Biomedical Sciences,
James Cook University

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published and unpublished work of other has been acknowledged in text and a list of references is given.

Desniwaty

May 2011

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LIST OF GENERAL ABBREVIATIONS

AI	Avian Influenza
AIV	AI Virus
APMV	Avian Paramyxovirus
APMV-1	Avian Paramyxovirus serotype 1
ANOVA	Analysis of Variance
AQIS	Australian Quarantine Inspection Services
AQAI	Agricultural Quarantine Agency of Indonesia
BLAST	Basic local alignment search tool
BHQ	Black Hole Quencher
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CEK	Chicken Embryo Kidney
cRNA	Complimentary RNA
Ct	Cycle threshold: The number of qPCR cycles taken for a reaction to produce a fluorescent signal that is statistically significantly above background
DBA	Digestion Buffer Additive
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EID	Egg Infectious Dose
ELISA	Enzyme linked immunosorbent assay
GuSCN	Guanidium isothiocyanate
GnHCl	Guanidine hydrochloride
HA	Haemagglutinin
HPAI	Highly Pathogenic AI
HPNAI	Highly Pathogenic Notifiable AI
HRM	High Resolution Melt
ICPI	Intracerebral Pathogenicity index
IVPI	Intravenous Pathogenicity Index
JCU	James Cook University
LB	Lysis Buffer

LPAI	Low Pathogenic of AI
LSDB	Liquid Sample Digest Buffer
M	Matrix
MDCK	Madin Darby Canine Kidney
MDBK	Madin Darby Bovine Kidney
MDT	Mean Death Time
mRSB	Modified RNA Safe Buffer
NA	Neuraminidase
NC	Nucleocapsid
ND	Newcastle disease
NDV	Newcastle disease virus
NDVs	Newcastle disease viruses
NP	Nucleoprotein
NTC	No template control
NS	Non Structural
PA	Polymerase A
PB	Polymerase B
PBS	Phosphate buffered saline
PC6	Protein convertase 6
PCR	Polymerase chain reaction
p.i.	Post inoculation
qPCR	Quantitative (real-time) polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT-qPCR	Reverse-transcriptase quantitative (real time) polymerase chain reaction
TAE	Tris-acetate ethylenediamine tetraacetic acid
TCB	TE Carrier Buffer
TE	Tris EDTA
<i>T_m</i>	Melting temperature: The temperature at which half of a DNA sample is single stranded
SA	Sialic Acid
SAN	Specific Antibody Negative
SARS	Special Administrative Regions
SDS	Sodium Dodecyl Sulphate

SPF	Specific Pathogenic Free
STDB	Solid Tissue Digest Buffer
WA	Western Australia
WHO	World Health Organization
vRNA	Viral RNA
VBB	Viral Binding Buffer

LIST OF NUMERICAL UNIT ABBREVIATIONS

Bp	Base pairs
Cl.	Confidence limits (typically 95%)
cm	Centimetre
<i>g</i>	Relative centrifugal force
g	Grams
hrs	Hours
kb	Kilobase
M	Molar
mg	Milligrams
min	Minutes
ml	Millilitres
ng	Nanograms
RPM	Revolutions per minute
sec	Seconds
STDV	Standard deviation
V	Volts
°C	Degrees Celsius
µg	Micrograms
µl	Microlitres

ABSTRACT

Limited infrastructure in developing countries makes rapid and accurate diagnosis of Avian Influenza (AI) and Newcastle disease (ND) difficult. This project has examined a method for collecting and transporting samples that eliminates the problems associated with previous ways of managing viral outbreaks. This method, verified using Real time (quantitative) reverse transcriptase polymerase chain reaction (RT-qPCR), also overcomes quarantine restrictions between countries. An epidemiological study was then carried out using the collection and transportation method and the results of this study can be used to support further epidemiological studies of AI and ND and to standardise assays between countries.

Diagnostic assays for AI and ND based on RT-qPCR have been developed and published. These assays use the deoxyribonucleic acid (DNA) binding dye, SYBR Green, or dual labelled TaqMan probes, (van Elden *et al.*, 2001; Spackman *et al.*, 2002; Tan *et al.*, 2004; Ward *et al.*, 2004; Pham *et al.*, 2005; Payungporn *et al.*, 2006b; Ong *et al.*, 2007). However, SYBR Green and TaqMan probes have limitations. SYBR Green can inhibit PCR reactions because it degrades PCR products, while TaqMan probes have less sensitivity due to the specificity of the probe sequences. Therefore, a SYTO 9 RT-qPCR was developed for the AI and ND diagnosis.

SYTO 9 RT-qPCR for AI virus (AIV) and ND virus (NDV) assays were successfully used. A series of new primers were evaluated and developed in these assays such as M+4100 forward and M-4220 reverse for ND detection of Class II NDVs (chicken) (Wise *et al.*, 2004) and NDV MGB1 for ND detection of Class I NDVs (wild birds). For the AI screening test, a few pairs of primers based on the M gene were also evaluated (Ward *et al.*, 2004; Heine *et al.*, 2005). Subsequently, these diagnostic assays were used to evaluate sample collection and transportation without a cold chain.

Further studies were also performed to evaluate the sequences of NDVs. This sequencing was intended to show the viral pathotyping and phylogenetic relationships of NDV. Primers that targeted a 343 bp Fusion sequence were designed and successfully used to amplify six Australian NDV isolates, both NDV Class I and Class II. Based on the amino acid sequence in the Fusion cleavage site, the six isolates were confirmed as low virulence of NDV. The motif of the cleavage sites were S-G-G-E-R-Q-E-R-L-V, S-G-G-E-Q-Q-G-R-L-I and S-G-G-G-K-G-R-L-I.

Evaluations of suitable buffers showed that Solid Tissue Digest Buffer (STDB) and Lysis Buffer (LB) were effective buffers for sample collection and transportation. Further studies showed that there was no significant difference between these two buffers. However, viability experiments showed that the LB deactivated both AI and NDV while STDB failed to fully inactivate NDV. Therefore, the lysis buffer was chosen for further sample collection and transportation without a cold chain.

A protocol for the extraction of samples collected without cold chain was evaluated. This modified protocol was successfully integrated into the nucleic purification system using a robotic device and ribonucleic acid (RNA) extraction protocol (Corbett Research, Brisbane). However, extracted and purified RNA may be degraded by ribonuclease contamination. Therefore, this project also evaluated buffers for RNA storage after extraction.

Evaluation of two modified buffers, the Tris EDTA (TE) carrier buffer (TCB) and modified RNA safe buffer (mRSB), showed that both buffers can prevent RNA degradation with no significant difference in cycle threshold (Ct)-Value detected by RT-qPCR.

The method of collection and transportation samples without a cold chain was applied by collecting live viruses and also viral antigens in Indonesia and transporting to Australia. Due to the time limitation, viral isolates were collected only from AI viruses representing four different regions in Indonesia (Timika, Ambon, Tangerang, and Sukabumi). For ND commercial antigens (Balitvet and Pusvetma) were purchased. The AI and ND samples were processed using a lysis buffer in the Quarantine Agency Laboratory of Indonesia and transported to James Cook University (JCU) in Townsville, Australia.

Transported AI and ND viral RNA were successfully amplified using SYTO 9 RT-qPCR. This study confirmed that not only viral RNA but also inactivated viral RNA can be preserved, transported and detected after a month of processing. This transportation also verified that viral RNA can be recovered and detected after chemical inactivation.

SYTO 9 RT-qPCR and TaqMan dual labelled probed RT-qPCR assays were carried out to screen the presence of AI viruses in the transport buffer. These assays used published protocols (Ward *et al.*, 2004; Heine *et al.*, 2005). The assays successfully detected viral RNA. However, the TaqMan dual labelled probe assay for H5 (Heine *et al.*, 2005) failed to detect viral RNA in the Sukabumi isolates.

Further studies of Indonesian AI isolates using High Resolution Melt assay recognised at least two populations from four different regions. Normalization data demonstrated that Timika and Ambon isolates were very similar while Tangerang and Sukabumi isolates represented different populations. These results suggest that there is continued evolution of AI viruses in Indonesia.

The ability of lysis buffer to preserve AI viral RNA was confirmed by amplification of 590bp and 1370 bp products from the Haemagglutinin (HA) gene. These products were successfully sequenced demonstrating that the viral RNA transported using lysis buffer was not fragmented.

As a result, a molecular epidemiology study of AI and ND isolates was successfully carried out using the samples transported without a cold chain. However, at this stage, the method of collection and transportation of samples without a cold chain has not been applied to field and clinical samples. The value of the technique would be enhanced if it could be demonstrated that it works equally well for swabs and tissues collected in the field.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

1. AI and ND are similar avian diseases both caused by RNA viruses. While both diseases vary widely in pathogenicity they can produce high mortalities. A diagnostic protocol to accurately carry out a differential diagnosis is therefore important.

Misdiagnosis of AI as virulent ND delayed the diagnosis of an outbreak of AI in Vietnam and Indonesia in 2005. However, accurate and reliable diagnosis is not available in most developing countries. Many developing countries have inadequate infrastructure and expertise to diagnose and differentiate these two diseases accurately. Thus, efficient transportation of viruses in suspected clinical samples to overseas reference laboratories is required to obtain a rapid and accurate diagnosis and to help evaluate the internal laboratory quality control by comparing the results of diagnosis assays. However, most reference laboratories are located overseas and the distance causes delays in transportation, thus compromising viral viability.

Transporting viruses to references laboratories overseas also involves quarantine restrictions designed to keep out exotic diseases. For example, the AQIS seeks to prevent the introduction and establishment of major diseases such as foot and mouth disease, AI and ND and so protect Australia's environment, agriculture and indigenous biodiversity. Maintaining environmental health is also essential to protect the extensive agricultural and animal trade industry. However, quarantine delays of imported viral samples can also mean that the viruses degrade.

Maintenance of viral viability is essential to efficient transportation. AIV and NDVs are thermo-sensitive. Thus, due to their thermo-sensitivity, they need to be carefully collected, stored and distributed with a suitable cold chain. Cold chains are difficult to establish and maintain.

This study evaluated a series of buffers which are components of the extraction protocols from Corbett Robotics (Prototype kits) or Sigma chemicals catalogue Number XTR1 or XTR2.

ND and AI disease viruses can be diagnosed using conventional methods for isolating the virus. Alternatively, the genome can be detected using RT-PCR.

PCR and RT-PCR have also been used to replace some of the gold standard cell culture and serological assays (Mackay *et al.*, 2002; Mackay, 2004). However, nucleic acid detection using PCR technologies requires an appropriate method for nucleic acid purification. In this study the transport buffers were integrated with the subsequent extraction protocols.

Sequencing of PCR products can provide valuable phylogenetic information as well as an indication of the presence or absence of virulence markers such as protein cleavage sites in the genes coding for the surface viral proteins of both ND and AI viruses.

JCU has a comprehensive set of molecular diagnostic tools including an automated nucleic acid extraction robot, a liquid handling robot and real-time PCR. Collaborative projects with other laboratories in Australia have guaranteed that JCU has RNA from reference viruses that can be used to standardise diagnostic assays for both AI and ND.

1.2 Aims of the research project

The specific aims of this research project are as follows:

1. To standardise a set of diagnostics tests based on real-time RT-PCR for AIV and NDV.
2. To develop methods for collecting and transporting RNA viruses without the need of a cold chain.
3. To develop methods for RNA virus storage following viral RNA purification procedures.
4. To apply these assays to reference samples and samples collected from infected birds in Indonesia.
5. To collect samples from outbreaks of AI or ND in Indonesia and extract their viral RNA in a suitable buffer that will allow transport without the need for a cold chain.
 - a. To demonstrate that the RNA can be used in routine diagnostic assays
 - b. To carry out genotyping studies on AIV and NDV samples to aid epidemiological studies.
 - c. To determine the potential pathogenicity of the isolates by sequencing protein cleavage sites.

CHAPTER 2

LITERATURE REVIEW

2.1 Avian Influenza Disease

AI Disease is a global problem for both wild birds and the poultry industry. This disease is caused by AIV. These viruses are important pathogens of poultry in Southeast Asia, African, Europe (de Jong *et al.*, 2006); in particular, a number of AIV outbreaks have been reported in Indonesia, China, Thailand and Vietnam (WHO, 2007). The viruses have created an epidemic in Asia since 1996 when there was an outbreak in geese in Guangdong, China. This outbreak resulted not only in high mortality of geese but also in the spread of the virus throughout Guangdong province (Chen *et al.*, 2004)

AI is not only a threat to poultry, but also to humans and there have been increasing fears of a human pandemic since the first human was infected. The first human cases of AI H5N1 occurred in 1997 when the viruses caused severe outbreaks in poultry in the Hong Kong Special Administrative Regions (Hong Kong SARS). Eighteen people were infected clinically and six deaths were reported in this outbreak. In 2002 and 2003, AI viruses were isolated in Hong Kong in a range of wild birds and it spread to the Republic of Korea in early December, 2003. These viruses spread and caused outbreaks not only in South Korea but also in others countries surrounding South Korea. In this outbreak, 35 human cases were reported and 23 deaths (Morris and Jackson, 2005). Overall, 522 people have been infected and 314 deaths have been reported (WHO, 2007).

In Indonesia, AI disease was first reported in 2003 in several districts in Central Java. Since then, the number of cases has been increasing and the disease is now present in 23 provinces and 151 districts and cities. The first human cases were noticed in 2005 in Tangerang. Total infections by 2007 were 74 and 57 deaths were reported (de Jong and Hien, 2006; WHO, 2006). However, in the early stages of the outbreak in Indonesia, the clinical signs were confusing and this disease was diagnosed as very virulent ND before it was confirmed as AI disease. Therefore, precise diagnostic assays for AI are required to prevent continuing severe outbreaks.

2.1.1 Virus Overview

AI viruses are classified in the family Orthomyxoviridae. In this family, Influenza viruses are classified into A, B, and C and AIV is classified as Influenza A. Influenza A virus is differentiated from Influenza B and C because of the differences in the major internal protein antigens: the nucleoprotein (NP) and M protein (Webster *et al.*, 1992)

Influenza viruses are small (80 to 120 nm in diameter), negative sense, single stranded RNA, pleomorphic, enveloped and segmented. These viruses have eight segments of RNA which encode 10 proteins: Polymerase A, B1, B2 (PA, PB1, and PB2), HA, NP, Neuraminidase (NA), M protein (M1 and M2) and Non structural protein (NS1 and NS2). These proteins can be categorised as surface antigens or internal proteins (Webster *et al.*, 1992).

The surface antigens of influenza viruses consist of HA and NA glycoproteins. HA is the major surface protein of the Influenza virion. This protein is embedded in the host derived lipid bilayer and integrated with the membrane protein. HA is responsible for binding the virion to the host cell receptor. Also, HA facilitates the fusion of the virion envelope between the virus and the host cell. The haemagglutinin protein is translated from RNA segment 4.

Protein translation of RNA segment 4 is followed by posttranslational processing, namely proteolytic cleavage. In this process, the new synthesized HA is cleaved to facilitate transportation to the cell membrane. This cleavage can be accomplished due to the presence of host-produced trypsin-like protease. Then, the cleavage of HA results into two subunits: HA1 and HA2 with HA1 forming the globular head on a stalk of natured HA protein, while HA2 and also HA1 form the stalk (Webster *et al.*, 1992).

HA cleavage is required for infectivity. HA becomes infectious when the free amino terminus of HA2 mediates virus-cell fusion. This protein is responsible for the neutralization by host antibodies (Thomas and Swayne, 2007). However, due to the receptor binding cavity and the antigenic sites of the molecule, HA is the major target of the host immune response.

Moreover, the HA antigen is subject to a very high rate of mutation due to its error prone viral RNA polymerase (Webster *et al.*, 1992). Thus, due to the high rate of

mutation, HA protein can change due to antigenic drift and so becomes unrecognisable by the host immune cells (Thomas and Swayne, 2007).

Another surface antigen of the influenza virus virion is NA. This protein is an integral membrane glycoprotein which is encoded and translated by RNA segment 6. This protein cleaves sialic acid from glycoproteins or glycolipids. NA protein separates the virions from the host cell receptors and facilitates the spread of the virus. Like HA, NA protein is highly mutated and a target of the host immune response (Webster *et al.*, 1992)

The internal antigens of Influenza viruses are formed by NP, M, PA, PB1, and PB2. These antigens are the basis of Influenza A, B and C differentiation. NP is translated from RNA segment 5. This protein is transported into the host cell nucleus to bind and encapsidate viral RNA. NP switches the viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesized. NP is abundantly synthesized and phosphorylated in host cells but depends on viral host restriction. This protein is also a host cytotoxic T-cell immune response target (Webster *et al.*, 1992).

Important internal proteins are M proteins. The M proteins, M1 and M2 form a cover surrounding the virion nucleocapsid which is encoded and translated from RNA segment 7 (Webster *et al.*, 1992). The M2 is found in a small quantity in Influenza A viruses. This protein serves as a signal for transport to the cell surface and occupies an ion channel to regulate the internal pH of the virus. Its function as an ion channel is vital for uncoating the virus in the early stages of replication (Webster *et al.*, 1992; de Jong and Hien, 2006)

Other internal proteins are RNA polymerase complexes. These polymerase complexes consist of three polymerase proteins which are PA, PB1 and PB2. PA protein is encoded and translated from RNA segment 3. This protein is limited in the infected cell nucleus. PA is believed to have a role as a protein kinase or as a helix-unwinding protein. However, the function of this protein in viral RNA synthesis is not yet known (Webster *et al.*, 1992).

The second RNA polymerase is PB1 protein and is encoded by RNA segment 2. This protein is responsible for elongation of the primed nascent viral mRNA. PB1 also serves as a template for cRNA and vRNA synthesis (Webster *et al.*, 1992).

Another RNA polymerase is PB2. This protein is translated by RNA segment 1. The PB2 provide viral RNA dependent RNA polymerase activity in the initiation process of viral mRNA protein transcription. This protein recognises and binds the host cells mRNA and uses it as viral mRNA transcription primers. PB2 is also believed to synthesise the full length template cRNA and new negative sense viral RNA (vRNA). However, the precise process by which this is done is still unknown (Webster *et al.*, 1992)

RNA segment 8 encodes two non-structural proteins: NS1 and NS2. These proteins play a role in viral replication. Non-structural protein 1 occurs largely in the infected nucleus cell while NS2 is found predominantly in the cytoplasm. Non-structural protein 1 is collinear with vRNA while NS2 originates by splicing. These proteins are involved in viral replication (Webster *et al.*, 1992)

Viral RNA segments of Influenza A are located inside the viral envelope. These segments are linked with NP and three viral polymerase subunits, PA, PB1, PB2 (de Jong and Hien, 2006). The viral RNA segments are protected and covered by a helical structure, Nucleocapsid (NC), associated with NP (Abed *et al.*, 2003). RNA segments, NP and viral RNA subunits form the Ribonucleoprotein (RNP) complex which is important in replication and transcription (de Jong and Hien, 2006)

The surface antigens of Influenza viruses are important for pathogenicity differentiation. HA antigens, which have 16 subtypes (H1-H16), and NA antigen with 9 subtypes (N1-N9) create a combination and cause different types of infection. Based on pathogenicity, Highly Pathogenic AIV is traditionally caused by H5 and H7 subtypes while other subtypes cause a milder disease which is considered as Low Pathogenic AIV (Capua and Alexander, 2004).

Office International des Epizootica defines AI disease as Notifiable AI (NAI). The NAI is classified according to the virulence of the virus: the Highly Pathogenicity Notifiable AI and Low Pathogenicity Notifiable AI (OIE, 2004). The Highly Pathogenic Notifiable

AI (HPNAI) viruses have an intra venous pathogenicity index (IVPI) greater than 1.2 in 4-8 week old chickens or cause 75% mortality in 4-8 week old chickens. Also, subtypes H5 and H7 virus isolates should also be considered as potential HPNAI. These isolates cause mortality less than 75% or IVPI less than 1.2 and have amino acids present in a cleavage motif similar to HPNAI isolates (OIE, 2009).

As far as molecular makeup is concerned, the pathogenicity of Influenza viruses is associated with multiple basic amino acids (arginine (R) or a lysine (K)) sequence at the H0 cleavage site. The specific motif consisting of a series of basic amino acid sequence at the cleavage site has been studied with motif X-X-R-X-R/K-R (X = A non basic amino acid) or R/K-X-R/K-R. Studies of H5 viruses show that Low Pathogenic H5 viruses have the amino acid motif PQRETR*GLF while the H5 highly pathogenic viruses have varied cleavage sites such as PQRKRKTR*GLF for the 1994/5 Mexican isolates (H5N2), PQRKKR*GLF for Chicken/Scotland/59 isolates (H5N1) and amino acid PQRRRKKR*GLF for Poultry/Italy/97 isolates (H5N2). Furthermore, most Low Pathogenic AI H7 viruses have motifs of either PEIPKGR*GLF or PENPKGR*GLF, while AI H7 with high pathogenicity have amino acid motifs of either PEIPKKKKR*GLF, PETPKRKRKR*GLF, -PEIPKKREKR*GLF, or PETPKRRRR*GLF. However, the amino acid sequences of Influenza virus pathogenicity are varied in different places and different subtypes. Also, influenza viruses' pathogenicity is influenced by protease such as enzyme access in host cells, whether it is trypsin like enzyme, furin or protein convertase (PC6) (Steinhauer, 1999; Zambon, 1999; Alexander, 2000b; Ito *et al.*, 2001; OIE, 2004)

2.1.2 Pathogenesis of AI Disease

Mechanism of AI viruses' infection can be divided into three steps which are initiation of infection, viral attachment and entry, viral replication, protein synthesis and new progeny released then spread in the host cell and cause clinical signs.

Initiation of infection required accessible, susceptible and permissive cells for the viruses at the site of infection, and the absence of or ineffective local host anti viral immunity.

The titre of virus required to initiate infection can be influenced by many factors. Environmental factors such as osmotic shock, pH changes and sunlight can affect viral

viability. Also, rapid dilution can reduce viral concentration especially infection from contaminated water. Aerosol dispersed infection required high concentration of virus, favourable environmental conditions and close contact. However, even though the infection may fail due to low viral concentration, it is not known yet how many virions are required to initiate and maintain the infection (Flint *et al.*, 2004).

Initiation of influenza viral infection and life cycle is started when the virions attach to the analogous receptors on host airway epithelial cells. This attachment is facilitated by HA which recognises the host cell receptor sialic acid (SA) residue or α -sialosides on the target cell surface. Sialic acid residue is a glycosylated oligosaccharides which are bound to glycans through α 2, 3 or α 2, 6 linkage by sialyl transferases (Meng *et al.*, 2010).

There are two host species specify types of sialic acid receptor on glycosylated cell surface molecule which will be recognised by HA antigen. These host species sialic acid specificity are SA α 2, 6 galactose β -1, 4-N-acetylglucosamine (SA α 2,6-Gal) or SA α 2,3 – galactose β -1, 3-N-acetylglucosamine (SA α 2,3-Gal). AI viruses have a preference to bind α 2, 3- linked sialic acid while human influenza viruses strain bind via receptor α 2, 6 linked sialic acid. However, a single amino acid mutation in the HA can change the HA receptor specificity of avian strain from α 2, 3 linked sialic acid to SA α 2,6-Gal (Connor *et al.*, 1994; Thompson *et al.*, 2006; Yamada *et al.*, 2006).

Viral attachment is followed by viral entry into the host cell by endocytosis. This endocytosis triggers HA cleavage due to the low pH of the endocytotic vesicle. The cleavage facilitates insertion of the hydrophobic free amino terminus of HA2 into the vesicular membrane. Then, the contents of the virion will be released into cell cytoplasm (Webster *et al.*, 1992)

The next step is viral replication and protein synthesis. This step is started when the nucleocapsid of the parent viruses migrate into the host cell nucleus. This migration will be associated with polymerase complexes to begin the primary transcription of mRNA. The primary transcription will be used for viral protein translations which are predominantly NP and NS1 in the early stage of translation. Then, the new NP and NS1 migrate to the nucleus and trigger the mRNA shift to synthesis of cRNA and vRNA using the viral genome. This new vRNA will be encapsidated in NP within nucleus and

become a template for secondary transcription of viral mRNAs. Afterwards, M1, HA, and NA protein are produced in the principal translation of infection. HA and NA proteins are then processed and transported posttranslational to the cell surface and integrate into cell membrane (Webster *et al.*, 1992)

Viral replication and protein synthesis is then followed by the assembly and release of progeny virions. The build up of M1 protein is followed by migration of nucleocapsid out from the nucleus. Then, a viral core of nucleocapsid will be encased in M1 shell protein and form the viral surface glycoprotein. The nucleocapsid will bud outward through the cell membrane and enclose itself within a bubble of membrane as its own envelope. This budding is initiated by the interaction between M1 and the cytoplasmic domain of HA, NA or M2. Moreover, NA activity will release new progeny virions outside of host cells (Webster *et al.*, 1992)

Finally, the last step is virus maturation. This takes place outside the cell and requires the cleavage of HA0 into HA1 and HA2. Cleavage of HA of AI viruses requires proteolytic activities of host cell proteases to cleave the HA precursor protein HA0 into HA1 and HA2. The host cell protease enzymes which are capable of this cleavage are trypsin like enzymes, elastase, thermolysin and furin. However, these enzymes are located in different cells which can lead to the differentiation of the viral pathogenicity. Highly pathogenic of AI viruses are cleaved by ubiquitous or intercellular proteases such as furin and PC6 while low pathogenic avian influenza (LPAI) viruses are cleaved by extracellular trypsin like enzymes. Therefore the cleavage site and differences in pathogenicity will result in differences in clinical signs (Webster *et al.*, 1992)

2.1.3 Clinical signs of Avian Influenza disease

Clinical signs of AI disease depend on animal species and viral pathogenicity. Clinical signs in birds vary from asymptomatic infections to mild respiratory disease through to severe and fatal systemic disease. Infection with HPAI in poultry results in clinical signs of decreased egg production; respiratory signs; extreme lacrimation; oedema of the head, eyelid, comb, wattles, and hocks; diarrhoea; neurological symptoms and deaths (de Jong and Hien, 2006).

Highly pathogenic AI viruses which cause sudden death frequently produce few clinical signs. However, clinical signs of highly pathogenic AIV disease may range from sudden death with no or little signs to more characteristic signs.

In humans, AI virus infections cause disease with similar clinical signs to seasonal flu such as fever, malaise, nasal discharge, coughing, laryngitis and body aches. H5N1 infections cause a rapid onset of lower respiratory tract disease which may result in pneumonia which is not responsive to antimicrobial therapy. However, the spectrum of clinical signs in humans is broad including encephalitis, chest pain, nose and gums bleeding and gastrointestinal signs. Infected people in Vietnam and Thailand have expressed signs of fever, respiratory symptoms, diarrhoea, lymphopaenia, and thrombocytopenia. Moreover, it was reported that some human cases had symptoms of fever and gastrointestinal signs without respiratory signs. Clinical signs of pneumonia and respiratory failure have been a feature of most of the fatal cases (Maines *et al.*, 2005)

2.1.4 Epidemiology of avian influenza

AI disease has been known since 19th century. This disease caused a severe and highly contagious poultry disease in Northern Italy in 1878. In that time AI disease was known as “fowl plaque”. Since then, fowl plaque has caused outbreaks throughout the world particularly in 1901 to 1930. In 1901, the causative agent of fowl plaque was demonstrated to be filterable. However, there was minimal understanding of the aetiology of AI for more than 50 years after the first event in Northern Italy.

Since the first outbreak in Italy, AIV has caused outbreak in many places including Asian (1957), Hong Kong (1968), Russia (1977) with different strains of AIV those are

H2N2, H3N2, H1N1 (Harimoto and Kawaoka, 2005). A further 12 countries suffered outbreaks of H5 and H7 from 1997 to 2004. These outbreaks also caused millions of birds to die. In Pakistan there were at about 3.2 million birds dying caused by infection with influenza H7N3. These cases predominantly occurred in broilers, breeders and layers.

AI outbreaks in many countries are caused by viruses of different pathogenicities. Outbreaks in Europe and United States have been caused by low pathogenic AI (LPAI) viruses. The LPAI viruses were also isolated in European countries such as Italy (1997 to 1998; 2002-2003), Northern Ireland (1998), Belgium (1999). In these countries the LPAI H7, H5N2, H5N9 and H7N1 subtypes were identified in infections of backyard chickens, turkey breeders and poultry breeders. The LPAI were also infected chickens and turkeys in north America (1997 to 2004), Virginia (2002), Connecticut (2003), Delaware and Maryland (2004), Canada (2001). However, in some countries in Europe the LPAI has mutated to become highly pathogenic AI (HPAI). The H7N1 subtype was confirmed as HPAI cases in Netherlands, Belgium and Germany in 2003 while in Italy, the cases were confirmed from 1999 to 2001 (Capua and Alexander, 2004).

In Asia, there is a panzoonotic caused by H5N1 subtype. This subtype initially caused outbreaks in geese in Guangdong. Infection of H5N1 in geese also occurred in the People's Republic of China in 1996. The infection then spread to Hong Kong in 1997 and H5N1 circulated in aquatic poultry in China from 1997 to 2002. In China, the subtype H5N1 was also isolated from ducks. While H5N1 caused clinical signs with high mortalities in poultry, the same viruses were isolated from healthy ducks in China and geese in Vietnam (Morris and Jackson, 2005).

The H5N1 strain has caused zoonotic transmissions to humans associated with poultry outbreaks in Asian countries such as Vietnam, Thailand, Indonesia and other East Asian Countries. These transmissions have been happening since early 2004. In 2004, human infection predominantly occurred in Vietnam and Thailand. However, human infections have increased in Indonesia, China and Turkey since mid 2005. Overall the numbers of human infections to 2008 are 382 cases with 241 are lethal cases (WHO, 2008)

Influenza A viruses has a wide range of hosts such as wild birds and domestic animals. Wild birds are natural reservoirs of AIV. Ducks, shorebirds and geese are the common

wild bird species acting as natural reservoirs of AIV. AI viruses have been also discovered in other animals such as pigs, horses, mink and marine mammals (Fouchier *et al.*, 2000).

As a natural reservoir of AI viruses, wild birds have a role in transmission of these viruses. Mainly, the transmission of AI is through faeces. Then, the viruses spill over to other birds and aquatic mammals. Migratory wild birds can transmit infection through the faeces to domestic poultry. Also, the infection with these viruses in aquatic birds can result in viral reassortment and pathogenic alteration posing a threat to poultry (Zambon, 1999).

In humans, transmission of influenza viruses occurs by inhalation of or dust. The transmission can be through direct and indirect contact onto the upper respiratory tract or conjunctival mucosa or possibly direct immolation of small particles directly into the lungs. Some cases have been shown to have had contact with birds up to a week before the onset of illness. However, human to human transmissions have not yet reported (Zambon, 2001).

2.2 Newcastle Disease

Another important viral disease in poultry industry is ND. This disease not only has a big impact to poultry but also to other birds all over the world. This disease causes mortality up to 100%. Similarly with AI disease, this disease is responsible for a loss of production and deaths and there may be a need for vaccination or eradication and quarantine programs. Newcastle disease is not a zoonosis as is AI disease. However, the virus can spread rapidly and economic impacts can include trading restrictions and embargoes on the infected country (Spradbrow, 1987; Alexander, 1995; Alexander, 2001).

Since ND was firstly recognised in 1926 in Jakarta, Indonesia and Newcastle-upon-Tyne, England, this disease continues to spread in many countries. Several panzootics have been reported with the first true panzootic being recognised at the end of 1960 in USA. This panzootic occurred when the world poultry industry was developing and lead to the commercialisation of poultry food production.(Alexander *et al.*, 2004). In Australia, this virus was firstly acknowledged in 1930. This outbreak was controlled and Australia appeared to be free from ND viral infection until the mid-

60s when an avirulent strain was isolated. Subsequently similar strains were recognised in Australia and in neighbouring countries such as New Zealand and Papua New Guinea (Spradbrow, 1987).

ND causes a serious problem to the many countries in Asia with the disease being endemic in Korea, India, Sri Lanka, Philippines, Malaysia and Indonesia. This is complicated by factors such as wild birds as carriers, legal and illegal movement of infected birds, and the strong reliance on village poultry (Spradbrow, 2001).

2.2.1 Virus Overview

ND is caused by avian Paramyxovirus type 1. This virus is assigned to the genus Avulavirus in the subfamily Paramyxovirinae, family Paramyxoviridae, and order Mononegalovirales (Mayo, 2002).

ND virus is a single stranded (ss), enveloped, non segmented, negative sense RNA virus. This virus has a genome size of 15,186, 15,192 or 15,198 bases (Czegledi *et al.*, 2006). The RNA genome of NDVs consists of six genes encoding three envelope and three core proteins. The envelope proteins are HA-neuraminidase (HN), Fusion protein (F), and M Protein. The core proteins of ND are RNA polymerase or large protein (L), Phosphoprotein (P) and NC protein (NP) (Mohan *et al.*, 2005). The genome position of ND viral proteins from 5' to 3' are L, HN, F, M, P, and NP (Peeters *et al.*, 1999; Gould *et al.*, 2001; Wakamatsu *et al.*, 2006). In addition there are two proteins V and W identified as non structural proteins (NSP) (Peeters *et al.*, 1999; Wakamatsu *et al.*, 2006), 1999 #1747} generated by RNA editing of alternative mRNA of the P gene (Huang *et al.*, 2003).

The viral proteins of NDV have different roles throughout the replication cycle of the virus. The F and HN proteins are two important glycoproteins for viral infectivity and virulence. Fusion protein of NDV is related to the pathogenicity. The pathogenicity of this virus is determined by the ability of host cellular protease to cleave the F0 protein into disulfide-linked polypeptides F1 and F2. The HN Protein has both receptor binding and neuraminidase functions that aid the release of the virus from the cell. Following receptor binding the F protein allows the virus to penetrate the host cells (Peeters *et al.*, 1999; Kobasa *et al.*, 2004).

Other proteins, NP, Phosphoprotein and Large protein are involved in replication. These proteins use viral RNA as a template for the production of viral M-RNA, Double strand RNA and progeny viral RNA (Romer-Oberdorfer *et al.*, 1999)

The two additional proteins, V and W proteins play a role in determination of NDV virulence and pathogenesis. These proteins are important to evade the action of interferon (IFN). Moreover, the virulence of NDV is also influenced by the ability of this virus to encode and alter the V protein being an IFN antagonist (Huang *et al.*, 2003).

2.2.2 Pathogenesis of Newcastle disease

The mechanism of ND infection in birds begins when the virus is exposed to the host cells. It attaches and penetrates into host cells where it replicates. Subsequently progeny viruses are distributed to a variety of organs. Clinical signs may indicate the damage caused by the virus or the systemic effects of cytokines on the host

Viral attachment is mediated by the HN attaching to receptors. Fusion then is mediated by the cleaved dimer of F1 and F2 polypeptides.

The cleavage of F0 is mediated by host cells protease and is vital for the production of infectious particles (Aldous and Alexander, 2001). The host cell proteases required to facilitate the F0 cleavage vary due to the amino acid sequence of the Fusion cleavage site. The high virulent ND viruses are cleaved by ubiquitous intracellular furin-like protease while avirulent and viruses of low virulence are cleaved by trypsin-like enzymes which may be outside the cells (Collins *et al.*, 1993; Aldous and Alexander, 2001).

The F, HN, and P genes have also a potential function in ND virulence. The alteration of HN protein glycosylation sites can either increase or decrease the pathogenicity of ND. Furthermore, V protein as a product of P gene which is rich in cystine in its carboxyl terminal region and zincs binding domain occupy may have the action of an interferon blocker. This antiviral cytokine acts as the first line of host defence and inhibits viral replication and dissemination (Collins *et al.*, 1993).

The replication of NDV occurs in a variety of cells. Normally, replication occurs in respiratory and intestinal tracts and then spreads into the spleen and bone marrow and other organs such as lungs, intestinal lymphoid tissues and CNS (Quinn *et al.*, 2002). The locations depend largely on the cleavage of the F0 protein. Those viruses with a multi-basic cleavage site that is capable of being cleaved by furins are found in a wide range of locations including respiratory tract and brain while those viruses requiring trypsin cleavage are found predominantly in the spleen, proventriculus and the region of the caecal tonsils.

Pathogenesis studies of viral distribution and lesion in tissue show that velogenic or very virulent pathotypes produce severe clinical signs. These virulent pathotypes infect multiple tissues resulting in gross and histological lesions. On the contrary, Mesogenic and lentogenic viral pathotypes produce less prominent clinical signs. However, some gross and histological lesions are found in infections produced by mesogenic viruses. Also, lentogenic viruses may have minimal replication in the myocardium (Brown *et al.*, 1999; Wakamatsu *et al.*, 2006).

2.2.3 Clinical signs of Newcastle disease

Chickens infected with NDV show a range of clinical signs dependent on the pathotype of virus infecting the birds. There are three major pathotypes of ND those are Lentogenic, Mesogenic and Velogenic (Callan *et al.*, 1995; Aldous and Alexander, 2001; Gould *et al.*, 2001; Wakamatsu *et al.*, 2006). Lentogenic pathotypes are the low virulence viruses which produce no symptoms or symptoms of mild respiratory or enteric infection. The least pathogenic of the lentogenic viruses are considered to be avirulent producing virtually no clinical signs (Alexander *et al.*, 2004).

Mesogenic pathotypes have a moderate virulence. This viral pathotype is associated with low mortality, primarily acute respiratory disease and some neurologic signs in birds. The velogenic pathotypes have a high virulence and can also be divided into neurotropic velogenic NDV and viscerotropic velogenic NDV. Neurotropic velogenic NDV causes respiratory and neurologic signs with high mortality. Viscerotropic velogenic NDV generates acute lethal infection with hemorrhagic lesion signs which are mainly apparent in the gastrointestinal tract (Seal *et al.*, 1998; Piacenti *et al.*, 2006)

Many factors influence the variance of clinical signs of NDV infection including viral strains, host species, the age of the host, immune status, secondary infection, and environmental stress (Alexander *et al.*, 2004). Sudden death is frequently observed in birds infected with velogenic pathotypes.

Viruses that produce severe disease in chickens may be less virulent in other species of birds (Roy *et al.*, 1998).

Generally, clinical signs of ND are depression, diarrhoea, prostration, and oedema of head and wattles. Also, infected poultry show clinical signs such as nervous and respiratory signs. Torticollis and paralysis are typical of nervous disease. In addition there may be declining egg production, termination of egg laying and death. However, there are frequently no pathognomonic clinical signs and frequently there are overlapping syndromes

2.2.4 Epidemiology of Newcastle disease

ND cases firstly were reported in Newcastle-on-Tyne, England. It was reported by Doyle when the first outbreak in the spring in 1926 occurred on the farm near to Newcastle-upon-Tyne. In that time, ND was known as Fowl pest. Also, the cases of NDs were reported in Java, Indonesia in March 1926. It was considered that transportation from South East Asia to the port of Newcastle responsible for the initial outbreaks and the introduction of NDVs into England (Alexander *et al.*, 2004). However, a similar outbreak was reported in Central Europe earlier before this disease was noted. Also, there were also highly virulent viral infections appearing in other locations in Europe at around the same time. Initially the disease was thought to be fowl plaque or HPAI.

Highly pathogenic outbreaks of ND in Poultry had been reported in Asia and Europe in the middle of the 20th century. Chickens and most avian species were shown to be susceptible to this virus. However, even though other avian species have been infected by this virus, infections with this disease rarely produce the severe clinical signs reported in the poultry. For instance, clinical signs are infrequently reported in turkeys and pigeons although generalised disease may develop (Spradbrow, 1999a; Alexander *et al.*, 2004)

ND has also caused problems and economic losses in village chickens in many areas. Many countries such as Malaysia, Sri Lanka, Uganda, Nigeria, Bangladesh, Central Africa, and Myanmar are endemic for NDV particularly in Village chickens (Spradbrow, 1999a). In China, this disease was first described in 1946 as an endemic disease in village chickens and required an intensive vaccination program (Liu *et al.*, 2003). In Vietnam, ND was reported in scavenging and backyard chickens particularly in rural areas such as Dong Thap, Soc Trang, Giang province with outbreaks reoccurring on an annual basis (Phan *et al.*, 2003; Quoc, 2003; Vui *et al.*, 2003). ND is probably endemic in all provinces of Indonesia (Darminto, 1995).

Various factors influence the persistence of NDV in village poultry. The cycle of infection between poultry and other domestic birds such as ducks, turkeys, doves, geese and guinea fowl maintains the virus. The role of carrier chickens is not clear. These factors provide a reservoir for NDV (Spradbrow, 2001). Wild birds can also act as a reservoir for NDV (Martin, 1999)

Another factor influencing viral survival is physical environment. NDVs can survive for three months at temperatures of 20°C to 30°C and for weeks to months at tropical temperatures. This virus has increased survival at cooler temperatures (Alexander *et al.*, 2004).

Transmission of NDV can be by aerosols, feed, ingestion of contaminated water and contact with contaminated equipment. The virus may also persist in the carcasses of affected birds (Martin, 1999; Quinn *et al.*, 2002).

In many countries, ND viruses have been isolated from wild birds. A variety of species such as migratory waterfowl to the aquatic birds can be infected. NDVs infected double-crested cormorants (*Phalacrocorax auritus*) in Scotland in the late 1940s. This infection also occurred in Quebec in 1975 and cormorants in Western Canada spread this disease to domestic turkeys (Alexander, 2001)

In Asia, wild birds also have a role in the spread of NDVs. Species such as Passerine birds, waterfowl, natatorial birds particularly herons, storks, cranes and shore birds have all been implicated (Hua *et al.*, 2005).

In Australia, the first panzootics of exotic virulent NDV were identified in chickens in 1930 and 1932 in Melbourne. The disease was eliminated by a combination of slaughter and quarantine control (Alexander *et al.*, 1986; Gould, 2004). However, viruses of low virulence were assumed to be still circulating among domestic poultry. The low virulence NDV in Australian chickens was considered to be similar to duck and feral waterfowl isolates from other countries. The second outbreak in Australia occurred in 1998. Initially, virulent NDV was recognised at Dean Park, New South Wales. Quarantine, slaughter and vaccination policies were applied to control the spread of this disease (Kattenbelt *et al.*, 2006b).

NDVs have been reported in ducks. An outbreak occurred in a flock of 300 ducks in Indonesia. The virulent viruses have been also isolated from ducks in Vietnam. However, in Pekin ducks isolation of NDV was contaminated with AI viruses. In Tanzania, the cases of ND were associated with chickens in the same location (Spradbrow, 1999b)

2.3 Molecular Epidemiology and Phylogenetic of NDVs

ND or avian paramyxoviruses serotype 1 (APMV-1) are classified in the genus Avulaviruses, family Paramyxoviridae. Avian paramyxoviruses have 9 serogroups (APMV1-APMV9), while APMV-1 is divided into two classes with genotypes within the class.

However, molecularly, the pathogenicity of NDV isolates was correlated with the sequence of the cleavage site of Fusion (F) protein. This molecular correlation is generated from the ease by which cellular proteases cleave the fusion protein. As described previously in the Chapter 2.2.2, in apathogenic viruses the F0 can be cleaved by trypsin-like proteolytic enzymes while the F0 precursor protein of pathogenic viruses is cleaved by furin like enzymes (Collins *et al.*, 1993; Ballagi-Pordany *et al.*, 1996; de Leeuw *et al.*, 2003; Lee *et al.*, 2004; Panda *et al.*, 2004).

Phylogenetically, NDVs, described by Czeglédi (Czeglédi *et al.*, 2006) have two classes (Class I and Class II) division. This division was determined regarding to the complete Fusion (F) and RNA polymerase (L) genes.

Based on Fusion (F) protein (Figure 1), Class II viruses have 9 genotypes (I-IX) with 2 different sub lineages. The first sub lineage known as ‘early NDV (<1930’s)’ have genome size of 15.186 nt. This sublineage is comprised of genetic group I-IV and has been identified with early NDV outbreaks in the 1930s. A second sublineage has a longer 15.192nt genome size. This sub lineage consists of genotypes V-IX and they are responsible for more recent outbreaks (>1960) (Czegledi *et al.*, 2006; Kim *et al.*, 2007a; Kim *et al.*, 2007b; Wu *et al.*, 2010).

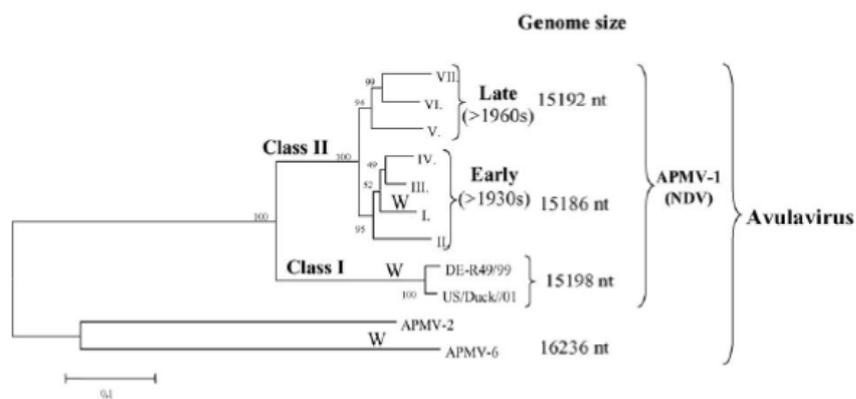


Figure 2.1. The phylogeny of NDV based on complete Fusion gene (Czegledi *et al.*, 2006).

Class I viruses have a genome of 15.198 nt and nine genotypes are recognised (1-9) (Kim *et al.*, 2007a; Kim *et al.*, 2007b; Kim *et al.*, 2008; Liu *et al.*, 2010). This class was identified in wild water birds, waterfowl, shorebirds or poultry in live birds market (Collins *et al.*, 1998; Aldous *et al.*, 2003; Gould *et al.*, 2003) and also in ducks (Lee *et al.*, 2004; Liu *et al.*, 2007). In contrast, Class II viruses are predominantly found in chickens (Liu *et al.*, 2003). However, occasionally Class I NDVs are also found in domestic poultry while the Class II NDVs in particular genotype one are frequently isolated from waterfowl and wild bird species (Aldous *et al.*, 2003; Kim *et al.*, 2007b).

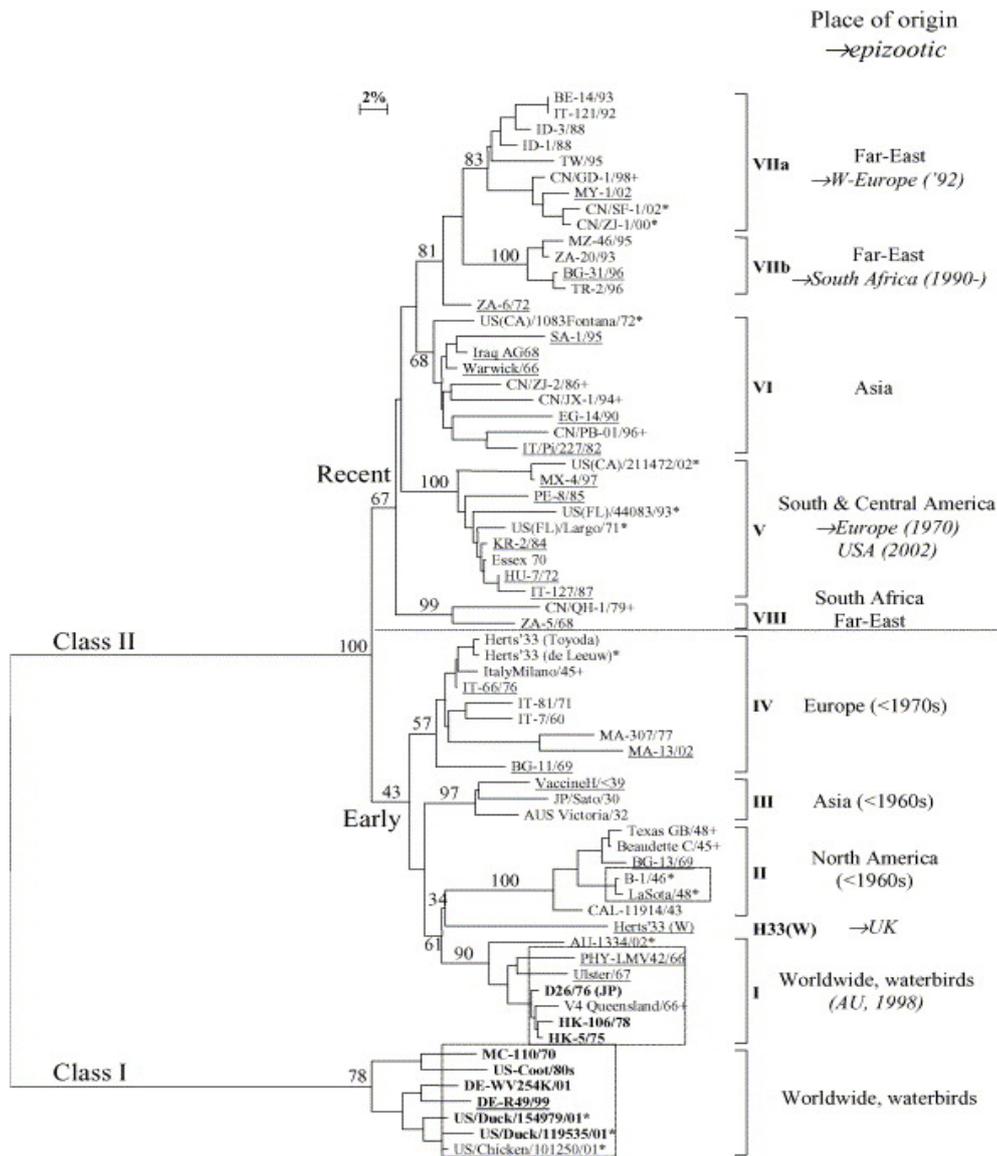


Figure 2.2. Genomic analysis of ND based on partial F sequences (Czegledi *et al.*, 2006).

It can be seen on Figure 2 that genotypes II, IV and V of NDV Class II were predominant in North America and Europe respectively while genotypes VI and VII caused outbreaks in Middle East, Asia and Far East. The genotype VIII also emerged in the Far East and South Africa (Herczeg *et al.*, 1999; Czegledi *et al.*, 2002; Huang *et al.*, 2004; Czegledi *et al.*, 2006).

In Australia, NDVs have been isolated from poultry and from wild birds. The isolate known as V4 belongs to Class II genotype I (Czegledi *et al.*, 2006; Wu *et al.*, 2010). In Western Australia (WA), the isolates of NDVs were divided into two groups based on monoclonal antibodies reactivity: V4-like (Class II NDV) and MC110-like (Class I NDV). These isolates were found mainly in Charadriiformes and Passeriformes (V4-like), and Charadriiformes and Anseriformes (MC110-like).

2.4 Laboratory Diagnosis of Avian Influenza and Newcastle Disease

Infections with AI and NDV can be confirmed by laboratory diagnostic assays including viral isolation, antigen detection, serology, pathology, and genome detection.

2.4.1 Samples for laboratory diagnosis

Samples from dead birds include oronasal swabs, lung, kidney, intestines, spleen, brain, liver and heart tissues. Samples from live birds consist of tracheal and cloacal swabs as well as fresh faeces (OIE, 2004). Samples obtained from live birds should be placed in buffer solutions such as phosphate buffered saline (PBS) with antibiotics. Then these samples should be processed immediately or stored at 4°C to prevent autolysis and bacterial contamination (OIE, 2004).

2.4.2 Virus and viral antigen detection

Samples from animals infected with AIV or NDV can be confirmed by culturing and inoculating the samples into the live cells or embryonated eggs which is the gold standard for viral detection. Cultivation can provide stock viruses for further research or vaccine production. However, viral isolation is time consuming and requires viable virus in the sample.

Samples can be inoculated into the allantoic cavity of 9 to 11 day embryonated specific pathogenic free (SPF) or specific antibody negative (SAN) chicken eggs (OIE, 2004). Some of the more virulent viruses cultivated in the allantoic cavity will kill the embryo of SPF or SAN eggs. The cells lining the allantoic cavity contain trypsin like enzymes and can support the growth of viruses of low virulence. The viruses that can be cleaved by furin can invade and kill the embryo (Collins *et al.*, 1993; Aldous and Alexander, 2001).

There are numerous cell lines have been used to replicate avian AIV and NDV such as Madin-Darby canine kidney (MDCK) cells, Madin-Darby bovine kidney (MDBK) cells, Chicken embryo kidney (CEK) cells and tracheal epithelial cells (Sahle *et al.*, 2002; Wambura *et al.*, 2006; Zaffuto *et al.*, 2008). It may be necessary to provide exogenous proteases to facilitate viral protein cleavage (Sahle *et al.*, 2002; Zaffuto *et al.*, 2008).

2.4.3 Haemagglutination Assay

The growth of viruses in allantoic fluid of embryonating eggs or cell culture is confirmed by HA. This assay is a tool for screening both AIV and NDV. The viruses bind to the N-acetylneuraminic acid-containing proteins on mammalian and avian erythrocytes. However, other agents such as bacteria also have haemagglutinating proteins. Moreover, the HA assay does not only detect viable viruses. It will also detect HA on degraded or inactivated particles. Therefore, the HA test has limitations when used as a viral identification test (Spackman, 2008).

2.4.4 Serological assay

Serological assays can be used to determine the immune responses to these viral infections. The most commonly used assays are haemagglutinin inhibition (HI) and enzyme linked immunosorbent assay (ELISA).

2.4.4.1 Haemagglutination inhibition

Haemagglutination inhibition is used to classify and subtype the haemagglutinating viruses and is applied to evaluate the antigenic relationship between viral subtypes recognised by specific antibodies (OIE, 2004; Spackman, 2008).

The assay is based on the inhibition of haemagglutination by specific antibody. The HI titre is the highest dilution of serum causing complete inhibition (OIE, 2004).

The assay is inexpensive compared with other assays and uses standard laboratory equipment. Moreover, this assay is approved as a World Health Organization (WHO) gold standard assay for AIV sub typing. However, HI requires specific antibody and reference reagents when working with uncharacterized viruses or antibodies (Spackman, 2008).

2.4.4.2 Enzyme linked immunosorbent assay

Indirect ELISA is based on the reaction of test antibodies with specific antigens attached to a plastic plate. Antichicken antibodies conjugated to a suitable enzyme are detected by the addition of a suitable substrate and chromogen which results in a colour change. This colour change can be read on a spectrophotometer (Alexander *et al.*, 2004)

The specificity of indirect ELISA depends on the purity of the antigens. The presence of contaminants may lead to poor specificity (Burgess, 1988; McCullough, 1993).

Sandwich ELISA is a configuration used to detect viral antigens. A capture antibody is adsorbed to the plate. An indicator antibody can be conjugated to an enzyme of detected by and antisppecies antibody conjugated to the enzyme. This assay frequently uses a polyclonal capture antibody and an unlabelled monoclonal indicator antibody detected by an antimouse conjugate. The specificity is determined by the monoclonal antibody (McCullough, 1993). Monoclonal antibody panels have been produced allowing this assay to be used to type the isolates (Crowther, 1993).

Monoclonal antibodies are mainly produced using avirulent NDV isolates. Based on the monoclonal antibodies, NDVs isolated from wild bird species can be classified into group labelled G, L and I while NDV in water birds are largely in group H (Alexander, 1995; Ballagi-Pordany *et al.*, 1996; Aldous *et al.*, 2003; Czeglédi *et al.*, 2006).

2.5 Molecular or Genome Detection

Molecular genome detection techniques are becoming the assay of choice. They can detect viral genome directly from animal samples and can also be used to facilitate the genetic characterization of the isolates.

2.5.1 Polymerase chain reaction

Polymerase chain reaction is a powerful technique for the detection of viral genome. It has the potential to have high sensitivity and is now accepted as the gold standard for nucleic acid detection and research (Mackay *et al.*, 2002). The principle of the assay is the cyclical replication using polymerase enzymes (Albert *et al.*, 2004).

However, PCR requires DNA as a template and the target viruses in this study have RNA as their nucleic acid. Therefore, RNA viruses require a reverse transcription step to produce single stranded complementary DNA (cDNA) through reverse transcriptase using a specific oligonucleotide primer and viral RNA as a template (Mackay *et al.*, 2002; Mackay, 2004; Turner *et al.*, 2005).

Reverse transcription polymerase chain reaction (RT-PCR) is used to detect RNA viruses such as AIV and NDV which are negative and single stranded RNA viruses. There are two different configurations of the RT-PCR assay. In the two step RT-PCR configuration, the cDNA is synthesized in a different tube before performing PCR assay. In contrast a one step RT-PCR firstly synthesises the cDNA. The reverse transcriptase is inactivated and the polymerase is activated simultaneously and the PCR reaction is carried out in a single tube (Pfaffl, 2004). This is rapidly becoming the assay of choice.

Several important steps need to be considered in developing an RT-PCR protocol. The first aspect is the RNA extraction. This needs to be an efficient process that can extract RNA from the samples even when it's in low concentrations and eliminate contaminants that will degrade the RNA (Pfaffl, 2004).

Another important aspect is the gene being targeted and the choice of primers. This can have a profound effect on the efficacy of the assay (He *et al.*, 1994). Poorly design primers can result in mispriming and the amplification of non-specific products or the

formation of primer dimer (Singh and Kumar, 2001; Abd-Elsalam, 2003; Pfaffl, 2004). The assays may be used to detect the organism, place it into appropriate genotypes and even determine the pathogenicity of the isolates.

Matrix genes are relatively conserved and many oligonucleotide primer sets have been used for the detection of NDV and AIV (Seal, 1995; Creelan *et al.*, 2002; Spackman *et al.*, 2002; Wise *et al.*, 2004; Spackman *et al.*, 2005).

However, screening test using M primers are not used to differentiate genotype ND and AI virus or to determine the pathogenicity based on the sequence of F and HA genes respectively. Therefore, it is crucial also to develop and evaluate set of primers based on fusion genes for NDV and haemagglutinin genes for AIV in order to confirm the pathogenicity of those viruses (Collins *et al.*, 1993; Collins *et al.*, 1994; Seal, 1995; Collins *et al.*, 1996; King and Seal, 1998; Heine *et al.*, 2005).

2.5.2 Real time (quantitative) polymerase chain reaction

Real time PCR (qPCR) is a configuration of PCR used to quantify and monitor the accumulation of amplicon in real time. This amplicon accumulation can be monitored by labelling the primers, oligonucleotide probes or amplicon.

qPCR is rapidly becoming the assay of choice since this method does not require an additional electrophoresis step using agarose gel. In conventional PCR there is an endpoint reaction which is then visualised using agarose gel electrophoresis where the DNA is stained using an intercalating dye such as ethidium bromide. This dye can be carcinogenic (Bustin and Nolan, 2004; Pfaffl, 2004; Bustin and Mueller, 2005).

The sensitivity of qPCR can be comparable to nested PCR without the accompanying problems of contamination and false positive results. Nested PCR is relatively cumbersome requiring two successive reactions with different sets of nested primers with the PCR products being visualised in gel electrophoresis (Singh and Kumar, 2001).

Another advantage of qPCR is that a melt curve can be incorporated into the analysis. Melting curve analysis can characterise and distinguish the amplified sequence based on their apparent melting temperature (T_m). This melting temperature is largely determined by the base composition and the length of the PCR product (Wilhelm and Pingoud, 2003).

The intercalating dyes SYBR Green and SYTO 9 are frequently used. They fluoresce when they are bound to double strand DNA and allow melting curve analysis to be carried out as the two strands come apart during the heating process (Wilhelm and Pingoud, 2003).

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Newcastle Disease and Avian Influenza Viruses

The reference viruses use in this study are outlined in Table 3.1

Table 3.1 Reference viruses of ND and AI used in this study

Virus	Names, Strains, Subtypes	Source		
		Country	Institution	Correspondence
ND	V4	Australia	TropBio Pty. Ltd	Dr. Jan Smith
ND	FP1	Australia	TropBio Pty. Ltd,	Dr. Jan Smith
ND	AP1	Australia	TropBio Pty. Ltd,	Dr. Jan Smith
ND	SP3	Australia	TropBio Pty. Ltd,	Dr. Jan Smith
ND	WA Domestic Duck Isolate WA3245	Australia		(Mackenzie <i>et al.</i> , 1984; MacKenzie <i>et al.</i> , 1985)
ND	Ibis	Australia	Department of Primary Industry	Dr Ibrahim Diallo
ND viral antigen	Balivet, Pusvetma	Indonesia	Balivet, Pusvetma	
AI	A/chestnut teal/Victoria/2004/H4N4	Australia	The Primary Industries Research Victoria	Dr Simone Warner
AI	H11N9/STS (Sharp-tailed Sandpiper)	Australia	WHO, Parkville, Australia	A. C. Hurt (Hurt <i>et al.</i> , 2006)
AI	H4N8 (Red Necked Stint = RNS)	Australia	WHO Parkville, Australia	A. C. Hurt (Hurt <i>et al.</i> , 2006)
AI	Timika, Ambon	Indonesia	Laboratory of Indonesian Agricultural Quarantine Agency	Syukur Iwantoro, Catur Putra Budiman
AI	Tangerang, Sukabumi	Indonesia	PT. BioTek Indonesia	Sudirman

3.1.1 ND viruses

ND viruses in this study were obtained from two different locations which are Australia and Indonesia. Australian strains of NDVs were attained from five different strains which were kindly provided by TropBio Pty Ltd-JCU. Those strains are V4, FP1, AP1,

SP3 and the WA Domestic Duck Isolate 3245. An Ibis NDV isolate was supplied by Dr Ibrahim Diallo DPI Brisbane.

The other strains were collected from Indonesia as inactivated RNA derived from Balitvet and Pustvetma antigens which were processed using the transport buffer and transported to Australia without a cold chain.

3.1.2 Avian Influenza viruses

AI viruses in this study were also attained from Australia and Indonesia. There were five different Australian isolates of influenza viruses used in this study.

The isolate A/chestnut teal/Victoria/2004/H4N4 was kindly provide by Dr Simone Warner, the Victorian Institute of Animal Science, Attwood and H11N9/STS (Sharp-tailed Sandpiper = STS), H4N8 (Red Necked Stint = RNS) which were isolated from Australian shorebirds species were provided by the WHO reference laboratory Melbourne, Victoria.

AI viruses were collected in Indonesia. Allantoic fluid was inactivated in the transport buffer and the RNA transported to Townsville.

These viruses were provided and processed in the Centre of Standard Testing of Indonesian Agricultural Quarantine Agency. The viruses were collected from Ambon and Timika outbreaks. Others samples were kindly donated by Sudirman, DVM, PT. BioTek Indonesia Jakarta. These viruses were Tangerang and Sukabumi. Samples from Indonesia were suspected to be AI subtypes H5N1. All were imported subject an AQIS permit.

3.2 Propagation of Stock Viruses

3.2.1 Viral culture

AIV and NDV were inoculated into specific pathogen free chicken eggs. Viruses were propagated following the procedure standardised by OIE(OIE, 2004) and (Webster *et al.*, 2002; Spackman, 2008) using 9-11 days old embryonated eggs inoculate into the allantoic cavity and incubated at 37°C.

3.2.2 Harvesting

Eggs were candled daily and embryos dying within 24 hours post inoculation were discarded. Eggs were chilled at 4°C overnight or -20°C for two hours then placed at 4°C for additional two hours before harvesting. Allantoic fluid was collected and centrifuged at 1,500 g for 10 minutes and tested for HA activity.

3.3 Haemagglutination Test

3.3.1 Preparation of 0.5% chicken red blood cells

The 0.5% chicken red blood cells were prepared as described by (Shortridge, 1982; Spackman, 2008), OIE and CSIRO (Anonymous, 2004). Whole chicken blood was collected from the wing vein of specific pathogen free chickens and diluted 1:4 in sterile Elsevier's solution (Appendix 2).

Aliquots of the cells were washed three times with phosphate buffered saline by centrifugation at 1,500 g for 10 minutes. The packed cell volume was noted and the cells were resuspended in PBS at a dilution of 0.5%

3.3.2 Antigen Titration

1. Serial, two-fold dilutions of the test viruses in 50 µl of PBS were carried out from columns 1 to 11 of a round bottomed microtitre plate with column 12 containing only PBS.
2. Aliquots of 50 µl of 0.5% chicken erythrocytes were added to all wells and the plates incubated at 4°C for 45 to 60 minutes.
3. The plate was examined for HA by tilting the plate and observing the presence or absence of RBC tear shaped streaming. The endpoint was the highest dilution at which there is complete agglutination without streaming. At this dilution the virus is said to contain 1 haemagglutination unit (HAU) per 50 µl

3.3.3 Bacterial contamination detection for quality control of stock viruses

The infected allantoic fluids were tested for bacterial contamination. Samples were streaked onto blood agar plates.

Allantoic fluids collected from eggs contaminated with bacteria were either filtered through 220 nm filters or treated with antibiotic (penicillin (2000 units/ml); streptomycin (2 mg/ml); gentamicin (50 µl/ml) and mycostatin (1000 units/ml)).

3.4 Confirmation of the presence of NDV using ELISA

Specimens of allantoic fluid containing NDV were confirmed by ELISA using strain specific monoclonal antibodies using kits provided by TropBio Pty Ltd, JCU, Townsville. ND antigens were screened using the “Trop-Ag NDV Screening kit (Catalogue No 03-001-01) and typed using the Trop-Ag NDV Typing kit (Catalogue No 03-001-03) following the manufacturers recommendations.

3.5 Estimation of viral titre by 50% egg infectious dose

The titre of the viruses was estimated by inoculating SPF chicken eggs and expressed as 50% embryo infective doses (EID₅₀) following the procedures describes by Mahy and Young (Mahy, 1985; Young *et al.*, 2002).

1. Serial 10 fold dilutions of stock viruses were suspended in PBS and aliquots of 100 µl were inoculated into the allantoic cavity of 10 day old embryonated eggs with 5 eggs per dilution.
2. The eggs were incubated at 35-37°C for 4 days and candled every day to check for embryo viability. Embryos dead at 24 hours were discarded. All remaining eggs were cooled at 4°C and harvested.
3. Titres were calculated using the method of Reed and Muench Method (Reed and Muench, 1938).

3.6 Polymerase Chain Reaction

3.6.1 RNA extraction

Viral RNA from 180 µl of allantoic fluid was extracted using a Corbett X-tractor Gene automated RNA/DNA extraction system (Corbett Robotic, Brisbane, Australia). The protocol followed the viral RNA/DNA purification protocol beta test (CorProtocol™ No. 25101).

3.6.2 Primers, probes and DNA binding dye

Primers and probes were synthesised by either Sigma Genosys or Biosearch Technologies (USA).

Where appropriate primers were designed using Vector NTI 10 (Invitrogen, Australia) or Beacon Version 6 (Premier Biosoft International) and AlleleID 6 or primers from published studies were used. Details are presented in Tables 3.2 and 3.3.

Lyophilized primers were suspended in 1× TE Buffer to produce a 100 µM stock solution stored at -80°C and this was diluted tenfold in nuclease-free water to make a 10 µM working stock that was stored at -20°C.

Table 3.2 Primers and probes for AI viruses' used in this study

Primer Name	Sequence (5'-3')	Target sequence	Position of primers	Product length	References
Screening test for AI					
IVA-D161M (Forward)	AGATGAGYCTTCTAACCGAGGTCG	AI Matrix	38 to 61	101 bp	(Heine <i>et al.</i> , 2005)
IVA-D162M (Reverse)	TGCAAANACATCYTCAAGTCTCTG	AI Matrix	115 to 138		
IVA-Ma (FAM-Probe)	FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1				
Ward MF	AAGACCAATCCTGTACCTCTGA	AI Matrix	183 to 205	95 bp	(Ward <i>et al.</i> , 2004; Munster <i>et al.</i> , 2005)
Ward MR	CAAAGCGTCTACGCTGCAGTCC	AI Matrix	256 to 277		
AI-Universal	AGCAAAAGCAGG	AI Matrix	1 to 12		(Hoffmann <i>et al.</i> , 2001)
IVA-D148H5-F	AAACAGAGAGGAAATAAGTGGAGTAAAATT	AI H5 short length	1568 to 1599	121 bp	(Heine <i>et al.</i> , 2005)
IVA-D148H5-R	AAAGATAGACCAGCTACCATGATTGC	AI H5 short length	1664 to 1689		
AI sequencing					
Pay H5F3+ Forward	AACAGATTAGTCCTTGCGACTG	AI H5 short length	1001–1021	102 bp	(Payungporn <i>et al.</i> , 2006b)
Pay H5R2+ Reverse	CATCTACCATTCCCTGCCATCC	AI H5 short length	1124–1103		

Table 3.3 Primers and probes for NDVs used in this study

Primer Name	Sequence (5'-3')	Target sequence	Position of primers	Product length	References
Diagnostic primers					
Wise-NDVM /M+ 4100 (Forward)	AGTGATGTGCTCGGACCTTC	NDV Chicken Matrix	811-830	120 bp	(Wise <i>et al.</i> , 2004)
Wise-NDVM / M-4220 (Reverse)	CCTGAGGAGAGGCATTTGCTA	NDV Chicken Matrix	911-931		
NDV-MGB1-F (Forward)	GGAACCGCATGTTATCCGATTG	NDV Duck Matrix	891 to 915	109 bp	Gene bank ID. AY626266 M
NDV-MGB1-R (Reverse)	GTGTGCCAGCTTGAATGATCAC	NDV Duck Matrix	979 to 1000		
Cr-NDV-Ch-F (Forward)	GGTGAGTCTATCCGGARGATAACAAG	NDV Chicken Fusion	4829 to 4893	202 bp	(Creelan <i>et al.</i> , 2002)
Cr-NDV-Ch-F (Reverse)	TCATTGGTTGCRGCAATGCTCT	NDV Chicken Fusion	5008 to 5031		
St-NDV-Du-M (Forward)	AGGACGCTTACAACCCTCC	NDV Duck Fusion	302 to 315	294 bp	(Stanislawek <i>et al.</i> , 2001)
St-NDV-Du-M (Reverse)	CTGCATCTTACCTACGGCAAC	NDV Duck Fusion	589 to 596		
Sequencing primers					
NDV-Duck-M2-F	CGTTTACAAGATTCCAACCGCAGC	NDV Duck Matrix	555 to 578	548 bp	GenBank ID. AY626266 M
NDV-Duck-M2-R	GCGAGTGCTTACTTCTTGAACGGG	NDV Duck Matrix	1080 to 1103		
NDV-F294-F	AGGACAYTGACTACTTTGCTC	NDV Chicken Fusion	4799 to 4819	293 bp	Consensus Chicken NDV Sequences
NDV-F294-R	CTGCATCTTCCCAACTGCCAC	NDV Chicken Fusion	5072 to 5092		

3.6.3 Conventional PCR

Conventional PCR in this study included reverse transcriptase (RT) for RNA and PCR for DNA. A variety of reagents were used Qiagen One-step RT-PCR kit (Catalogue No. 210210) while two step RT-PCR were carried out using various Fermentas, Bioline and Qiagen kits.

3.6.3.1 One step reverse transcriptase polymerase chain reaction

Extracted viral RNA (50 to 100 ng of template RNA) was amplified using Qiagen One-step RT-PCR kit (Catalogue No. 210210) in an Eppendorf Master Cycler using 1.5 mL tubes and 50 µl of the reaction mix following the manufacturers' instructions. Specific primers were used at a concentration of 0.4 µM.

3.6.4 PCR amplification

PCR amplification of DNA produced in the one step kits was carried out in some cases using either Fermentas polymerase or GoTaq colourless or GoTaq Green (Catalogue no. M7121) Master Mixes (Promega).

Template DNA was amplified in an Eppendorf Master Cycler which was set up according to the following reaction: initial denaturation step at 95°C for 2 min and 35 cycles of denaturation at 95°C for 45 sec, annealing at 5°C below the calculated melting temperature of the primers for 45 sec, extension reaction at 72°C for 1 min and a final extension at 72°C for 5 min.

3.6.5 Analysis and purification PCR product

All the PCR products were analysed by agarose gel electrophoresis utilising 2.5% w/v agarose gels containing 0.5 µg/ml ethidium bromide. A 100 bp plus DNA ladder (Fermentas, USA) was used as a markers.

PCR products were visualised in InGenius LHR Gel Documentation and Analysis system (Syngene, USA) with Gene Snap Software version 06.08.04 and Gene Tools analysis software version 03.07.03 (Synoptic. LTD, UK).

3.6.6 Extraction of DNA fragments

The DNA fragments were recovered and concentrated from agarose gels using Real Genomics™ (Real Biotech Corporation) kit, HiYield™ Gel/PCR DNA Extraction (catalogue no. YDF100).

3.7 Sequencing

Purified PCR products or PCR products without purification were sent to Macrogen, South Korea to obtain targeted sequences. PCR products were sequenced utilising the primer pairs used to produce the amplicons.

Sequences were analysed by assembled using ContiqExpress Project, Vector NTI Advanced 10 (Invitrogen), and Sequencher version 4.7 (Gene Codes corporation, USA). Sequences were compared to the available GenBank sequences database using Basic Local Alignment Search Tool (BLAST). GeneDoc and Mega 3.1 to 5 were used to perform alignments and produce phylogenetic trees.

3.7.1. Phylogenetic analysis

Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2007). The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The optimal tree was shown with the sum of branch length at about 1.14919081. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) to the branches (Felsenstein, 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and in the units of the number of base substitutions per site. The ME trees were searched using the Close-Neighbour-Interchange (CNI) algorithm (Kumar *et al.*, 2008) at a search level of 0. The Neighbour-joining algorithm (Saitou and Nei, 1987) was used to generate the initial trees. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated, and then phylogenetic trees were produced.

CHAPTER 4

Development and evaluation of real time RT-PCR assays for the detection of ND viruses

4.1 Introduction

Assays for the detection of AIV based on detection of the M gene (Heine *et al.*, 2007) were previously evaluated by others at this School. It was considered that these assays would be appropriate for screening the AI isolates to be used in the study.

It was anticipated that both Class I (duck strains) and Class II (chicken strains) of NDV would be encountered in this project (Czegledi *et al.*, 2006). There were several publications describing RT-PCR assays for the Class I strains of NDV but there are only limited published techniques for the detection of the Class 2 strains of NDV. Multiplex assays for the simultaneous detection of these viruses had not been described at the time this work was carried out.

4.2 Literature Review

Many primers have been used in qPCR for AIV and NDV. Pairs of primers designed by Spackman (Spackman *et al.*, 2002) have been widely used and modified for the detection of AI. These primers were also modified by Heine (Heine *et al.*, 2005) and this modification has been widely used in Australia as a standard technique for screening AI disease by RT-qPCR and for detection H5N1 isolates. Moreover, study of these primers (Long, unpublished thesis) has shown that these primers can be successfully used in SYBR green qRT-PCR (Long, 2007). However, published primer (Ward *et al.*, 2004) were used in this study with a SYTO 9 AI RT-PCR diagnostic assay. Ward primer were also compared with other published primers (Heine *et al.*, 2005) in this study.

A variety of molecular assays for NDV have been developed with primers and probes targeting both Class I and Class 2 viruses (Creelan *et al.*, 2002; Stanislawek *et al.*, 2002; Wise *et al.*, 2004). Assays based on these primers vary substantially in sensitivity and specificity and in this study they were evaluated using SYTO 9 RT-qPCR.

A set of primers that targeted the M gene were designed and evaluated as individual assays and in a duplex format combined with a set of primers designed by Wise (Wise *et al.*, 2004). These were evaluated using both Class I and Class 2 isolates which were anticipated to be similar to those that could be isolated from wildlife in North Queensland (Seal *et al.*, 1998; Wise *et al.*, 2004; Czeglédi *et al.*, 2006; Kim *et al.*, 2007b).

4.3 Aims

The specific aims of this project are as follows:

1. To develop a single one step RRT-PCR assay with SYTO 9 to rapidly detect NDV
2. To develop and evaluate a duplex one step RRT-PCR assay with SYTO 9 to rapidly detect both Class I and Class II NDV
3. To differentiate ND virus isolates based on melting curve analysis in one step SYTO 9 RRT-PCR

4.4 Materials and Methods

4.4.1 Propagation of viruses

ND V4, FP1, AP1, SP3; Duck isolate 3245 and ibis isolates as described in Chapter 3.1.1 and 3.1.2 were used. Viruses were propagated in embryonated eggs as described in Chapter 3.2.

Based on the classification, the isolates used in this study belonged to Class II genotype I (V4 and V4 like isolates: FP1, AP1, SP3) or they were Class I isolates (Duck #3425 and Ibis).

4.4.2 RNA extraction

Viral RNA was extracted using a Corbett X-tractor Gene automated RNA/DNA Extraction system (Corbett Robotic, Brisbane, Australia) as described in Chapter 3.6.1

4.4.3 Primer sets

The primers for ND were from published papers (Stanislawek *et al.*, 2001; Creelan *et al.*, 2002; Wise *et al.*, 2004) or they were designed using Vector NTI 10 (Invitrogen, Australia) and AlleleID (Premier Biosoft). The new pair of primers NDV-Duck-M2-F and R was designed using sequence accessed from GenBank database as described in Table 4.1

Table 4.1 Primer sets for ND virus diagnosis targeting M gene

No	Primer	Sequence	Position / Product Length	Viral Class	References
1	Wise-NDVM /M+ 4100-F	5'-AGTGATGTGCTCGG ACCTTC-3'	811-830 / 120 bp	Class II	(Wise <i>et al.</i> , 2004)
2	Wise-NDVM / M-4220-R	5'-CCTGAGGAGAGGCA TTTGCTA-3'	911-931 / 120 bp	Class II	(Wise <i>et al.</i> , 2004)
3	NDV-Duck-M2-F	5'-CGTTTACAAGATTCC AACCGCAGC-3'	891 to 915 / 109 bp	Class I	GenBank ID. AY626266 M
4	NDV-Duck-M2- R	5'-GCGAGTGCTTACTTC TTGAACGGG-3'	979 to 1000 / 109 bp	Class I	GenBank ID. AY626266 M

4.4.4 Reverse transcriptase real time PCR

A SYTO 9 RT-qPCR was performed in a RotorGene 3000 (Corbett Robotic, Australia). The SuperscriptTM III Platinum[®] One step Quantitative RT-PCR System (Invitrogen, catalogue no.11732-020) was used. The dye SYTO 9 was used at a concentration of 1.5 μ M and the primers were used at a concentration of 0.4 μ M

The cycling parameters were as follows 30 min at 48°C for the reverse transcriptase step. Then, 95°C for 10 min to activate the Hot start Taq DNA Polymerase. Then, 40 cycles of 95° C for 10 sec (denaturation) and 60°C for 45 sec (primer annealing and extension) were performed to amplify DNA.

The DNA was quantified using Quantization Cycling A-Green. This quantification calculated the cycle threshold (CT-Value) of normal fluorochrome. The Melting curve analysis was performed at 72°C to 95°C. Quantization Cycling A-Green and Melt A Green analysis was performed using Rotor-Gene 6000 Series Software series 1.7.

Initially four pairs of primers (two for M gene and two for fusion) were evaluated.

The two pair of M primers were chosen as the standard diagnostic primers. Initially they were evaluated as single pairs of primers and then as a duplex using SYTO 9 RT-qPCR and the products from NDV Class I and II viruses were differentiated by melt curve analysis.

4.4.5 Conventional PCR

Conventional PCR were performed as a quality control for the SYTO RT-qPCR for NDVs. Two steps Reverse Transcriptase was carried out in conventional PCR. The two steps RT-PCR were generated by firstly producing cDNA followed by PCR reaction.

cDNA was produced using Fermentas RevertAid™ First Strand cDNA Synthesis kit (catalogue no. #K1622). cDNA synthesis was carried out as described in Chapter 3.6.3. Synthesis of cDNA was then followed by PCR performed using Fermentas PCR kit and PCR conditions as briefly explained in Chapter 3.6.3. PCR products were analysed on gel electrophoresis and visualised using an InGenius LHR Gel Documentation and Analysis system (Syngene, USA) as described in Chapter 3.6.4.

DNA fragments were extracted from purified PCR products by using Real Genomics™ (Real Biotech Corporation) kit, HiYield™ Gel/PCR DNA Extraction (catalogue no. YDF100). The extraction of DNA fragments is described in Chapter 3.6.5. Where necessary additional cycles of PCR were performed on extracted DNA fragments of purified PCR products.

4.5 Results

SYTO 9 RT-qPCR assay was used to diagnose and differentiate NDV Class I and Class II isolates.

4.5.1 Evaluation of Class I and Class II NDV primers

Each of the pairs of primers detected their target genes.

The Wise primers detected Class II matrix gene. The Cr-NDV primers detected Class II fusion gene. The St-NDV detected Class I fusion and the NDV Class I primers detected the Class I M (Figure 4.1)

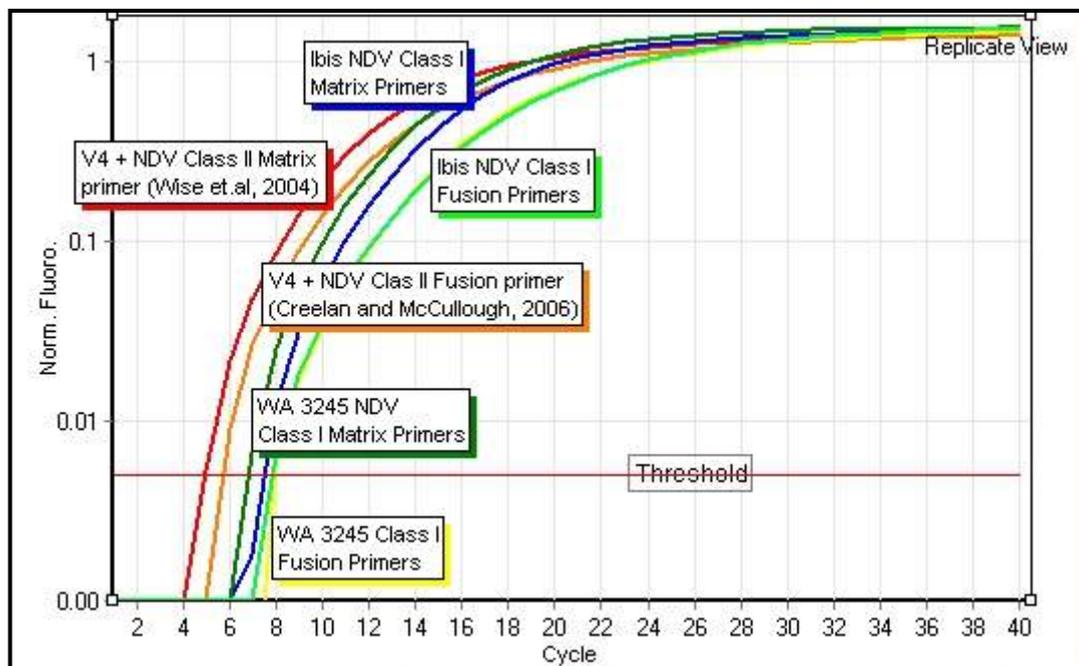


Figure 4.1 Real times PCR for Class I and Class II NDV's based on M and fusion genes. Four sets of primers in different tubes were evaluated to the Class I (WA 3245, Ibis) and Class II (V4) isolates. All primers successfully amplified their respective targets.

On the basis of this result, two of primers for each of the Class I or Class II NDV were selected based on M gene detection. These primers were then used for screening NDV

4.5.2 Evaluation of matrix primers for NDV Class I and Class II detection.

Evaluation of M primers was performed separately with homologous and heterologous ND viruses.

The evaluation of the two different M primers showed that the Wise primers (Wise *et al.*, 2004) specifically detected the Class II genotype 1 isolates but did not amplify the Class I NDV. The results of the SYTO 9 RT-qPCR can be seen in Figure 4.2.

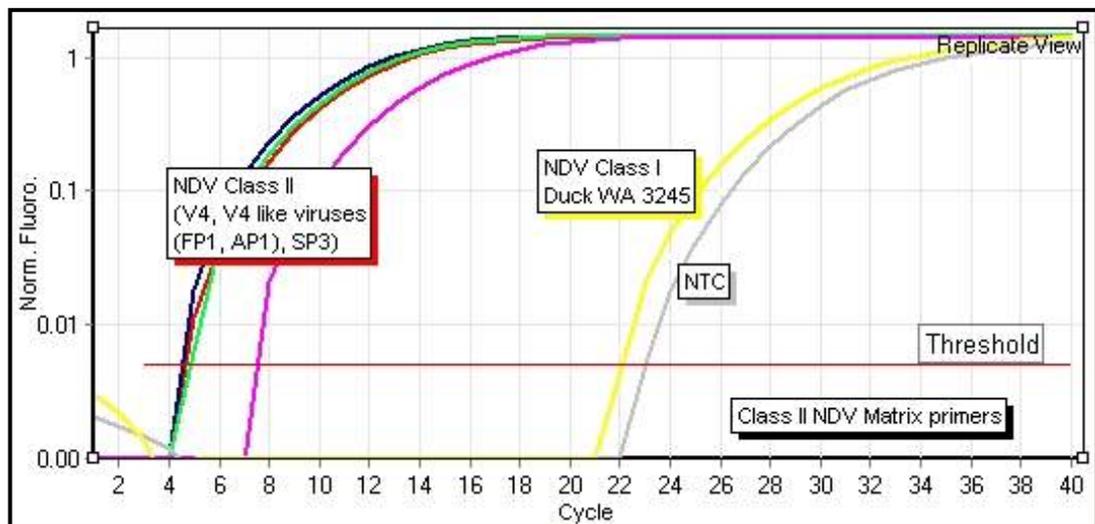


Figure 4.2 SYTO 9 RT-qPCR for detection of NDVs of Class I and Class II using the Wise M Class II primers (Wise *et al.*, 2004)

In contrast, The Duck (#3245, Class I virus) isolate was preferentially detected with the newly designed primers NDV-Duck-M2-F and R for ND Class I isolates. In addition, this pair of primers failed to react NDV Class II isolates as can be seen from SYTO 9 RT-qPCR result (Figure 4.3)

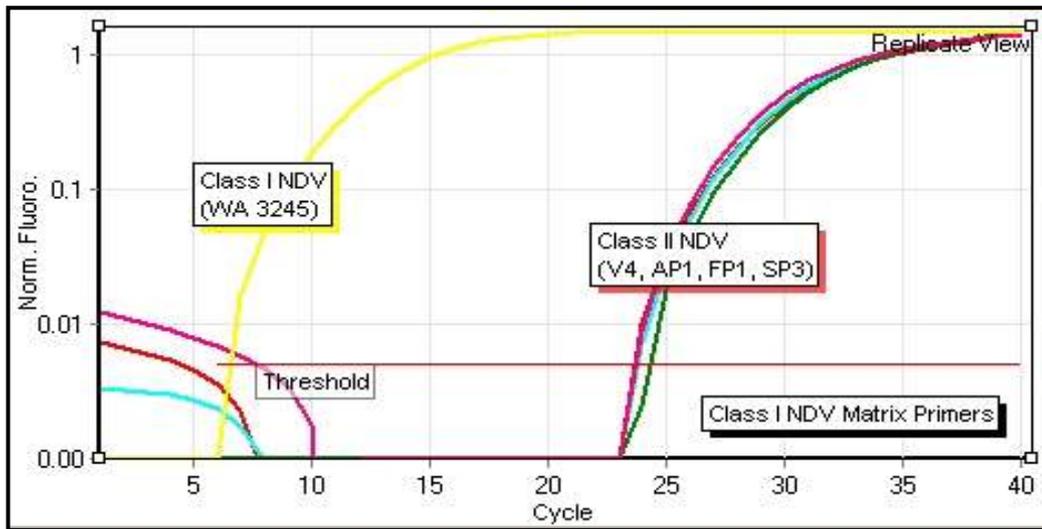


Figure 4.3 SYTO 9 RT-qPCR for detection of Class I and Class II NDV using the Class I M primers. The primers amplified the Class I NDV WA 3245 isolates but failed to detect the four chickens Class II NDV isolates V4, FP1, AP1, and SP3

4.5.3 A Duplex assay using two pairs of ND virus matrix primers

After successfully applying M primers in separated tubes to detect Class I and Class II, the two pair of M primers was evaluated in a duplex configuration. This was evaluated using tubes containing only Class II or Class I isolates as well as tubes containing a mixture of viruses.

A single product was produced by each of the pairs of primers. The Class I viruses in this project were WA3245 and an Ibis isolate. The Class II isolate was V4 (Figure 4.4).

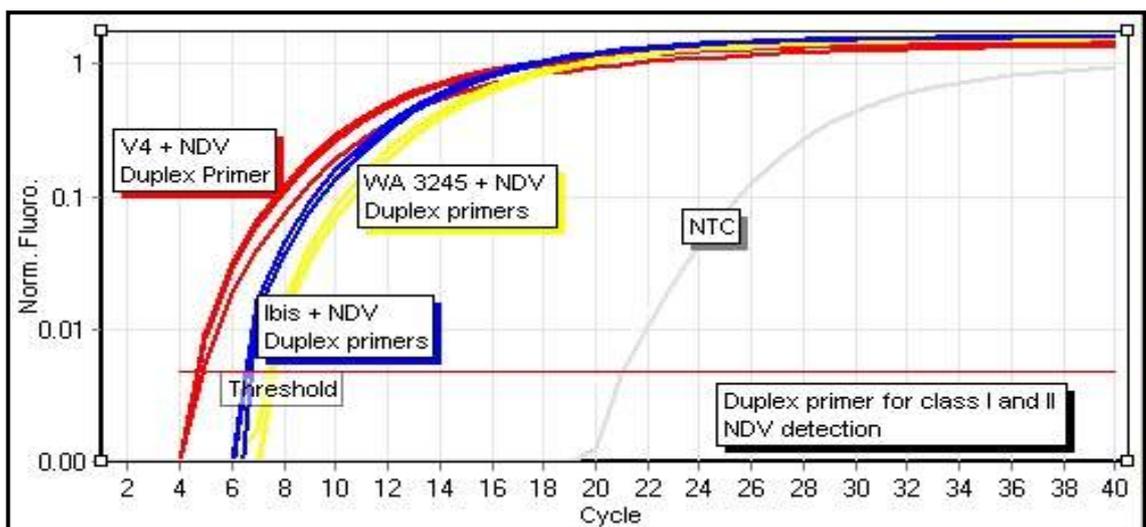


Figure 4.4 Fluorescence data of duplex SYTO 9 RT-qPCR assay Class I and Class II ND isolates.

4.5.4 Melting curve analysis of NDV Class I and Class II isolates

Melting curve analysis was performed and it was noted that there was a difference in melting temperature between the amplicons produced by each of the four pairs of primers (Figure 4.5)

NDV Class I (WA 3245) M and fusion required lower temperature to melt 50% of dsDNA than NDV Class II. NDV Class I M genes were melted at a temperature of $\pm 85^{\circ}\text{C}$ while NDV Class II M melted at $\pm 86.7^{\circ}\text{C}$.

Also, NDV Class I Fusion ($\pm 86.16^{\circ}\text{C}$) requires lower temperature than NDV Class II Fusion ($\pm 87^{\circ}\text{C}$) to dissociate double stranded amplified DNA to be a single stranded. The temperature differences, indeed, was affected by the length of sequence targets, M or Fusion genes, in NDV Class I or Class II as can be seen on Table 4.1 and 3.3.

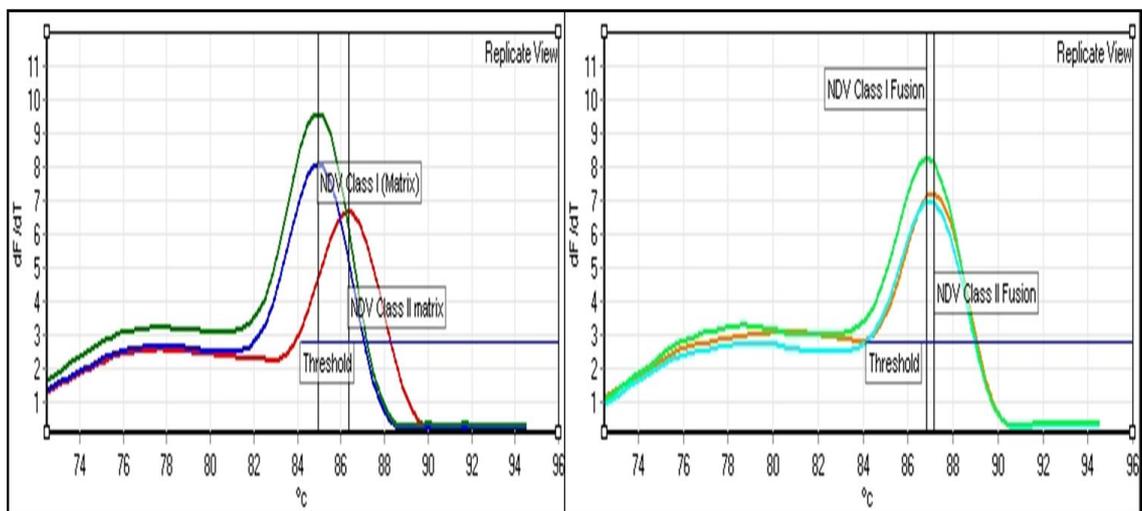


Figure 4.5 Melting Curve profile in different primers for different M and fusion sequence Class I and Class II of NDVs. Two classes NDV's M and fusion fragments have different melting points.

Melt curve analysis for M genes in duplex configuration confirmed that the two classes of NDV can be discriminated with Class II isolates represented by V4 having a higher melting temperature than the Class I isolates (Duck WA3245 and Ibis isolate) (Figure 4.7).

It was clear from the Figure 4.4 that different nucleotide sequence in the heterologous M genes produces different melting points. This different melting point, indeed, were used to differentiate Class I and Class II of NDV in single tube assay.

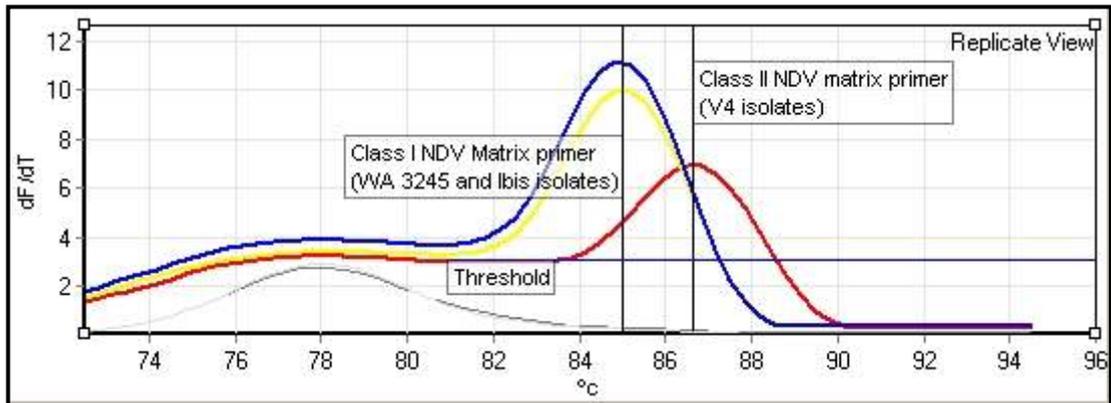


Figure 4.6 Multi curve profile of Class I and Class II NDV isolates in duplex RT-qPCR assay.

However, the melt curve analysis of amplified M sequences showed that slightly different peaks between NDV isolates in Class II could be observed. The V4 isolates had a similar melting point to the FP1 isolates while the AP1 and SP three isolates have a slightly higher melting curve (Figure 4.7). The result confirmed that different strains have different melting points even though they are in the same class and genotype. These viruses are all Class II genotype 1.

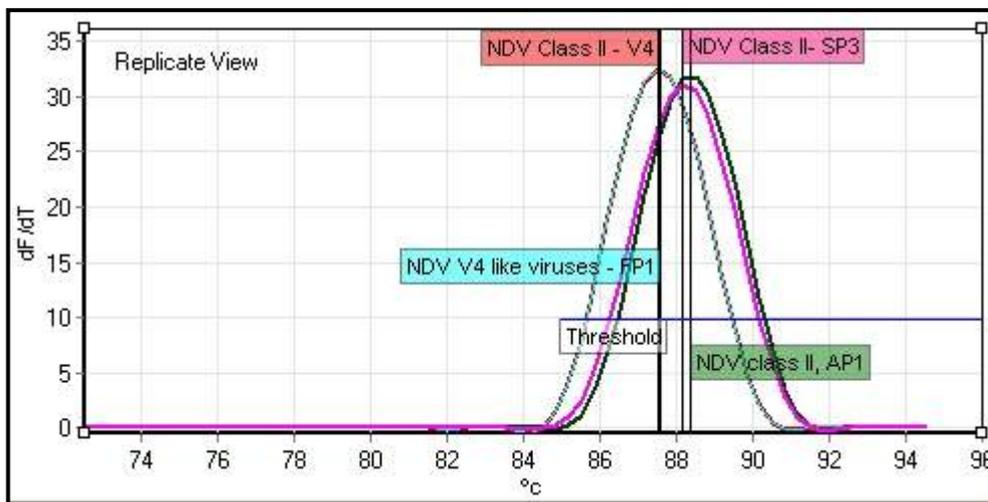


Figure 4.7 Melting curve profile of SYTO 9 RT-qPCR for Class II NDV's isolates using the M Primers (Wise *et al.*, 2004). There were minor differences in melting temperature (T_m V4 = 87.5; T_m FP1 = 87.56), (T_m AP1 = 88.34; T_m SP3 = 88.14)

4.5.5 Sensitivity of the test

The detection of template RNA of NDV V4 with tenfold dilution showed that SYTO 9 Real time RT assay can detect the RNA viruses of V4 up to a 10^{-5} dilution (Figure 4.8). No attempt was made to determine the number of copies being detected.

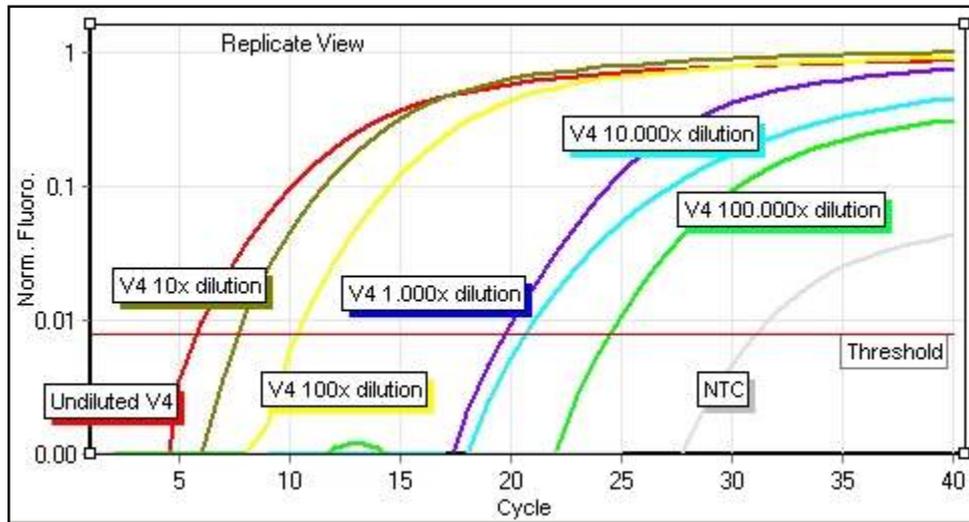


Figure 4.8 Performance of the duplex SYTO 9 RT-qPCR assay using tenfold dilutions of NDV RNA at a threshold 0.05.

4.6 Discussion

The RT-qPCR assay is a powerful tool that can be used to detect and quantify nucleic acids and is increasingly being used to diagnose infectious disease (Mackay, 2004; Suarez *et al.*, 2007). There are real advantages over the conventional two step assays as contamination can be avoided. Therefore RT-qPCR is perhaps the assay of choice to detect AIV and NDV genomes (Wilson, 1997; Spackman *et al.*, 2002). However, sensitivity and specificity of the assay can also be influenced by the choice of the primers.

The M and F proteins are the two important antigens in NDV. The M protein is relatively conserved and the corresponding gene is therefore a good target for diagnostics. A study of molecular evolution of the NDV M gene in the USA has noted 86% to 95% sequence identity between different isolates of Class II viruses (Seal *et al.*, 2000).

The traditional target has been the fusion protein gene and this protein is the important determinant of pathogenicity of NDV. This protein has a protein cleavage site which is cleaved by specific cellular protease (Seal *et al.*, 2000). The difference in virulence of NDV isolates correlates closely with the sequence of the cleavage site (Gould *et al.*, 2003). Most of highly virulent ND viruses have the fusion cleavage sequence ¹¹²R/K-R-Q-K/R-R¹¹⁶ whereas the viruses of low virulence have amino acid sequence in the same region of ¹¹²G/E-K/R-Q-G/E-R¹¹⁶ (OIE, 2004). Therefore this study evaluated both M and fusion primers using SYTO 9 RT-qPCR.

Previous studies aimed at developing M gene assay have successfully detected Class II NDV isolates but failed to recognise Class I NDV isolates (Kim *et al.*, 2007b). Further studies of the M gene sequences accessed from GenBank showed that there is substantial difference between Class I and Class II viruses. A divergence of 25%, with up to six mismatches in the probe site developed for the Class II isolates (Kim *et al.*, 2007b). Therefore a specific Class I M-gene assay was evaluated in this study and it successfully detected Class I NDV isolates and in a duplex format it could detect both Class I and Class II isolates.

A review of the literature indicated that the M primer set M+4100 forward and M-4220 reverse successfully detected most genotypes of Class II NDV isolates (Wise *et al.*,

2004). When used in real time RT-PCR these primers detected the reference Class II viruses V4, FP1, AP1, and SP3. The reference Class I viruses WA Domestic Duck Isolate 3245 (D1) and an Ibis isolate were not detected.

The primer pair NDV-MGB1 designed in this study preferentially detected the Class I NDV isolates. This pair of primers was designed using the published sequence (AY626266) accessed from the GenBank database

Fusion primers for NDV chicken (Class II) (Creelan and McCullough, 2006) and Class I viruses (Stanislawek *et al.*, 2002) were also evaluated. The Ct-values were higher than the corresponding results for the M primers. Therefore the M primers were chosen for use in the SYTO 9 RT-qPCR to monitor NDV.

The duplex RT-qPCR was evaluated using two pairs of M primers in one tube of master mix to detect both chicken and duck isolates of NDV. The two classes of virus could be differentiated on melt curve. No loss of sensitivity or specificity was noted in this format.

The duplex RT-qPCR for ND has the potential to save time and effort in the laboratory and it is cost effective. There is a potential for detection of non specific products and it is important to optimise the assay to avoid excessive amounts of primer dimer formation (Markoulatos *et al.*, 2002).

This assay was developed for screening wildlife infected with NDV isolates. It was anticipated that they would be infected with either Class I viruses or Class II genotype 1 viruses. The reference viruses chosen fit into these categories. For this study other genotypes of Class II viruses were not evaluated. However, the Class II primers had been previously shown to detect an extensive panel of Class II isolates (Wise *et al.*, 2004).

Melting Curve analysis can distinguish the two classes of NDV used to evaluate this assay. The Class II isolates had a higher melting temperature than did the Class I isolates. Using a high resolution melt analysis it was possible to distinguish between two subtypes of the Class II genotype 1 virus. Phylogenetic analysis confirms that V4 and FP1 cluster together and SP3 and AP1 cluster with the I2 vaccine strains (Chapter

5.5.3). These different melting temperatures can result from differences in sequence or length of PCR product (Wittwer *et al.*, 2001).

The SYTO 9 dye has advantages over SYBR green. It is a saturating dye that does not cause inhibition of the PCR reaction. It can be used in high resolution melt studies to detect minor sequence changes (White and Potts, 2006; Eischeid, 2011).

4.7 Conclusion

A SYTO 9 RT-qPCR using two pairs of M primers in a duplex configuration is a promising diagnostic assay for monitoring NDV infection in wildlife.

CHAPTER 5

MOLECULAR PHYLOGENETIC AND PATHOTYPING STUDY OF AUSTRALIAN NEWCASTLE DISEASE VIRUS ISOLATES

5.1 Introduction

Some strains of NDV can produce serious and fatal disease in poultry flocks and occasionally in wildlife (Seal *et al.*, 1998; Alexander, 2000a; Lee *et al.*, 2009). Pathotyping can require specific assays that are cumbersome and time consuming. Sequencing of the fusion gene and estimate of the amino acid sequence of the protease cleavage site can correlate closely with pathogenicity (Seal *et al.*, 1995; Seal *et al.*, 1998; Seal *et al.*, 2000; Seal *et al.*, 2002; Seal *et al.*, 2005).

In Australia, surveillance of NDV has generated several studies (King and Seal, 1997; Gould *et al.*, 2001; Chare *et al.*, 2003; Gould, 2004; Kattenbelt *et al.*, 2005; Kattenbelt *et al.*, 2006 (Spradbrow, 1987; Spradbrow *et al.*, 1995)).

The phylogenetic relationship and estimated pathogenicity of the reference viruses used in this study were determined by sequencing the F gene and predicting the sequence of the cleavage site.

5.2 Literature review

Pathotypes of NDV have been determined using conventional methods such as Mean Death Time (MDT), IVPI and Intracerebral pathogenicity index (ICPI). These assays utilise inoculated embryonated chicken eggs or chicks (Aldous and Alexander, 2001; Alexander, 2001).

ELISA based on monoclonal antibody has been also used for typing and for pathotyping NDV isolates (Li and Zhang, 2004; Lee *et al.*, 2006). However, there may cross reactions with epitopes common to other members of the Avulavirus genus such as PMV-3 (Alexander, 1990).

Molecular based techniques have been developed to predict the pathogenicity of NDV isolates (Jestin and Jestin, 1991; Suarez, 2003). RT-PCR can be used to amplify the F gene and the amino acid sequence predicted (Seal, 1995; King, 1996; Marín *et al.*, 1996; Seal *et al.*, 2000; Aldous *et al.*, 2001). Virulent isolates have the amino acid sequence ¹¹²R/K-R-Q-K/R-R¹¹⁶ at C terminus of F2 protein and F (Phenylalanine) at residue 117, the N-terminus of the F1 protein. Low virulence viruses have sequence of ¹¹²G/E-K/R-Q-G/E-R¹¹⁶ and L (Leucine) at residue 117 (Alexander, 1990; Alexander, 1995; Collins *et al.*, 1996; Aldous *et al.*, 2001; Gould *et al.*, 2001; OIE, 2004).

Molecular biology methods have been also used to evaluate the evolution of Avian PMV-1. Several studies have been conducted to determine evolution based on genotypes of NDV using sequence analysis of the F gene. Toyoda has classified the 11 NDV strains isolates from 1930's to 1970's into 3 lineages: A, B and C (Toyoda *et al.*, 1989) while Collins (Collins *et al.*, 1996) determined F sequences of Pigeon PMV-1 into 4 lineages (I to IV) and new genotypes (V). In Western Europe, Lomniczi classified 20 NDV isolates into 7 lineages (I to VII) (Lomniczi *et al.*, 1998) while genotypes VII b and VIII were proposed by Herczeg (Herczeg *et al.*, 1999) for NDV in Southern Africa and Southern Europe.

Then, Czeglédi (Czeglédi *et al.*, 2006) has re-classified NDV into two classes, Class I and Class II. NDV Class I and Class II have been divided into 9 genotypes: 1-9 (Class I) and I-IX (Class II) (Kim *et al.*, 2007a; Liu *et al.*, 2010). Class I of NDV was considered having a genomic length of 15,198 nt while NDV Class II has two sublineages with different genomic lengths (15,192 and 15,186 nt) (Czeglédi *et al.*, 2006).

There are two major reservoirs of NDVs in nature. The first reservoir is the primordial reservoir of NDV, the wild water bird species and the second is chickens or poultry. The Class I viruses predominantly infect wild water bird species while the Class II viruses predominantly genotype I infect wildlife with the remaining genotypes known as the pathogenic viruses infecting poultry (Czeglédi *et al.*, 2006; Kim *et al.*, 2007b; Liu *et al.*, 2010).

In Australia, the NDV strain V4 (Queensland V4) was isolated from chickens and has been identified as Class II genotype I. This virus is likely to have been transmitted to

chickens from wildlife and it was shown to be extensively distributed throughout the chicken population. Additional isolates of Class II NDV were reported (Kim *et al.*, 1978; Spradbrow *et al.*, 1995). Subsequently other Australian viruses were also recognised in water wild birds those were isolated in Victoria (VIAS 6 to 10), WA (wa1886 and wa2116) and MOURA Qld (Moura 1 and 2) in 1992. However those other Australian viruses were classified as NDV Class I.

Then, based on the phylogenetic and pathotyping studies performed by previous studies (Gould *et al.*, 2003; Czegledi *et al.*, 2006; Kim *et al.*, 2007a; Kim *et al.*, 2007b; Kim *et al.*, 2008), this project was conducted to sequence part of the F gene of the six Australian NDV isolates and predict their phylogeny and pathotypes.

5.3 Aims

The specific aims of this project are as follows:

1. Determine the sequence of the F gene of six Australian NDV isolates used as reference isolates in this study and compare these sequences with the published sequences of reference viruses.
2. Predict the amino acid sequence of the cleavage site and compare with reference viruses

5.4 Materials and Methods

5.4.1 Viruses

The ND virus isolates in this study were Class II NDV isolates (V4, FP1, AP1, SP3), and Class I NDV isolates (WA Domestic Duck Isolate 3245 and Ibis Queensland which were outlined in Chapters 3.1.1 and 3.1.2). These viruses were propagated as described in Chapter 3

5.4.2 GenBank Sequences

ND viral sequences used in this study obtained from GenBank. The accession numbers of the GenBank sequences can be seen in the figure of the phylogenetic tree later in this Chapter.

5.4.3 Primers

Primers were used in this study were designed using AlleleID (Premier Biosoft)

The Fusion gene Primers for Class I isolates were based on the sequences of WA Domestic Duck Isolate 3245 (Alexander *et al.*, 1986) and Ibis Queensland and the resultant sequence corresponded to sequences around the NDV F protein cleavage site, similar to the published primers (Collins *et al.*, 1998; Stanislawek *et al.*, 2002). The pair of primers was forward primer F302 (5'-AGGACGCTTACAACCCTCC-3') and reverse primer F596r (5'-CTGCATCTTACCTACGGCAAC-3') resulting in a 294 bp product.

The M gene primers for Class I were designed using aligned sequences. These sequences were accessed from Gen Bank with accession numbers AY626266, AY626267, AY626268, and DQ097393. These primers were forward -M2-F 5'-CGTTTACAAGATTCCAACCGCAGC-3' and reverse -M2-R 5'-GCGAGTGCTTACTTCTTGAACGGG-3' which amplified a 549 bp product.

Amplification of F sequence of Class II isolates was performed using forward primer F343 F 5'-CCCAAGGATAAAGAGGCGTCTGC-3' and reverse primer F343-R 5'-GCTGCATCTTCCCAACTGCCAC-3' which amplified a 343 bp product.

5.4.4 Reverse transcriptase PCR

Conventional Reverse transcriptase PCR was performed using One Step RT PCR and two step RT-PCR kits. Qiagen One Step RT-PCR (Catalogue No. 210210) was used in one step RT-PCR. The two-step RT-PCRs used Maloney Murine Leukaemia Virus (MMLV) Reverse Transcriptase with low RNase H activity (BioScript-Bioline catalogue No. BIO-27036) or RevertAidTM First Strand cDNA Synthesis kit (catalogue no. #K1622) for cDNA production while Fermentas, RBC Taq Polymerase or RBC

polymerase (RBC Bioscience, catalogue no. RT001) were used for PCR amplification. The Reverse Transcriptase PCR was described in Chapter 3.6.3

5.4.5 PCR product analysis and purification

All the PCR products were analysed by agarose gel electrophoresis utilising 2.5% w/v Agarose Standard low EEO Applichem (CAS No. 9021-36-6) as described in Chapter 3.6.4

Purification of PCR products was performed using Real Genomics™ (Real Biotech Corporation) kits, HiYield™ Gel/PCR DNA Extraction (catalogue no. YDF100) as described in Chapter 3.6.5

5.4.6 Sequencing

The PCR products and purified PCR products were sent to Macrogen, South Korea to attain the targeted sequences as described in Chapter 3.6.6. ContiqExpress Project, Vector NTI Advanced 10 (Invitrogen), and Sequencher version 4.7 (Gene Codes corporation, USA) were used to assemble the sequences. Then, Sequences were compared to the sequences in Gen Bank database using BLAST. Sequence alignment and phylogenetic tree was performed using GeneDoc and Mega 4

5.5 Results

5.5.1 ND Fusion and matrix gene detection for Class I isolates

A 294 bp product from the F gene of Class I viruses was amplified by the F302 and F596r primers (Stanislawek *et al.*, 2001) while the M gene was amplified by the M2 primers to produce a 549 bp product (Figure 5.4)

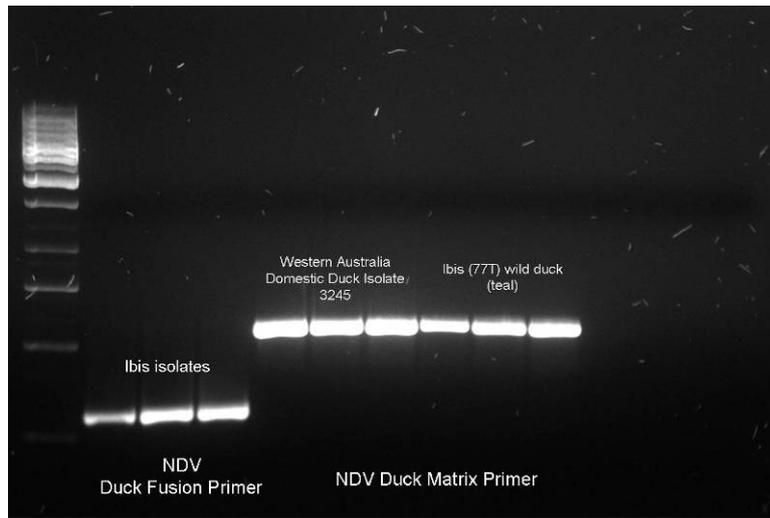


Figure 5.4 Gel electrophoresis analysis of PCR products of Class I NDVs. M gene was amplified using NDV-Duck- M2 while Fusion gene was amplified using F302 and F596r primers.

5.5.2 ND Fusion gene detection for Class II isolates

A specific 343 bp PCR products of the F gene of NDV Class II isolates was produced as outlined in Figure 5.5. These primers also reacted with the Class I isolates. However, the amount of PCR product appeared to be much less (Figure 5.5).

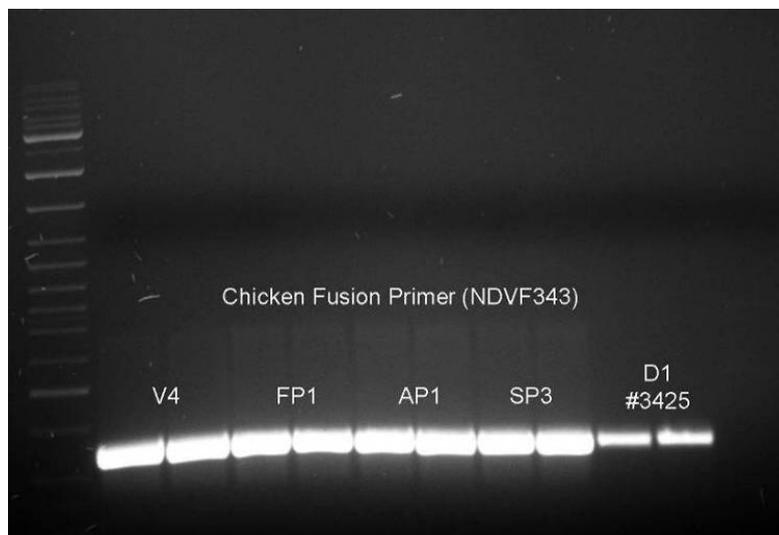


Figure 5.5 Gel electrophoresis analyses of the fusion gene of Class II isolates amplified using the F343 Fusion primers

5.5.3 Sequences Analysis

The analysis of the alignment of the sequences confirms that there are two distinct groups of isolates with the Class I isolates (3245 and the Ibis) clustering together and the Class II isolates (V4, AP1, FP1, and SP3) clustering together. Within the class two viruses, V4 and FP1 formed a cluster with AP1 and SP3 forming the other cluster (Figure 5.6).

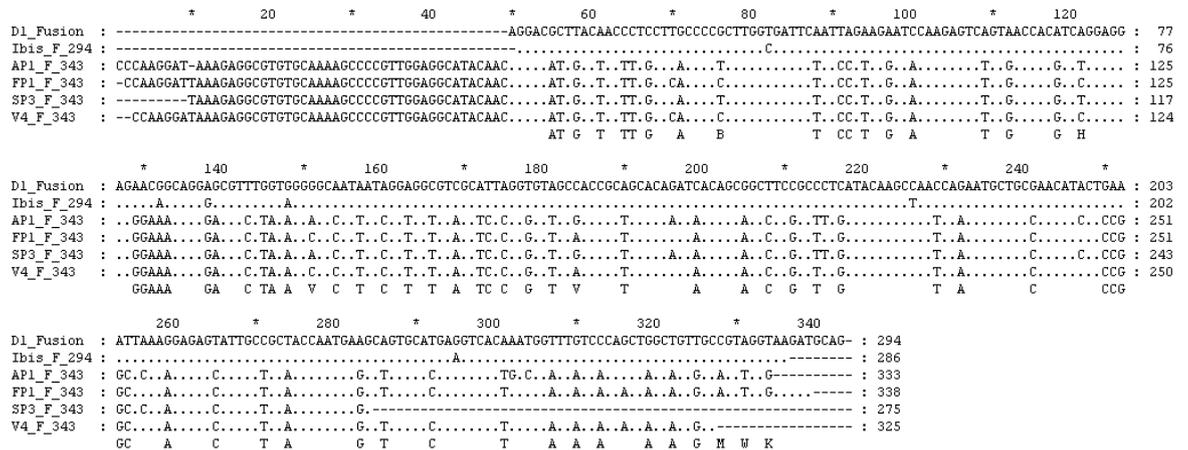


Figure 5.6 Alignment view of the fusion gene of six ND isolates including the Class I Duck Isolate WA 3245 and Ibis and Class II V4, AP1, FP1, and SP3.

Analysis to the Fusion sequences around the cleavage site confirmed that all of these six NDV isolates were lentogenic strains with motif of cleavage site of S-G-G-E-R-Q-E-R-L-V for the Class I WA 3245 (referred to as D1) and S-G-G-E-Q-Q-G-R-for the Ibis isolate. The Class II viruses (V4, AP1, FP1, and SP3) all had S-G-G-G-K-Q-G-R-L-I. (Figure 5.7).

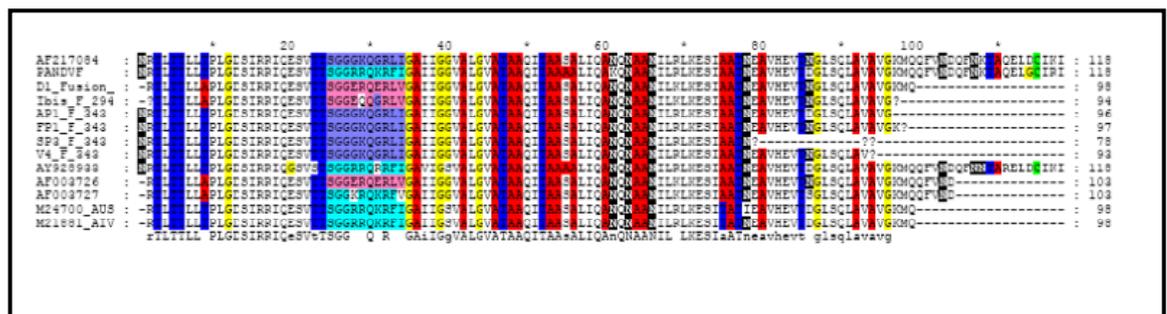


Figure 5.7 Amino acids translation of Fusion gene sequences of six Australian Newcastle disease viral isolates

A phylogenetic analysis of the F gene sequences indicates that the Australian Class I isolates cluster with other Class I isolates and that the Class II isolates clustered in the Class II genotype 1 cluster. Within the Australian isolates there are three separate clusters with V4 and FP1 being in one cluster and AP1 and FP3 clustering with the I2

vaccine. The third clusters of Australian isolates are the pathogenic isolates or their progenitors (Figure 5.8).

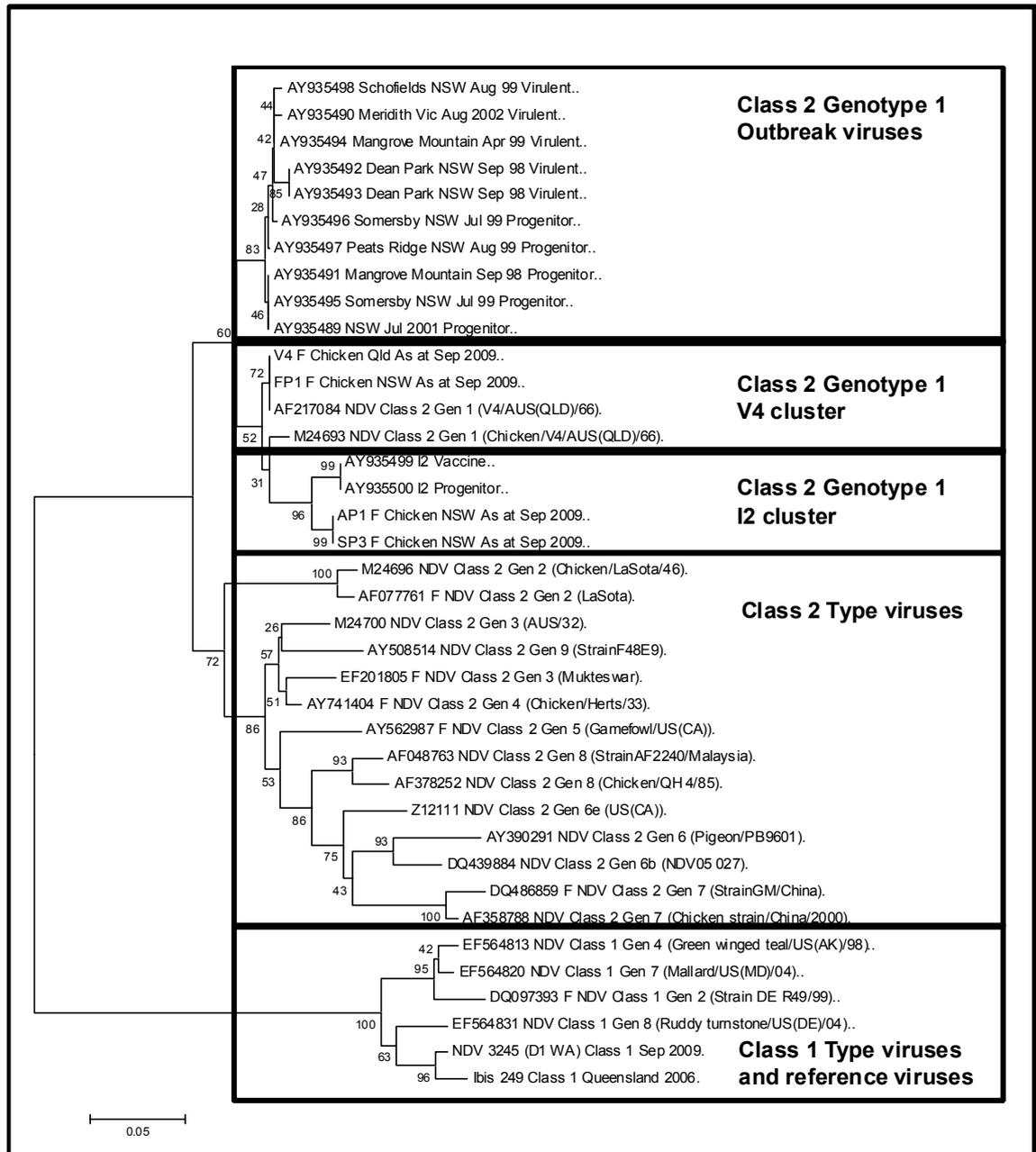


Figure 5.8 Phylogenetic trees of NDVs in Class I and II. Sequence was aligned using ContigExpress Project, Vector NTI Advanced 10 (Invitrogen). The Neighbour-joining algorithm (MEGA-5) was used to generate the initial tree. The analysis involved 38 sequences. There were a total of 263 positions in the final dataset.

5.6 Discussion

Both M and F genes of the six Australian reference viruses were amplified and sequenced. Analysis of the sequences confirmed that they were appropriate as reference viruses representing the two Classes (1 and 2) of NDV.

The four Class II viruses which were all isolated from chickens clustered in the genotype 1 cluster and this is suggestive that they all were derived from wildlife. It is reasonable to speculate that they were transmitted to chickens through close contact. Their role in respiratory diseases in chickens is not clear as they all appeared to be of low pathogenicity. They cluster with the two vaccine viruses V4 and I2 which are regarded as either apathogenic or lentogenic (Gould *et al.*, 2003; Kattenbelt *et al.*, 2006a)

CHAPTER 6

EVALUATION OF TRANSPORT BUFFERS FOR COLLECTION AND TRANSPORTATION OF SAMPLES WITHOUT A COLD CHAIN

6.1 Introduction

The investigation of AI outbreaks and NDs cases in many developing countries requires collection of samples. These samples must be transported to nearby laboratories to diagnose these diseases rapidly. However, obtaining good quality viable samples of AIV and NDVs can present significant problems and challenges. Therefore, effective sample collection and transport protocols can significantly improve the quality of the diagnostic outcome (Webster *et al.*, 2002).

Many developing countries have inadequate infrastructure to diagnose the diseases accurately. Delays and misdiagnosis reduce the effectiveness of prevention and control of AI virus outbreaks in Southeast Asia particularly in Indonesia in 2003. Improvements to transportation of samples to diagnostic and reference laboratories are required. However, the transportation of live viruses can be problematic as there can be loss of viability during transit and the movement of live viruses can be subject to quarantine restrictions. Therefore it is crucial to develop an integrated system of sample collection and transportation that overcomes these limitations.

Transportation of samples to overseas reference laboratories faces restrictions. Some countries do not allow the importation of infectious agents such as AI viruses. Therefore an integrated system of sample collection and safe transport will overcome the quarantine issues.

6.2 Literature Review

6.2.1 Collection and transportation of viruses with a cold chain

Viral transport media consists of isotonic solutions with protective proteins, antibiotics and buffers to control the pH. For instance Stuart transport media, Eagle MEM, Hanks BSS and tryptic soy broth are used for viral transport. These buffers maintain the survival of the viruses. However, different viruses have a different stability. Long distance of transportation and ambient temperature influence viral stability. Therefore, it is necessary to maintain the stability and infectivity of the viruses (Johnson, 1990).

Both AIV and NDV are thermo sensitive and the viruses can deteriorate when exposed to high temperatures. Light exposure especially UV may also affect the virus. It is recommended that samples collected for transport to a diagnostic laboratory be stored under refrigeration or in dry ice. This requires the establishment of a cold chain. It is also important to transport the samples to other laboratories as speedily as possible. However, it is difficult to maintain viral stability and infectivity in delivering samples to distant diagnostic laboratory or overseas reference laboratories. Therefore it is crucial that techniques to be developed that do not require a cold chain.

6.2.2 Collection and transportation of viruses without cold chain

There were many treatments to process the virus without a cold chain. For instance, viruses can be treated with chemical solution such as β -propiolactone, Binary ethylamine, and formalin (Scodeller *et al.*, 1984; Perrin and Morgeaux, 1995; Sagripanti *et al.*, 2011). Heat treatment is also a choice. This treatment is aimed to inactivate viruses in the field samples such as blood, serum, as well as cloacal and tracheal swabs. Viral inactivation will reduce the risk and eliminate the hazard of suspected materials from agent of diseases such as AIV or NDVs. However, some chemical solutions can harm the viruses. They may be harmful to humans and they may be toxic. (Johnson, 1990; King, 1991).

Diagnosing viral nucleic acid from clinical samples requires not only an appropriate viral inactivation but also nucleic acid separation. Nucleic acid detection frequently is inhibited by molecules in clinical samples. These molecules inhibit the enzymes which

are required to separate and amplify the viral nucleic acids. Indeed, it is important to remove the nucleic acid inhibitors. This inhibitor removal can be achieved by using appropriate extraction reagents and suitable buffers. However, even though many commercial viral extraction kits are available nowadays, some of the kits still fail to effectively remove the nucleic acid inhibitor and result in a poor yield of extracted RNA. Therefore, this study evaluated suitable buffers that could be integrated with sample collection, transportation and RNA extraction.

6.2.3 Important criteria for buffers to be used for the collection and transportation of samples without a cold chain

A suitable buffer for AIV and NDV collection and transportation has to meet specific criteria. Firstly, the buffer should be simple and effective. Simple means that the buffer requires no specialised or additional equipment to use the buffer. Effective means that the buffer should be integrated in the RNA extraction protocol methods. This protocol should be sensitive, rapid and reproducible. Secondly, the buffer should not only preserve RNA and inhibit RNase but also inactivate the infectivity of the virus. Heat stability is also an important criterion of a suitable buffer. Moreover, an appropriate buffer should allow enzymatic modification for nucleic acid purification. Finally, the risk of transmission to personnel and other samples must be small (Boom *et al.*, 1990)

6.2.4 Chemical components of buffers

A stable buffer is produced by mixing chemical components. There are many chemical components that have been used to stabilize the buffer including detergents, proteolytic enzymes, chaotropic agents, RNase inhibitors, and RNA carriers

6.2.4.1 Detergent and chelating agent

Detergent can disrupt cells and separate membrane proteins off the cells. This is achieved by interrupting the hydrophobic association and destroying the lipid bilayer (Albert *et al.*, 2004). One of the important detergents is Sodium Dodecyl Sulphate (SDS). SDS solubilises and denatures proteins by disrupting the cell membrane (Clark, 2005; Turner *et al.*, 2005). However, disrupting extracellular matrix requires chelating agents to remove the binding components in outer membranes. These chelating agents such as ethylene diamine tetraacetate (EDTA) chelate the Ca^{2+} on cell-cell adhesion to allow the cell separation (Albert *et al.*, 2002).

6.2.4.2 Proteolytic enzymes

Proteolytic enzymes are also required to support the detergent. Proteolytic enzymes may break down proteins into their basic amino acids and in doing so will also destroy enzymes such as RNase and DNase (Clark, 2005). Proteinase K is a commonly used proteolytic enzyme and it is an endolytic protease which cleaves peptide bonds and eliminates DNases and RNases. (Brown, 1995)

6.2.4.3 Chaotropic agents

Chaotropic agents are broadly used in RNA extraction. Chaotropic agents cause the molecular structure to be disrupted and dissolved. Then, the disruption and dissolving of proteins releases the nucleic acids. These chaotropic agents destabilize protein by disrupting water composition and allow hydrophobic groups to dissolve (Clark, 2005). Also, chaotropes can lyse the cells and inhibit nucleases and proteases. These agents provide hybridization stringency to the target analyte without altering the target chemically (Ness and Chen, 1991).

The commonly used chaotropic agent is guanidine. Guanidine is a crystal compound with strong alkalinity due to guanine oxidation. Guanidium hydrochloride and Guanidium isothiocyanate (GuSCN) are common chaotropic agents which have shown to be effective in the purification and detection of RNA and DNA due to their potential to lyse cells(Boom *et al.*, 1990). Guanidine chloride destroys the three dimensional structure of proteins. Guanidinium chloride or aminofomamide chloride is the first guanidinium salt to be used as an agent of deproteinization. However, guanidinium chloride is not powerful enough to denature proteins and extract the intact RNA from tissue which rich in RNase. Therefore, guanidinium isothiocyanate is used to effectively extract RNA from RNase rich tissue (Sambrook and Russel, 2001).

Guanidinium isothiocyanate is a stronger chaotropic agent. It can also reduce and break disulphide bonds and prevents cationic and anionic groups forming strong hydrogen bonds (Sambrook and Russel, 2001). This chaotropic agent will not only lies the cells but also rapidly inactive ribonucleases (Brown, 2000).

6.2.4.4 RNase Inactivator

RNase inactivators can inhibit the degradation of RNA by RNase and improve the recovery of RNA from a sample (Almarza *et al.*, 2006). Indeed, RNase inhibitor can prevent the inevitable loss of RNA that can be associated with the disruption of cells (Chirgwin *et al.*, 1979).

There are many RNase inhibitors commonly used to keep the activity of RNases such as Diethylpyrocarbonate (DEPC) and protein inhibitors of RNases. DEPC is an alkylating agent to inactivate RNases in buffers and on glassware. Protein inhibitors of RNases also inhibit RNases which bind tightly in the cytoplasm of virtually all mammalian tissues. However, DEPC and Protein inhibitors of RNases not only inhibit the RNases but also modify protein and nucleic acid or RNA (Chirgwin *et al.*, 1979; Sambrook and Russel, 2001). However, several manufactured sources of protein inhibitors of RNases cannot be used in the presence of denaturants such as SDS and guanidine due to lack of covalent complexes with RNases (Sambrook and Russel, 2001).

Another RNases inactivator is Dithiothreitol (DTT). DTT is a protective reagent for SH groups resulting in oxidation in air and reduction of disulfide bonds in proteins. In the presence of DTT, RNase is inhibited and ubiquitinated. DTT reduces disulfides in MetSO-RNase and maintains the essential sulfhydryls of some ubiquitinating enzymes which is important in causing intracellular protein degradation. (Dunten and Cohen, 1989). Also, DTT can be used with guanidine to reduce disulphide bonds (Chirgwin *et al.*, 1979).

6.2.4.5 Carrier RNA

The amount of purified nucleic acid attained from the nucleic acid extraction process is important for the accuracy of the diagnosis of disease agents. Nucleic acid extraction can be effectively carried out from diagnostic samples. However, there may only be very low yields of RNA which can easily be degraded. The addition of carrier RNA will provide an additional substrate for RNases and in turn stabilise the extracted RNA.

Many carriers have been used such as molecules, glycogen and RNA. Poly-A RNA/Salmon sperm DNA and tRNA are commonly used. (Kishore *et al.*, 2006).

6.2.5 Integration of transport buffer with nucleic acid extraction protocol

It is vital that the transport buffer be integrated with the RNA extraction protocol.

In this study, a range of suitable buffers that would be integrated with the nucleic acid extraction protocol from Corbett Robotics (Prototype kits) or Sigma chemicals catalogue Number XTRV, XTR1 or XTR2 was evaluated.

6.3 Aims

The specific aims of this project are divided into three parts as follows:

6.3.1 Pilot project

The pilot project aimed to evaluate four suitable buffers (Corbett Research, Australia) and select two buffers for further evaluation.

6.3.2 Definitive study

The definitive study aimed to evaluate the two chosen buffers and compare their ability to preserve the RNA using several replicates and statistical analysis.

6.3.3 Viral viability verification

The aim of viral viability verification is to confirm that the chosen buffers were able to inactivate viruses and in turn ensure the safety of the transport of samples. This was vital as the samples would need to be imported into Australia consistent with the policies of AQIS.

6.4 Materials and methods

6.4.1 General Methods

6.4.1.1 Viruses

AI virus H4N4 and NDV V4 were used in this experiment. They were considered to be representative of the target viruses. The details of these viruses are described in Chapter 3.11 and 3.1.2. The H4N4 and V4 viruses were propagated in allantoic fluid of 10-day-old chicken embryos as described in Chapter 3.2.

6.4.1.2 Buffers

Buffers were used in this study are the commercial buffers contained in the Corbett X-tractor pack (part no. XTR1, XTR2, and XTRV) at the time of the project produced by Corbett Robotic, Brisbane, Australia. The evaluated buffers were solid tissue digest buffer (STDB) (part no.Q3883, lot no 035K6168) with Digestion Buffer Additive (DBA) (part no 3508), lysis buffer (LB) (part no. C0616, lot no 035K6045) with LB Additive (part no Q3633), Viral Binding Buffer (VBB) constructed from Lysis buffer plus additional ethanol (part no C0616, lot no 035K6045 and sigma part no E7023), Liquid Sample Digest Buffer (LSDB) (part no B5810, lot no 035K6142).

STDB contained SDS, NaCl, Tris HCl, and EDTA while Digestion Buffer had additional Proteinase K. LB contained Guanidium Thiocyanate, Tris HCl, EDTA and Triton. Lysis buffer Additive which was composed of DTT was added to LB while Viral Binding buffer consisted of the Lysis buffer with additional ethanol but without DTT. The last evaluated buffer was Liquid Samples Digest buffer which contained Tris HCl, Guanidine HCl, EDTA, Tween 20 and Triton X100.

Buffers were used this study were as described in Corbett Protocol for the Viral RNA/DNA Purification Protocol Beta Test kit for the X-tractor GeneTM (CorProtocolTM No.25101 Version 01), Whole Mammalian Tissue DNA Extraction Protocol X-tractor GeneTM (CorProtocolTM No.14201 Version 04) and Universal Liquid Sample Protocol X-tractor GeneTM (CorProtocolTM No.14104 Version 01).

6.4.1.3 Design of the study

Viruses in allantoic fluid were processed in the commercial buffers as described above (Chapter 6.4.2). This laboratory experimental study used a factorial design with three levels of factors of treatments. The first level was buffer modification. The buffer modification was also generated with or without additional RNA carrier. The second factor was time and the third level was temperature.

A storage experiment for sample collection and transportation was performed in two steps. Firstly a preliminary study was carried out and this was followed by a definitive study using two selected buffers.

The quality of buffers was evaluated by measuring the amount of viral RNA in each sample. The assays used were an RT-PCR using SYTO-9 and expressed as Ct values.

6.4.1.4 A SYTO 9 RT-qPCR

RT-qPCR assay based on SYTO 9 was performed to determine the ability of buffers to preserve viral RNA. Viruses preserved in the selected buffers were extracted using the procedure described in Chapter 3.6.1. Then, an RT-qPCR assay based on SYTO 9 was performing as described in Chapter 4.

Two sets of primers were used in the RT-qPCR assays. The NDV RT-qPCR used the Class II M primers - forward primer 5'-AGTGATGTGCTCGGACCTTC-3' and 5'-CCTGAGGAGAGGCATTTGCTA-3' reverse primer (Wise *et al.*, 2004) while the AI H4N4 isolate was detected using AI M primers: forward primer 5'-AAGACCAATCCTGTACCTCTGA-3' and 5'-CAAAGCGTCTACGCTGCAGTCC-3' reverse primer (Ward *et al.*, 2004). Full information of the primers is outlined in Chapter 3.6.2

6.4.2 Pilot Project

A preliminary study was performed to eliminate the buffers which were least effective. This study was carried out to select two buffers which appeared to be most effective in preserving the RNA.

The preliminary study used three factorial levels of treatment including buffers, temperature and times.

The first level of the treatment was the buffers. Four buffers including STDB with Proteinase K, LB with DTT, LB with ethanol or known as VBB and Liquid Samples Digest Buffer were used. These four buffers were evaluated with and without carrier RNA.

The second level was temperatures. In this preliminary study, two temperatures were chosen: room temperature (25-27°C) and 37°C.

The third and last factor was times. In this pilot project the processed allantoic fluids containing either NDV V4 or AI H4N4 were stored for 1 day, 1 week and two weeks.

As this was a preliminary study only one replicate used for each level of treatments.

6.4.3 Definitive Study

It was noted that the carrier RNA consistently improve the stability of the stored RNA and two buffers containing carrier RNA were chosen for the definitive study. This study had three replicates each treatment and was extended to include a four-week observation. The Ct-values were recorded and used a statistical analysis.

6.4.3.1 Data Analysis

Data analysis was performed in definitive study using Ct-Values of the RT-qPCR assay. The data were generated from 16 observations with three replicates which totalled 48 data points. The data were analysed using the statistical package SPSS 14.0. The probability of Ct-value of treatments were compared and significance was set at a p-value of <0.05

6.4.4 Viral Viability Verification

The titre of the AI and ND viruses expressed as EID₅₀ of were measured as described in Chapter 3.5

Samples treated with the optimised buffer were inoculated into 10-day-old embryonated SPF eggs. Potentially the buffer could also be toxic for the embryos. This was determined by inoculating dilutions of the buffer into the allantoic cavity of 10-day-old embryonated eggs. Buffers as well as the stored samples in buffer were diluted as appropriate using PBS.

The embryonated eggs were incubated at 37°C and candled every day to confirm the embryonic viability. Allantoic fluid from the inoculated eggs was harvested and checked for HA activity and where necessary a serial passage was carried out to determine whether the allantoic fluid contained viable virus.

6.5 Results

6.5.1 Pilot Project

Evaluation of the four Corbett buffers as described in 6.4.2 after two weeks storage is shown in Figures 6.1 and 6.2.

The NDV RNA was best preserved in STDB and LB. The Ct values for the VBB and Liquid Samples Digest Buffer were higher suggesting that there was a lower titre of viral RNA (Figure 6.1). The difference is two to five cycles. Every three cycles is equivalent to approximately a tenfold difference in RNA titre.

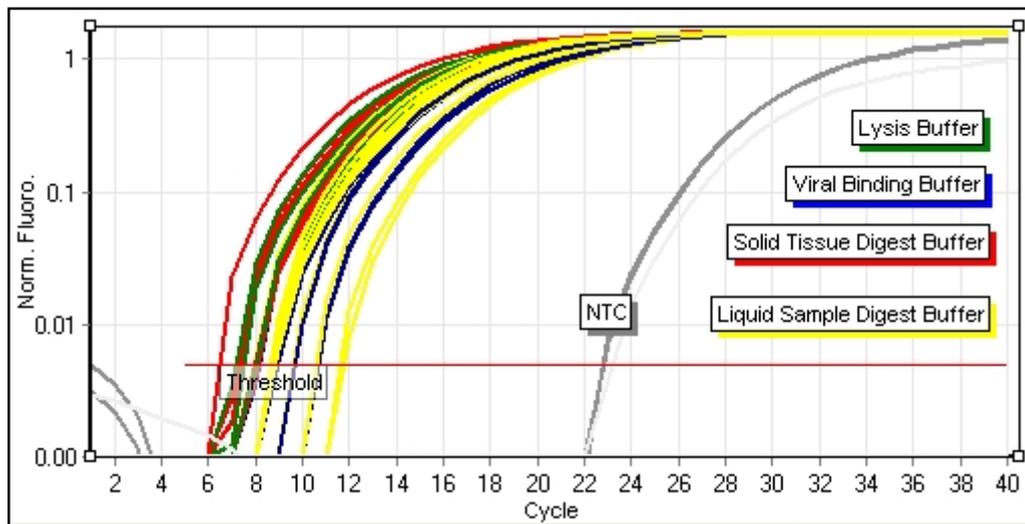


Figure 6.1 Quantitation analyses of NDV V4 RNA in four different buffers: STDB, LB, VBB, and LSDB. The data was produced for cycling A green at gain 5 and threshold 0.005 with reaction efficiency threshold 3%. The quantitation analysis was run on software version RotorGene 6.0.38. The colours indicate the buffer: 1). Red as STDB, 2). Green as LB, 3). Blue as VBB and 4). Yellow as LSDB. NTC stands for No Template Control.

Similarly, the preliminary storage experiment using AIV H4N4 resulted in better preservation in the STDB and the LB (Figure 6.2) which is consistent with the results obtained the NDV V4.

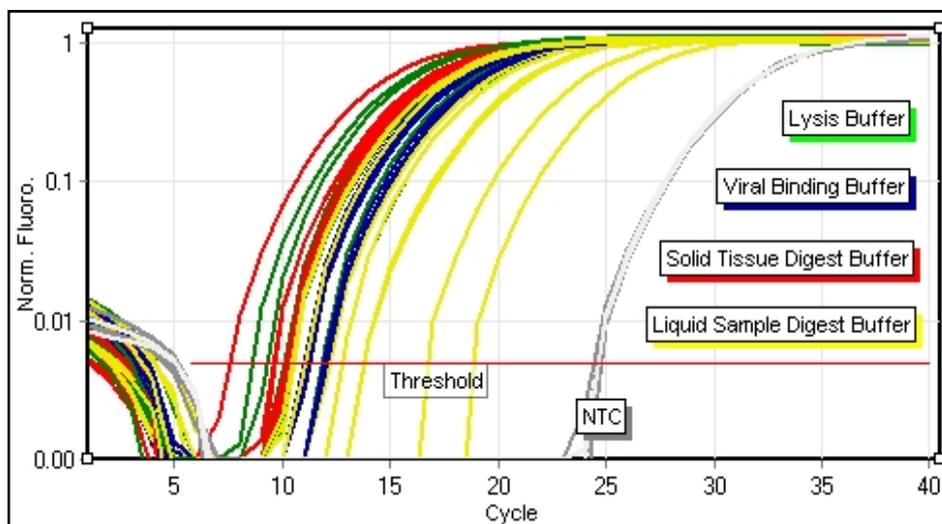


Figure 6.2 Quantitation analysis of AI H4N4 RNA in STDB, LB, VBB, and LSDB buffers. The data was analysed at a threshold 0.005 with reaction efficiency threshold 3%. The quantitation analyses was run on software version RotorGene 6.0.38 with channel cycling A green at gain 5. The colours indicate the buffer: 1). Red as STDB, 2). Green as LB, 3). Blue as VBB and 4). Yellow as LSDB. NTC stands for No Template Control.

6.5.2 Definitive Study

Based on the observations in the preliminary study the two buffers STDB and LB were evaluated.

The results of storage of NDV V4 RNA for four weeks at 25°C and 37°C are shown in Figure 6.3. There was a rise in Ct value between the 25 °C and 37°C treatments. However, this is still relatively small and it can be concluded that both of these buffers were able to stabilise the NDV RNA for at least a month at 37°C.

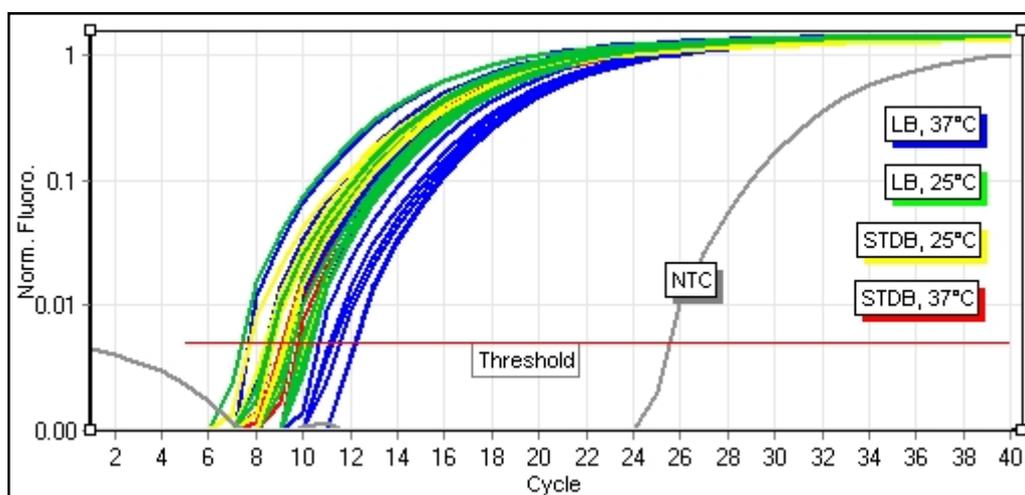


Figure 6.3 Quantization analysis of viral RNA V4 processed in STDB and LB at 25°C and 37°C. The normal fluorescence of SYTO 9 was performed in Cycling A green at a gain 5 and quantified at threshold 0.005. The quantitation analysis was run on software version RotorGene 6.0.38. The colours indicate viral RNAs were treated in: 1). STDB at 37°C (Red), 2). STDB at 25°C (yellow), 3). LB at 37°C (blue), 4). LB at 25°C (green)

Similar results were observed in the AIV H4N4 with both buffers stabilising the viral RNA and minor difference being observed between the two temperatures of 25°C and 37°C (Figure 6.4).

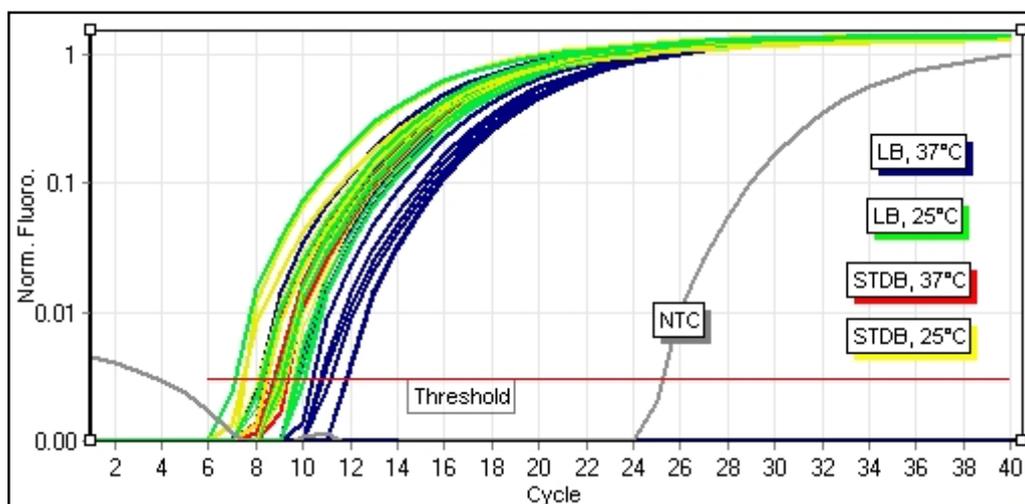


Figure 6.4 Quantization analysis of AIV H4N4 RNA in STDB and LB at (25°C and 37°C). The Ct-Values were analysed at a threshold 0.005, with efficiency threshold 3%. Cycling A green with gain at 5 were used in measure the amount of normal fluorescence emitted by DNA binding dyes SYTO9. The colours indicate viral RNAs were treated in: 1). STDB at 37°C (Red), 2). STDB at 25°C (yellow), 3). LB at 37°C (blue), 4). LB at 25°C (green)

6.5.2.1 Statistical Analysis

6.5.2.1.1 Analysis of Ct-Values of PCR products of viral RNA NDV V4

Ct-Value data of PCR product of viral RNA V4 measured in RT-qPCR after storage in the two buffers were analysed statistically using Univariate Analysis of Variance (ANOVA). Statistical analyses of the data are shown in Figure 6.5. No significant difference was determined that the two buffers (p -value = 0.836)

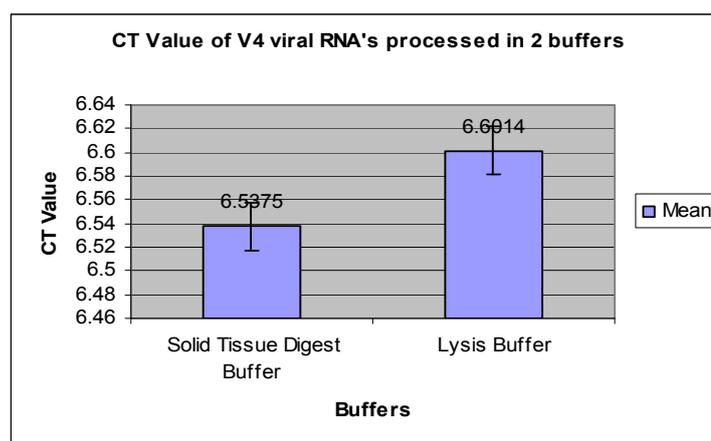


Figure 6.5 The mean of Ct-Values of NDV V4 RNA preserved in STDB and LB analysed with ANOVA. Significance were set at p -value of <0.05

Statistical analysis to the Ct-value of RT-qPCR of NDV V4 viral RNA stored in the two buffers at different times of storage is shown in Figure 6.6. No significant differences

were determined (p -value = 0.083) at 1 day, 1 week, 2 weeks and 4 weeks of storage time.

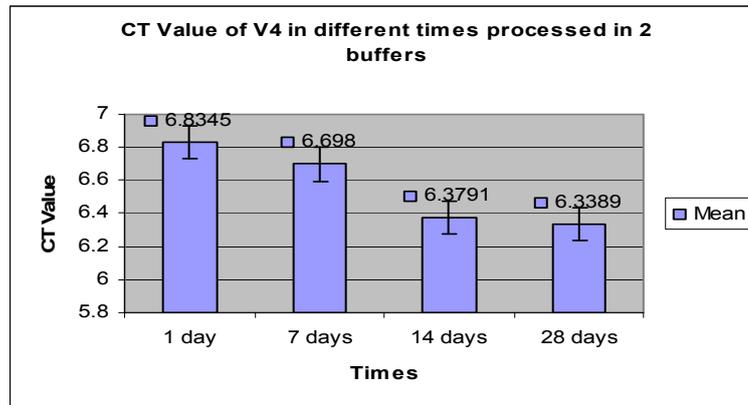


Figure 6.6 The mean Ct values of NDV V4 RNA at four different times analysed with ANOVA with significance at p -value of <0.05

It can be seen from Figures 6.5 and 6.6 that the solid tissue digests buffer slightly outperformed the lysis buffer and there was a gradual decrease in titre with time. However, the differences are not significant.

It can be seen also from Figure 6.5 that the two buffers have preserved viral RNA V4 effectively for four weeks. There was a gradual decline of titre with time.

Further analysis compared room temperature and 37° and there is a significant difference in the Ct values (p -value = 0.021) (Figure 6.7).

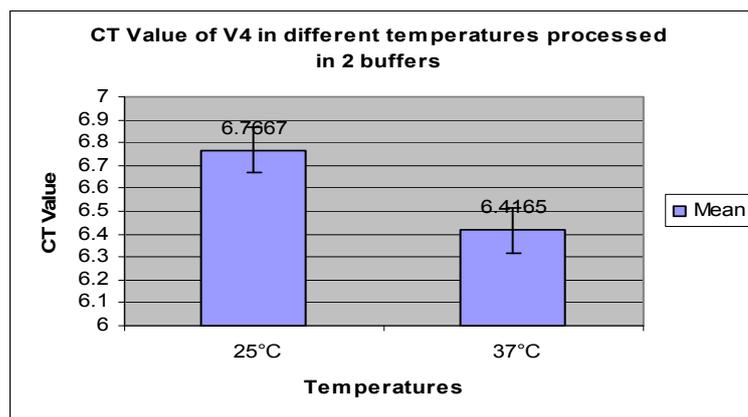


Figure 6.7 The mean of Ct-Value of NDV V4 RNA in the two buffers with a comparison between storage at 25°C and 37°C .

However, the difference in Ct-value while statistically significant was still relatively small and the overall results suggested that both of the buffers were able to preserve the NDV V4 RNA for at least a month and this would be sufficient to detect the viral RNA in clinical samples.

6.5.2.1.2 Analysis of -values of PCR product of viral RNA AI H4N4

Ct-Value data for the storage experiments of AI H4N4 viral RNA were analysed statistically using the Kruskal-wallis and Mann Whitney Test.

A significant difference was noted for the two buffers with solid tissue digest buffer having a significantly lower Ct value than the lysis buffer (ρ -value = 0.008) (Figure 6.8). Further details are available in Appendix 6.4.

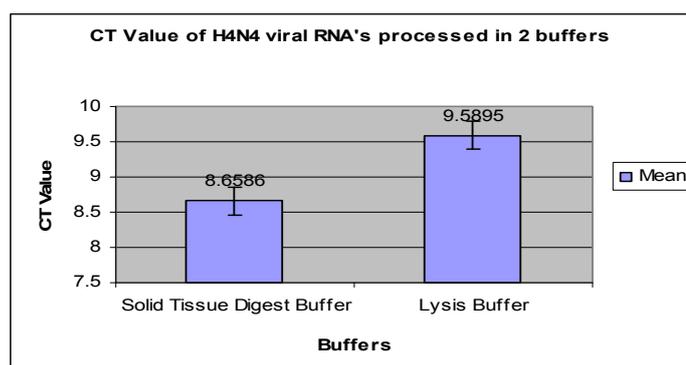


Figure 6.8 The mean of Ct-Value of H4N4 viral RNA processed in two different buffers, STDB and LB. The data were analysed using the Kruskal-wallis and Mann Whitney Test with significance level at ρ -value of <0.05

The data analysis indicates that the Ct-value increased over the four weeks of storage. However, no significant differences were determined (Figure 6.9).

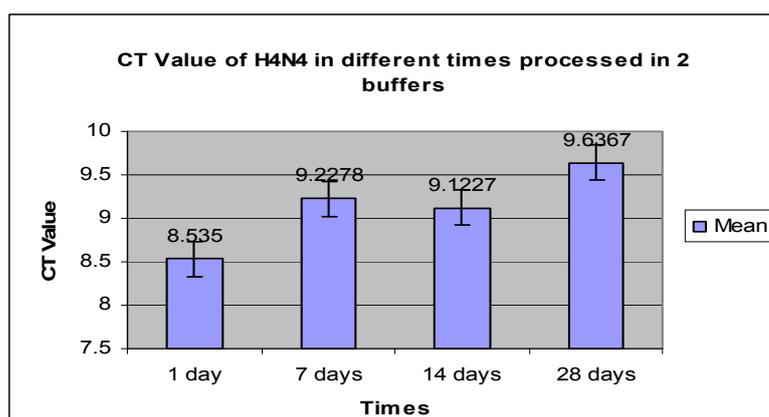


Figure 6.9 The mean of Ct-Values of AI H4N4 RNA preserved in STDB and LB over the four weeks of the experiment analysed with Kruskal-wallis and Mann Whitney Tests with significance level at ρ -value of <0.05

While there was an increase in Ct-value between the 25°C and 37°C observations this difference was not significant (ρ -value = 0.087) (Figure 6.10).

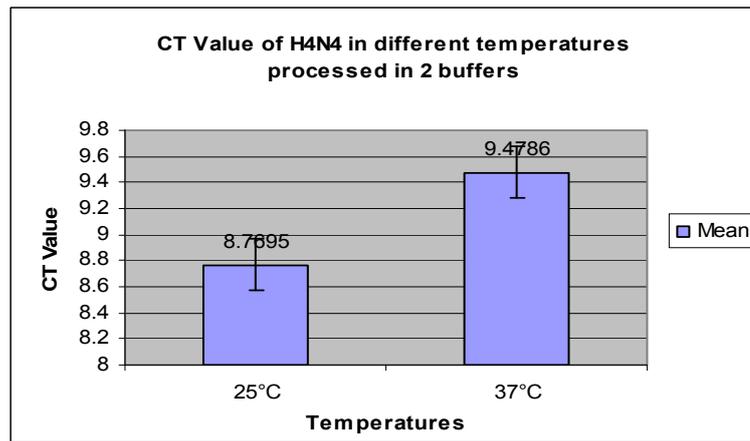


Figure 6.10 Ct-Value means of processed viral RNA V4 in STDB and LB at the two temperatures. The data was analysed using Kruskal-wallis and Mann Whitney Tests with a significance level at p-value of <0.05

It was therefore concluded that either of the buffers were capable of preserving AI RNA for up to 4 weeks at 37° with minimal loss in titre.

6.5.3 Viral Viability

Measurement of the titres of the two viruses expressed in egg infectious doses 50 indicated that at the commencement of the experiment the NDV V4 allantoic fluid contained EID₅₀ 10^{8.75} /ml and the AIV H4N4 had a titre of EID₅₀ 10⁶ /ml.

Undiluted LB plus DTT killed embryos on day 1 after inoculation. Some embryos also died after being inoculated with the 10⁻¹ dilution. Embryos inoculated with more dilute samples remained viable. It was concluded that death of embryos inoculated with allantoic fluid in undiluted or 10⁻¹ dilution of lysis buffer would not be a reliable indication of viable virus.

In contrast embryos inoculated with undiluted STDB plus Proteinase K died within 24 hours but the embryos inoculated with the 10⁻¹ dilution all remained viable.

Embryos inoculated with NDV V4 diluted in solid tissue digests buffer in most cases remained viable. However, passage of the allantoic fluid clearly indicated that this buffer had failed to completely inactivate the virus.

The lysis buffer killed the embryos inoculated with undiluted buffer. All of the other embryos remained viable and no live virus was demonstrated.

Embryos inoculated with AIV H4N4 dilute it in solid tissue digests buffer all remained viable and no HA was detected on serial passage.

The AIV H4N4 stored in lysis buffer killed the embryos at the first dilution and the remainder of embryos remained viable. No live virus was demonstrated (Table 6.1).

It was concluded that while both of these buffers were able to preserve both NDV and AIV stored for four weeks at 37°C some viable virus could be demonstrated in the solid tissue digests buffer indicating that it failed to completely inactivate all of the virus. Whereas LB inactivated both NDV and AIV.

Table 6.1 Verification of the viability of the two viruses stored in solid tissue digests buffer or lysis buffer. Stored viruses were inoculated into 10-day-old embryonated eggs which were checked for viability and the allantoic fluid screen for HA activity.

Virus	Buffer	Embryos after 4 days post inoculation			HA test (HA titre)			HA test (2nd passage)		
		Undiluted	Diluted		Undiluted	Diluted		Undiluted	Diluted	
			(10 ¹)	(10 ²)		(10 ⁻¹)	(10 ⁻²)		(10 ⁻¹)	(10 ⁻²)
NDV V4	STDB	Dead & Live	Live	Live	2 ⁹ to 2 ¹⁰	2 ¹⁰	2 ⁹ to 2 ¹⁰	2 ⁹ to 2 ¹⁰	2 ⁹ to 2 ¹⁰	2 ⁹ to 2 ¹⁰
	LB	Dead	Live	Live	Neg	Neg	Neg	Neg	Neg	Neg
AI H4N4	STDB	Live	Live	Live	Neg	Neg	Neg	Neg	Neg	Neg
	LB	Dead	Live	Live	Neg	Neg	Neg	Neg	Neg	Neg

6.6 Discussion

Integrated systems for the collection, storage, transport and extraction of viruses capable of causing disease in domestic and wild animals are important for both developed and developing countries. In developing countries it is extremely important that these transport systems do not require a cold chain. If samples are to be sent to reference laboratories in developed countries it is vital that there is minimal quarantining risk for the receiving country.

Laboratory proficiency programs are vital to maintain expertise and diagnostic capabilities in laboratories (Gilbert and Patey, 1998). The ability to send samples without a cold chain with minimal risk to quarantining significantly improves these quality assurance and proficiency programs.

Preservation of RNA is much more difficult than DNA as it is more susceptible to degradation and is very sensitive to minor changes in pH, temperature and exposure to heavy metals. The sources of ribonucleases contamination can be exogenous and endogenous ribonucleases. Exogenous ribonuclease contamination resulted from careless and aseptic techniques, finger grease, airborne bacteria or fungus and dust particles while endogenous ribonuclease resulted from cells and tissue (Sambrook and Russel, 2001; Kudlicki *et al.*, 2007).

The preliminary study indicated that some of the buffers were less efficient than others at preserving the viral RNA. The LSDB and VBB appear to be less efficient than the LB and STDB.

Guanidine hydrochloride (GnHCl) is a denaturing and solubilising agent for proteins. GnHCl has an ability to abolish the non-covalent inter- and intramolecular interaction of polypeptide chains. Related to this ability, guanidine hydrochloride has been also used as a tool for determining the molecular weight of oligomeric protein subunits (Ullmann *et al.*, 1968). Moreover, GnHCl is able to unfold ribonuclease (Greene and Pace, 1974). However, guanidine hydrochloride has about 2.5 fold less effectiveness on a molecular basis than does guanidine thiocyanate as an equilibrium denaturant. (Chirgwin *et al.*,

1979). Consequently, LSDB preserved viral RNA of AI and ND less than others buffers.

Guanidine isothiocyanate is a stronger denaturing agent compared with guanidine hydrochloride (Chirgwin *et al.*, 1979).

The preliminary experiment confirmed this finding and that the buffer containing guanidine isothiocyanate appeared to be more effective at preserving the RNA than the buffer containing guanidine hydrochloride.

The ability of guanidine isothiocyanate to preserve the viral RNA was improved in the presence of DTT which has the ability to reduce disulphide bonds (Chirgwin *et al.*, 1979; Basehore, 2006). However, DTT can react with the thiocyanate anion to produce hydrogen sulphide and a green colour (Chirgwin *et al.*, 1979).

Ethanol and DTT can be mixed with guanidium isothiocyanate. This combination is called Nuclease inhibitor cocktail (Kudlicki *et al.*, 2007). The nuclease inhibitor cocktail was presented in all four buffers in this experiment. However, the combination of Guanidine thiocyanate, DTT and ethanol was presented in VBB. This buffer also is a modification of LB with the addition of ethanol.

Alcohols such as ethanol can be used to precipitate DNA and protein (Chomczynski, 1993). Alcohol destabilises hydrophobic interactions between non polar residues to destabilise proteins. Alcohol disrupts hydrogen binding between amide groups in the secondary protein structure. Alcohol also interrupts the hydrogen bonding between side chains in tertiary protein structure in an assortment of amino acid mixtures. However, the addition of alcohol to aqueous solvents affects the charge state of protein. Alcohol lowers the dielectric constant of protein and its denaturation temperatures (Velicelebi and Sturtevant, 1979).

STDB was shown to be the best performing preservative for the viral RNA. This buffer contains SDS which is a detergent. However, SDS has also an ability to refold protein and prevent the complete denaturation cytochrome C by urea (Xu and Keiderling, 2004). It is also reported that several oligomeric enzymes undergo renaturation after denaturation with SDS (Weber and Kuter, 1971).

It was expected that the SDS would completely remove the viral envelope from the NDV and render the virus completely inactivated. Clearly this was not the case. Viable NDV was recovered from the allantoic fluid indicating that this buffer had failed to completely inactivate the virus. This indicates that STDB would not be suitable for the importation of viral RNA into a country that is free from velogenic NDV as is Australia.

This buffer may be suitable for use within Indonesia. The presence of some residual live virus may not be critical. However, it would be irresponsible to use this buffer when samples are being exported to a reference laboratory. ND virus and in particular the V4 strain is relatively thermostable being able to resist 56°C from 3 to 9 hours (Ideris *et al.*, 1987). Strain V4 has a heat stable haemagglutinin. The infectivity of strain V4 is relatively heat resistant even though this virus is apathogenic in chickens (Spradbrow, 1987). This heat resistance may be important in determining the stability of the virus in the STDB. In addition this virus grows extremely well in the allantoic cavity and the allantoic fluid used for this experiment had a titre of $10^{8.75}$ EID₅₀.

Storage of nucleic acid without a cold chain has been studied in different ways. Several researchers has been tried to store nucleic acid on filter papers (Panteleeff *et al.*, 1999; Devost and Choy, 2001; Vincek *et al.*, 2001). ND and AI viruses have also been collected on filter papers (Brugh *et al.*, 1980; Wambura, 2006), formalin fixed and paraffin-embedded (Wakamatsu *et al.*, 2007). However, collecting viruses on filter papers lacked sensitivity. This lack of sensitivity is a result of the small amount of virus that can be stored on filter paper even though viral nucleic acid was identified 30 days after collection (Perozo *et al.*, 2006). Other studies have indicated that collection of viral RNA onto filter papers can have a relatively poor clinical sensitivity.

The dilution of samples in lysis buffer provides an opportunity to the transport of a relatively large amount of sample in a format that is ready to be added to an extraction buffer and is compatible with the first stages of viral RNA extraction. Viable virus is completely inactivated making it suitable for importation into countries free of the disease. The stability of the samples guarantees that the inactivated RNA should not be degraded when these samples are transported without a cold chain.

Therefore, lysis buffer is highly recommended to be used as transport buffer for sample collection and transportation without a cold chain

6.7 Conclusion

Lysis buffer could be used to preserve RNA. Viable virus was completely inactivated and a relatively large sample could be safely moved without the need for a cold chain. These samples are compatible with the early stages of nucleic acid extraction making it a highly desirable protocol for transporting specimens in the absence of a cold chain.

CHAPTER 7

STORAGE OF EXTRACTED VIRAL RNA

7.1 Introduction

Extraction and purification of nucleic acid such as RNA is a common procedure in molecular laboratory to analyse the genome. This genome analysis can be used to diagnose diseases such as AI and ND. However, this extracted and purified RNA can be easily degraded and fragmented by conditions such as thawing and long term storage (Imamura, 2000; Thompson *et al.*, 2007).

Ribonucleic acid is more susceptible to degradation and fragmentation than is DNA as a result of ribonuclease from both exogenous and endogenous sources. Indeed to prevent this contamination, an effective method of RNA extraction is required. However, long term storage and usage of extracted RNA can be affected by the physical conditions such as temperature fluctuations in temperature as well as freeze thawing (Sambrook and Russel, 2001; Kudlicki *et al.*, 2007). Therefore, it is crucial to develop a system for RNA storage that is integrated with the extraction and purification of RNA.

7.2 Literature Review

The extraction of RNA in this project was based on the use of Guanidine isothiocyanate (Chirgwin *et al.*, 1979; Boom *et al.*, 1990; Chomczynski, 1993; Chomczynski and Mackey, 1995; Chomczynski and Sacchi, 2006). Guanidine isothiocyanate has been also widely used to extract and purify RNA from AIV and NDV (Seal, 1995; Seal *et al.*, 1998; Briggs *et al.*, 2003; Collins *et al.*, 2003; Wei *et al.*, 2006). However, there are limited studies devoted to the subsequent storage of the purified RNA.

Tris EDTA buffer has been used to elute purified RNA from the glass fibres (Boom *et al.*, 1990; Chomczynski and Sacchi, 2006; Santella, 2006) and TE buffer is also commonly used to store DNA or RNA. This buffer contains Tris (hydroxymethyl) aminomethane – hydrochloride (Tris-HCl) as a buffer and EDTA which is a strong chelating agent which chelates the Ca^{2+} , Mg^{2+} , and other divalent metal ions. The chelating action of EDTA is ensured by Tris-HCl to properly bind the divalent cations. Together with Tris-HCl, chelating ability of EDTA suppresses the ion and nuclease

activity causing RNA and DNA degradation processes (Maes *et al.*, 2004). However, the presence of EDTA potentially causes a problem in PCR reactions which require Mg^{2+} . Therefore, it may be necessary to evaluate and compare other buffers for routine storage of RNA.

FORMAZOL® is one possible RNA storage buffer. This buffer is formulated from purified and stabilized formamide. FORMAZOL® is claimed to be superior to other commercial formamide buffers. Moreover, this buffer can be used as an RNA solubiliser without additional purification for two years at 4°C or -20°C without the need to reduce the temperature to -70°C. It is claimed that FORMAZOL® protects the RNA from RNase degradation (<http://www.mrcgene.com/formazol.htm> (Mackey and Chomczynski, 1996)). However, the amplification yield of RNA stored in FORMAZOL® was poor. Even though RNA had not degraded, formamide in FORMAZOL® had an undesirable effect on the reverse transcriptase reaction. As a result, storing RNA in FORMAZOL® may adversely affect the PCR result (Swinson and Koban, 2005).

Another alternative is the RNA Safe Buffer. This buffer is formulated to contain Tween 20, Sodium azide, and carrier RNA. The RNA safe buffer has been used to conserve RNA used in interlaboratory standardisation tests. The stability of the RNA diluted in RNA safe buffer was confirmed by a freeze thawing experiment which resulted in no reduction in RNA titre after the RNA was subjected to freezing at -20°C and thawing 40 times. In addition RNA diluted in RNA safe buffers was stored at 37°C for up to 12 days with only moderate damage. This moderate damage was confirmed by a slight increase of Ct-Value in RT-qPCR (Hoffmann *et al.*, 2006).

In this project RNA safe buffer was evaluated and compared with TE buffer.

7.3 Aims

The specific aims of this project are as follows:

1. To develop methods for storage of extracted viral RNA to be intergraded with the RNA extraction protocol using samples collected and transported without a cold chain.
2. To carry out diagnostic assay from processed viral RNA.

7.4 Materials and Methods

7.4.1 Extracted Viral RNA

Extracted viral RNA of AI virus H4N4 and NDV V4 were used in this experiment. These viruses were described in Chapters 3.1.1 and 3.1.2. The H4N4 and V4 viruses were extracted using the standard procedure described in Chapter 3.6.1

Extracted viral RNA was diluted in Elution Buffer (TE buffer) (part no. C0241, lot no. 035K6044, Corbett Robotic, Australia)

7.4.2 Buffers

Two buffers i.e. TCB and mRSB were compared for the storage of viral RNAs of AIV and NDV.

TE ×100 concentrate (Fluka Biochemika 86377) was diluted to make ×1 concentrate TE Buffer pH approx. 8.0 at temperature 25°C. Then, 50 ng/μl carrier RNA was also added to make the TCB.

mRSB was constructed from RNA safe buffer (Hoffmann *et al.*, 2006). This mRSB contained 50 ng/μl carrier RNA, 0.05% Sodium Azide and 1% Tween 20 in RNase free water (mRSB) (Hoffmann *et al.*, 2006).

7.4.3 Experimental design

Experimental designs were carried out using the two evaluated buffers, the TCB and mRSB. In this study the RNA was divided into three groups namely undiluted RNA, a 10^{-1} dilution of RNA and a 10^{-3} dilution of RNA. Three groups of dilutions using two

different buffers were also divided into two experiments; freeze thawing and long storage experiments.

Extracted viral RNA was further diluted with TE Carrier RNA Buffer and MRSB. The extracted viral RNA was diluted at 10× and 1,000× dilution using these two buffers. The two diluted extracts of viral RNA were then stored at -20°C for 3 months and 9 months.

The diluted extracted viral RNA was treated in a freeze thawing experiment in 5× and 10× freeze thawing cycles at -20°C. Every treatment in thawing and times of storage used three replicates.

Then the RNA samples were checked using RT-qPCR

7.4.4 Real time (quantitative) RT-PCR

RT-qPCR assay was performed based on SYTO 9 RT-qPCR using a RotorGene 3000 (Corbett Research, Australia). This assay was carried out using NDV M primers (Wise *et al.*, 2004) for viral NDV V4 RNA and the AIV M primer set (Ward *et al.*, 2004) were utilised to identify viral AIV H4N4 RNA. Ct-values for the RT-qPCR were analysed statistically.

7.4.5 Data Analysis

Data of Ct-Value of RT-qPCR assay were analysed using the statistical program SPSS 14.0. The probability of Ct-value of treatments were compared and significance were set at a p-value of <0.05

7.5 Result

7.5.1 Dilution of extracted viral RNA's in two different buffers

Dilutions of extracted viral RNA in two different buffers have shown that viral RNA of NDV V4 can be identified in both TCB and mRSB.

The dilution of extracted viral RNA of NDV in TCB and mRSB were successfully detected using RT-qPCR. As expected the Ct values increased for each of the dilutions with the increase being approximately equal to 3 Ct values for every 10 fold dilution of the target RNA. Ct value for the 10⁻³ dilution was very similar to the no template control (Figure 7.1).

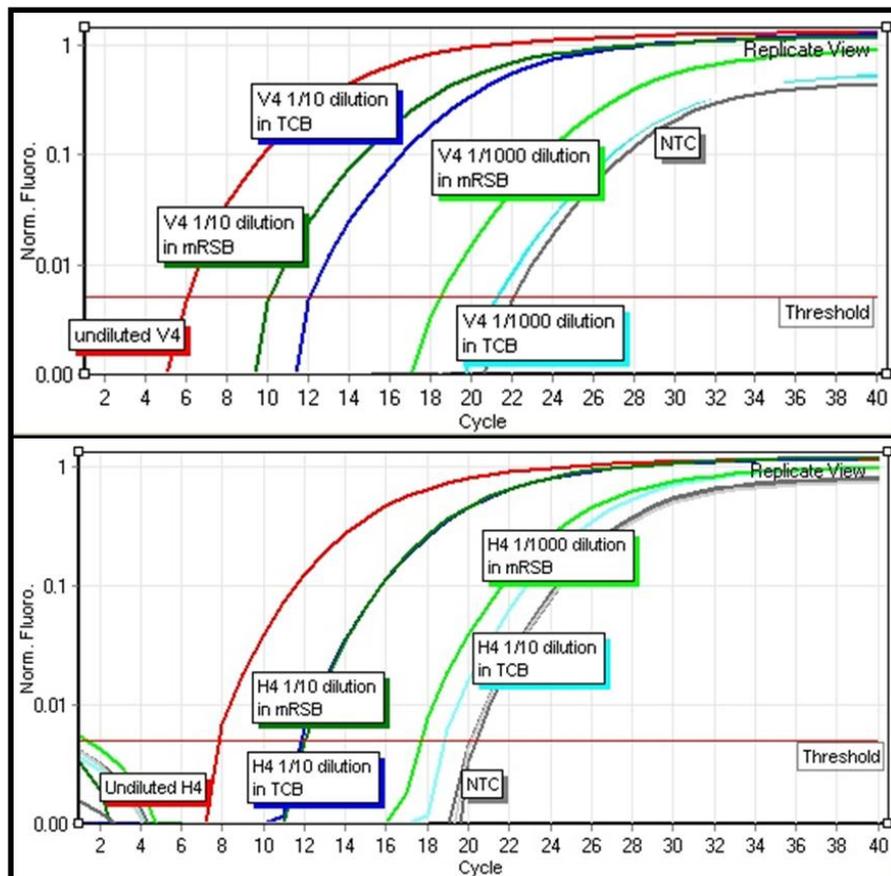


Figure 7.1 Quantitative analysis of V4 NDV and H4 AIV RNA stored in two different buffers

Amplification of viral RNA of AIV H4N4 in the two different buffers TCB and mRSB were also successfully generated whether it diluted in 10× dilution or 1000× dilution.

Melt curve analysis of PCR products generated from viral RNA diluted in TCB and mRSB showed that the amplified DNA of PCR products of viral RNA of NDV V4 and H4 AIV diluted in the two different buffers have similar melting points to the equivalent undiluted samples. It can also be seen on Figure 7.2 that viral NDV V4 RNA diluted 1000× in TCB had a similar melting point to the no template control (NTC).

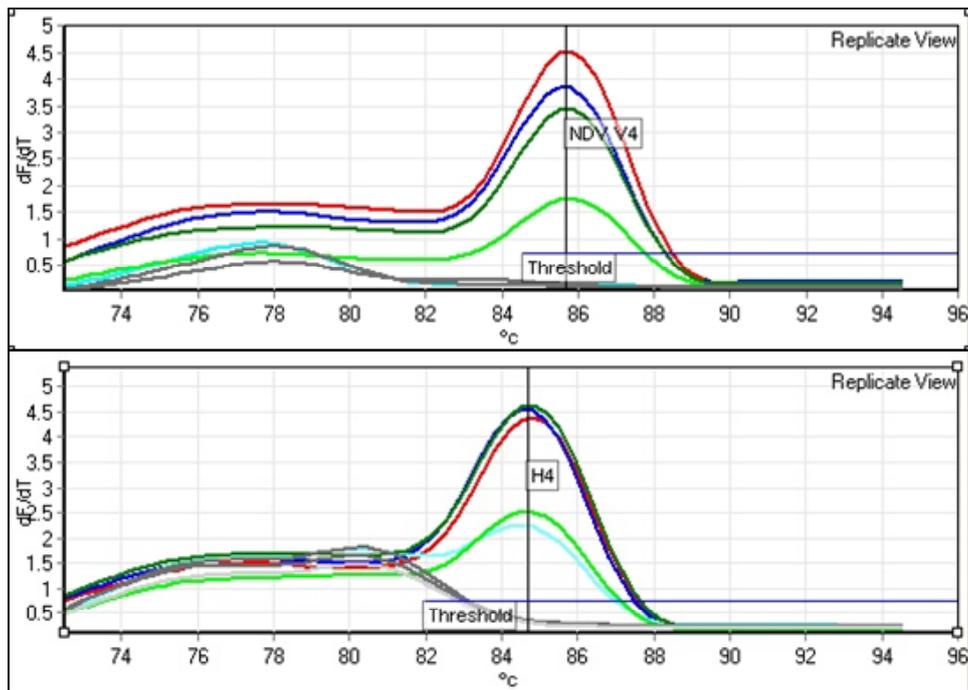


Figure 7.2 Melting curve profiles of undiluted and diluted NDV V4 and AIV H4 RNA in TCB and mRSB. The stored RNA in TCB had a similar melting point to the stored RNA in mRSB

Univariate ANOVA using SPSS 16.0 of different buffers with different dilutions based on the Ct-Value for the PCR products of V4 viral RNA showed no significant difference between buffers. However, the different dilutions had a significant difference. Also the interaction between buffers and dilutions were not significantly different compared with the interaction between buffers and interaction between dilutions.

However, even though the statistical analysis showed the insignificant difference, it can be seen from Figure 7.3 that preservation of diluted V4 viral RNA both of 10 \times and 1000 \times dilution using mRSB (Hoffmann *et al.*, 2006) were slightly better than using TCB. This can be seen in Figure 7.3 that the PCR products of diluted viral RNA in mRSB had a lower mean of Ct-Value's than PCR products of diluted viral RNA in TCB.

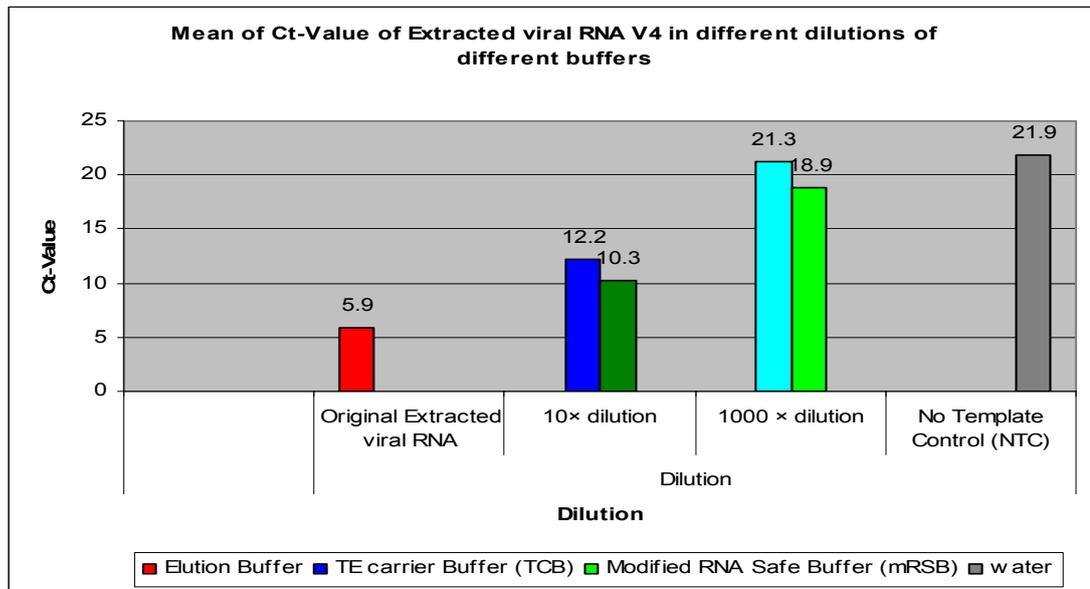


Figure 7.3 The mean of Ct-Values for the extracted V4 viral RNA samples diluted in two different buffers. The data were analysed using Univariate ANOVA with significance at a p-value < 0.05.

Analysis of Ct-Value of PCR products of AI viral RNA H4N4 did not demonstrate a significant difference between buffers. However, there was a significant difference between the original extracted viral RNA compared with the 10× and 1000× dilutions, negative control and NTC. Interaction between buffers and dilutions were also not significantly different.

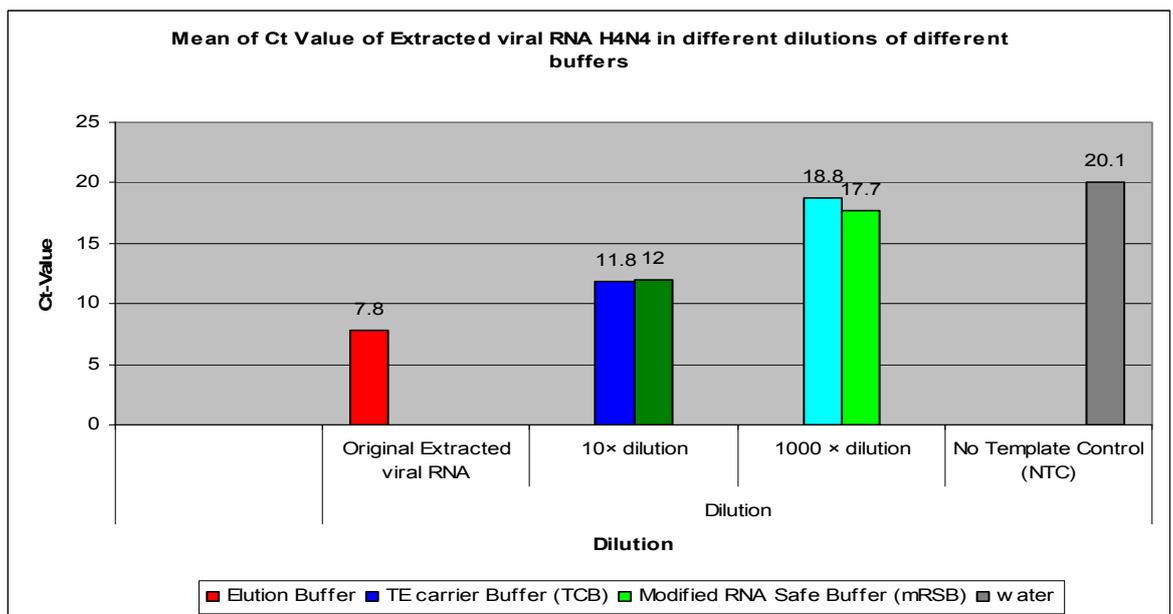


Figure 7.4 The mean of Ct-Values for the extracted AI viral RNA samples diluted in two different buffers analysed by Univariate ANOVA. Significances were set at a p-value < 0.05.

It can be concluded from the treatments in two different buffers and three different dilutions that there were no significant difference between the preservation of RNA in either of the two buffers.

7.5.2 Freeze-thawing experiment

Freeze thawing experiments with diluted viral RNA V4 in TE Carrier RNA Buffer and mRSB showed that extracted viral RNA was still detected after 5× and 10× freeze thawing at -20°C. This detection was confirmed by Ct-Value performed in SYTO 9 RT-qPCR.

It can be seen from Figure 7.5 that viral RNA has slightly better preserved in mRSB than it was in TCB. The quantitation analysis of PCR products of the extracted and diluted viral RNA V4 after 5× and 10 × freezes thawing at -20°C can be seen in Figure 7.5

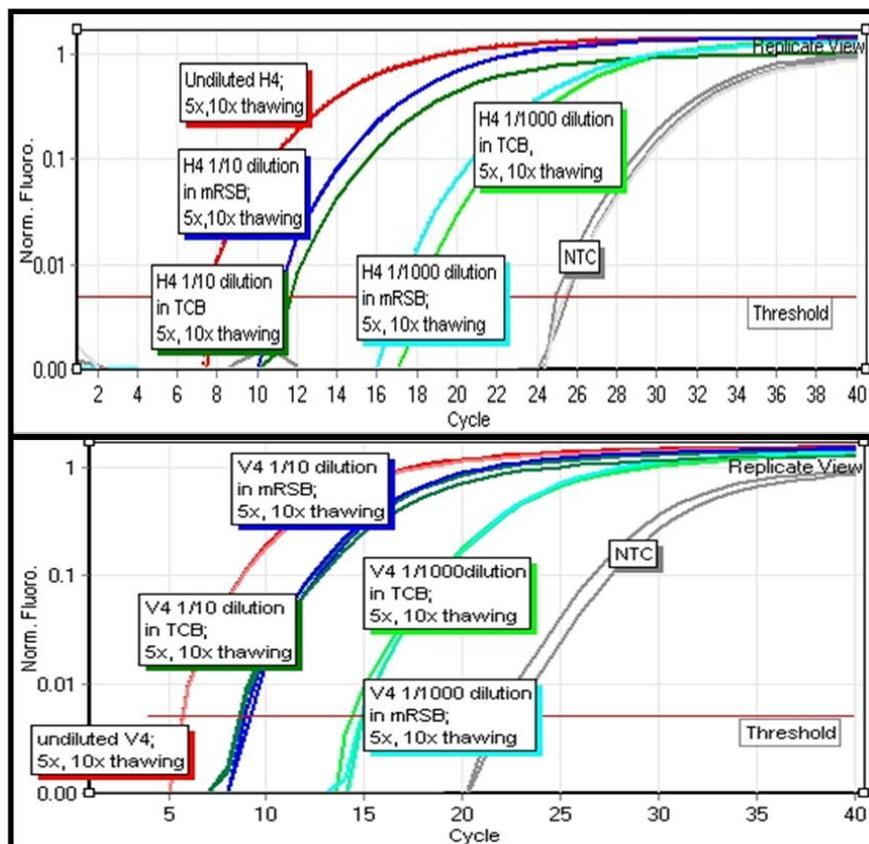


Figure 7.5 Quantitation analysis of diluted RNA of NDV V4 stored in two different buffers, TCB and mRSB with freeze thawing experiment. The Ct-Value were quantitated at the threshold 0.05

Similarly with extracted V4 viral RNA, extracted H4N4 viral RNA was still be able to be detected after 5× and 10× thawing experiments.

Univariate ANOVA showed that there was no significant difference between buffers. However, there was a significant difference within dilutions and between thawing times. Statistically analysis indicated that there was a significance difference between no thawing and 5 times thawing. However, no significance difference was demonstrated between 5 times and 10 times thawing.

The interaction between buffer and thawing and the interaction between thawing and dilution were not significantly difference. However, there was a significance difference in interaction between buffer, dilution and thawing.

It can be seen from the graph that extracted viral RNA preservation has been slightly better in mRSB than it was in TCB after 5× and 10× thawing. This better preservation can be seen from the lower Ct-Value observed in RT-qPCR of viral RNA V4 in mRSB than in TCB. The performance of Ct-Value can be seen in Figure 7.6.

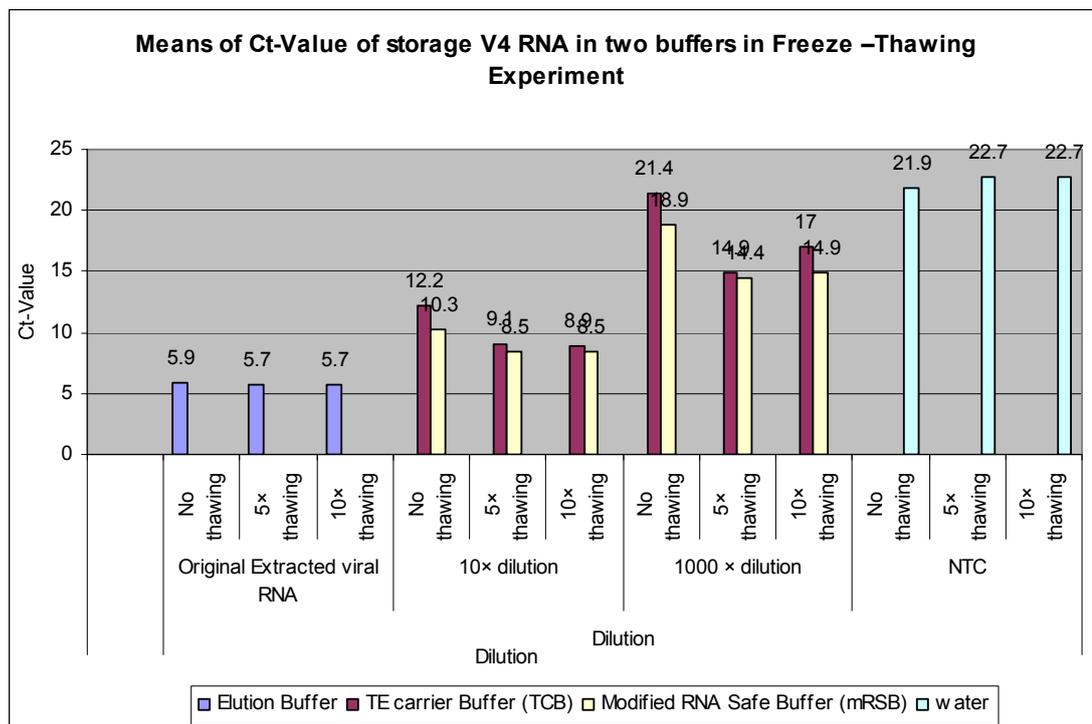


Figure 7.6 The mean of Ct-Value of thawing experiment of extracted V4 viral RNA in different buffers.

Similarly with viral NDV V4 RNA, statistical analysis using Univariate ANOVA showed that there was no significance difference between TCB and mRSB. However there is significant difference from different dilutions and different thawing.

The interaction between buffer and thawing or buffer and dilution was not significantly different. Also, the interaction between three factors; buffers, thawing and dilution were not significantly different.

The mean of Ct-Value can be seen on Figure 7.7

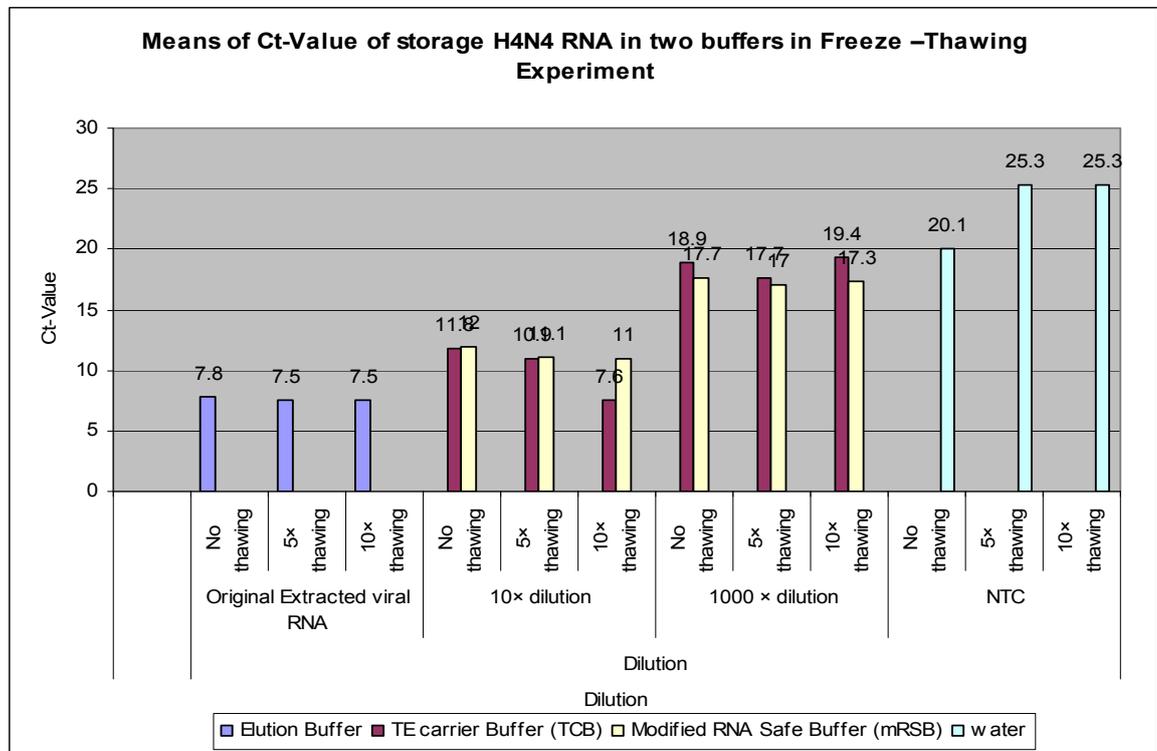


Figure 7.7 The mean of Ct-Value of thawing experiment of extracted H4N4 viral RNA in different buffers with different dilution. The data were analysed using Univariate ANOVA with significance at p-value < 0.05. Overall 5x and 10x freeze thawing at -20°C was not significantly affecting to the H4N4 viral RNA. Lower Ct -value means better fluorescence signal generated.

This experiment showed that a 5x and 10x freeze thawing at -20°C did not significantly affect the H4N4 viral RNA preserved in either buffer. Also, statistically analysis showed no significant difference between freeze thawing of H4N4 viral RNA either preserved in TCB or preserved in mRSB.

7.5.3 Long term storage experiment

Storage experiment of V4 viral RNA showed that the M gene of V4 viral RNA and AI H4 were still able to be detected after 3 months and 9 months storage at -20°C in TCB and mRSB. However, it can be seen from Figures 7.8 that the 10^{-3} dilution of V4 RNA in TCB was not preserved for the three and nine month storage periods.

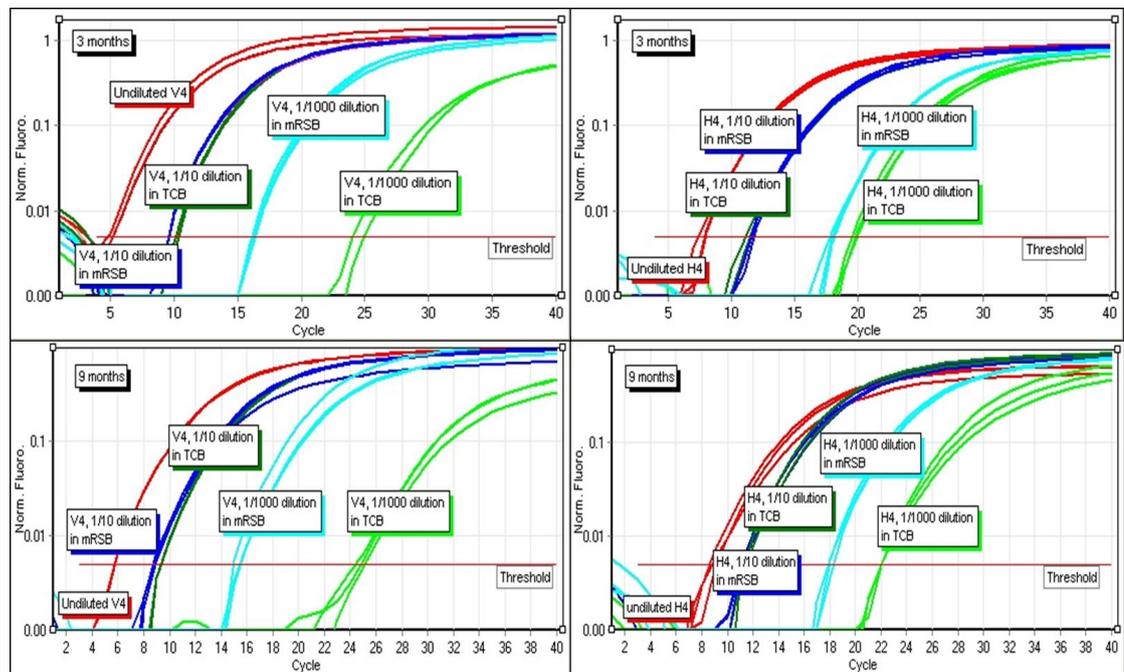


Figure 7.8 Quantitation Analysis of PCR Product of viral RNA V4 in 3 and 9 months storage at -20°C in different storage buffers, the TCB and modified mRSB. At threshold 0.05, the level of fluorescence of the RNA storage in mRSB is slightly better in $10\times$ dilution compared by in TCB. Also, in $1000\times$ dilution, storage in mRSB is much better than in TCB.

Statistically analysis of the Ct-values for V4 RNA was significantly different within buffers and within dilutions. However, different times of were not significantly different.

Interaction between buffer and times was not significantly different. However, interaction between buffer and dilution or between times and dilution were significantly different. The interaction of all of the treatment which is buffer, times and dilution was also significantly different (Figure 7.9).

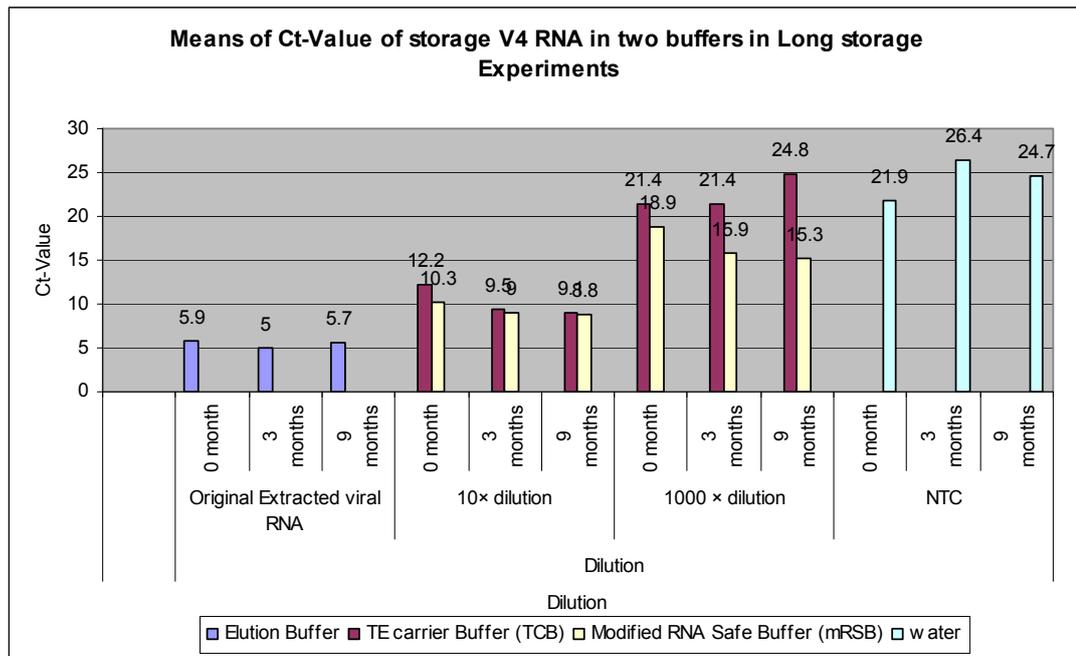


Figure 7.9 The mean of Ct-Value of extracted V4 RNA stored in different buffers with different dilution for 3 months and 9 months storage.

It can be seen from Figure 7.9 that V4 RNA was preserved in long term storage with both TCB and mRSB.

Statistically analysis to the Ct-Values determined that there was a significant difference within buffers, within dilution and within times. Univariate ANOVA demonstrated that elution buffer preserved H4N4 viral RNA significantly different from TCB or mRSB. Storage of H4N4 viral RNA for 3 months was also significantly different from storage for 9 months. Also, the interactions between buffer, times and dilution were also significantly different (Figure 7.10).

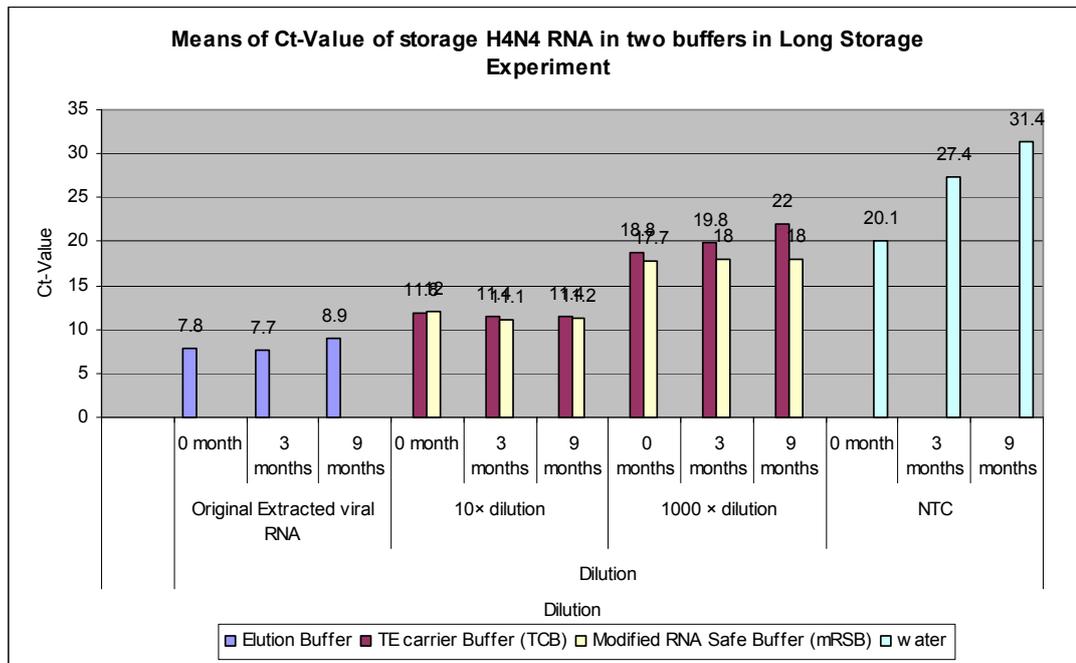


Figure 7.10 The mean of Ct-Value of storage extracted H4N4 viral RNA in different buffers with different dilution for 3 months and 9 months storage. The data were analysed using Univariate ANOVA with significance at p-value < 0.05. Preservation in mRSB is slightly better in the DNA amplification than in TCB even though in preservation either in mRSB or TCB were not affected significantly to the different of Ct-Value

7.6 Discussion

RNA may be stored frozen for a variety of purposes at temperatures of -20°C or -70°C . However, the RNA may degrade during the storage process.

Liquid crystallization is a physical factor during freezing condition causing the destruction of RNA. This liquid crystallisation results in the formation of ice crystals. The effects of the ice crystals are similar to dehydration due to sequestration of solvent water into the solid phase. This is more pronounced at temperatures below -10°C (Carpenter and Crowe, 1988; Karow, 1991; Arakawa *et al.*, 1993).

Another critical factor influencing the RNA degradation is endogenous and exogenous ribonuclease. This ribonuclease can be generated by careless, aseptic techniques and contamination of RNA during the extraction process (Chirgwin *et al.*, 1979; Sambrook and Russel, 2001; Maes *et al.*, 2004; Kudlicki *et al.*, 2007). Therefore, the appropriate choice of buffer can make a critical difference in the preservation of the RNA.

EDTA is a strong chelating agent which binds the divalent metal ions, magnesium and calcium. By binding calcium and magnesium as enzyme co-factors EDTA inhibits

ribonuclease and prevents the degradation of RNA and is commonly used in buffers for the dilution and preservation of RNA (Chomczynski and Sacchi, 1987; Boom *et al.*, 1990; Burgoyne, 1996; Maes *et al.*, 2004; Chomczynski and Sacchi, 2006; Santella, 2006). However, chelating calcium and magnesium can produce problems in the further PCR reactions as these divalent cations are important cofactors in the PCR.

Tween 20 is a non ionic detergent in the Tween family. The polyoxyethylene sorbitan esters of fatty acid of the Tween family behave as surfactants by replacing lipid bilayers in membrane proteins (Garavito and Ferguson-Miller, 2001; Arnold and Linke, 2007; Privé, 2007). Hence this detergent can be used as a component of the buffer. It is a mild detergent and is unlikely to interfere with the PCR reaction (Arnold and Linke, 2007).

Sodium azide is bacteriostatic and will block the action of a number of enzymes capable of degrading the RNA. In these experiments it would appear that the addition of sodium azide had minimal effect on the preservation of the RNA. It is likely to be of greatest assistance when there is a possibility of bacterial contamination of the samples. This is likely to occur in those samples that undergo multiple freeze thaw cycles as each step has the potential to introduce contaminating bacteria.

The results obtained in this study demonstrate that the RNA was protected from degradation by both buffers. The major difference between the two buffers is the addition of Tween 20 and sodium azide. If the RNA samples were contaminated with RNase enzymes prior to storage or if contamination with bacteria was introduced during the freeze thaw process is likely that the RNA safe buffer may have provided additional protection for the RNA.

7.7 Conclusion

The prevention RNA degradation in freeze thawing and long term storage for further use requires an appropriate storage buffer. Therefore in this study we proved that RNA can be stable despite freeze thawing and long term storage in either the original elution buffer or with either of the buffers evaluated.

It is speculated that contaminated samples would be more stable in mRSB as the sodium azide would inactivate contaminating bacteria.

CHAPTER 8

COLLECTION AND TRANSPORTATION OF SAMPLES FROM INDONESIA WITHOUT A COLD CHAIN

8.1 Introduction

Recent outbreaks of highly pathogenic AI virus in Indonesia have highlighted the importance of having appropriate diagnostics and suitable transport systems to allow responses to be based on up-to-date information on the distribution and prevalence of viruses capable of producing diseases in animals and humans. Transportation between laboratories in Indonesia and between Indonesia and overseas reference laboratories is vital for appropriate decision-making.

Sharing samples will be of benefit to Indonesia as it will provide information on the evolution of AI viruses (Fidler, 2008). However, some countries are reluctant to receive samples that could pose a threat of importation of highly pathogenic viruses. It is therefore vital that methods be developed for the safe transportation of nucleic acids that would allow the identification and sequencing of viruses without posing a quarantine threat to the importing country.

The traditional methods used to transport viruses such as AIV and NDVs or their RNA requires an extensive cold chain. It is vital that techniques be developed to overcome the need for the cold chain so that samples can be moved around Indonesia and delivered to reference laboratories overseas in a simple inexpensive way. It is important that samples can firstly be confirmed to be AIV or NDV and the RNA should be sufficiently intact for substantial amounts of sequence data to be obtained.

8.2 Literature Review

Highly contagious viruses such as AIV is of concern to a number of countries and specific conditions have been applied to the importation of material derived from this virus. The Agricultural Department of USA requires that samples be inactivated using chemicals such as phenol or formalin before being transported (Perozo *et al.*, 2006).

Phenol has been confirmed to inactivate viruses and preserve nucleic acid for amplification of RNA in RT-PCR (Jackwood *et al.*, 1996). Also Phenol precipitation has been used as a standard technique for viral inactivation and molecular characterisation (Purvis *et al.*, 2006). However, chemically inactivation of samples may interfere with the reaction and several additional steps may need to be taken to remove traces of phenol before PCR or RT-PCR can be carried out. Failure to do so may result in misdiagnosis (Purvis *et al.*, 2006).

Formalin has been also used as a fixative to allow the transport of samples. Formalin fixed – paraffin embedded tissue are frequently used as histological specimens (Masuda *et al.*, 1999; Lehmann and Kreipe, 2001; Wakamatsu *et al.*, 2007). However, nucleic acid may be degraded and the processing of the fixed tissue may present some problems (Coombs *et al.*, 1999).

Moreover formalin fixed tissue is resistant to some solubilising agents such as Proteinase K and chaotropic agents (Masuda *et al.*, 1999). The complicated processing restricts the use of formalin fixed tissues.

Beta-Propiolactone and Binary Ethylenediamine can be used to inactivate lipid and non-lipid coated viruses. However these chemicals are hazardous and while they have no effect on viral proteins they inactivate nucleic acids (King, 1991; Refaie *et al.*, 2004). Therefore these samples are unlikely to be useful in molecular diagnostics.

Filter paper collection and transport systems are compatible with processing of nucleic acid (Perozo *et al.*, 2006). However, without suitable additional treatment the nucleic acid will degrade rapidly.

The lysis buffer described in Chapter 6 is a promising technique for transporting samples throughout Indonesia and to reference laboratories. The nucleic acid should remain stable and the viruses will be totally inactivated. The commercial lysis buffer containing guanidinium isothiocyanate and dithiothreitol will precipitate all proteins and inactivate both viruses and bacteria (Alberti and Fornaro, 1990; Boom *et al.*, 1990; Boom *et al.*, 1991). This guanidinium viral inactivation is compatible with the further processing of the samples for diagnosis and sequencing.

Viruses such as influenza A continue to evolve and the decision to withhold live viruses from collaborating countries reduces the information available on the evolution of these viruses (Fidler, 2008). Transport of inactivated viral RNA may overcome a number of the objections to this process and supply vital information.

The simple application of melt curve analysis may allow outliers to be recognised and these will be suitable candidates to be given priority for sequencing (White and Potts, 2006; Gundry *et al.*, 2008).

8.3 Aims

The specific aims of this project are as follows:

1. To apply the newly developed methods for collecting and transporting RNA viruses without the need of a cold chain assays to reference samples and samples collected from infected birds in Indonesia.
2. To collect samples from outbreaks of AI or ND in Indonesia and suspend their viral RNA in a suitable buffer that will allow transport without the need for a cold chain.
3. To apply a new standardised set of diagnostics tests based on real-time PCR for the recognition of AIV and NDV in samples collected from infected birds in Indonesia.
4. To demonstrate that RNA fragmentation is not a problem in transported samples from Indonesia.
5. To characterise and pathotype NDV and AIV samples transported from Indonesia.

8.4 Materials and Methods

8.4.1 Samples

Samples in this study were collected in Indonesia and processed in the Centre of Standard Testing of Agricultural Quarantine Agency of Indonesia. Then, the samples were transported to JCU.

Samples from Indonesia included AI viruses and ND viral antigens. AI viruses were kindly provided by the Agricultural Quarantine Agency of Indonesia (AQAI). These viruses were collected from Ambon and Timika outbreaks and propagated in the Centre of Standard Testing of AQAI as their stock viruses. Other samples were derived from organs which were kindly donated by Sudirman, DVM, PT. BioTek Indonesia –Jakarta. These organs were collected from dead chickens suspected of being infected with H5N1 AI viruses and they were propagated in 10 day embryonated SPF eggs. However, to maintain confidentiality the source of the chickens was not divulged.

Samples for NDV RNA were purchased from Balitvet and Pusvetma. These samples were NDV antigens which had been treated with β -mercaptoethanol.

Both of AI viruses and NDV antigens were processed using the modified Corbett lysis buffer and transported without a cold chain.

8.4.1 GenBank sequences

AI and ND viral isolates in this study were compared to the references sequenced accessed from GenBank databases. The accession number of the reference sequences can be seen on the figure of the phylogenetic tree.

8.4.2 Permit certificates

A full description of the samples was prepared on letterhead and signed by the responsible scientist. An appropriate import permit was issued by the Australian Quarantine and Inspection Service (Permit No IP7021860) while Exportation permits were issued by the Directorate General Livestock Service (Certificate No. 632/PD.630/F.5/02/08) and Agricultural Quarantine Agency of Indonesia (Quarantine Certificate No. 0002595 M/E/110100/20080218/002268)

Furthermore, the transportation of the samples was facilitated by a Material Transfer Agreement between the Agricultural Quarantine Agency of Indonesia and the School of Veterinary and Biomedical Sciences JCU.

8.4.3 Buffer

The commercial lysis buffer (part no.C0616, lot no 035K6045) containing guanidium isothiocyanate and dithiothreitol (LB Additive, part no Q3633) was used to ensure that the samples being imported were free from infectious organisms.

8.4.4 Protocol of samples collection in transport buffer

The viral suspensions were initially processed in Indonesia by suspending at a 1:2 ratio of virus to lysis buffer making a 33% suspension of virus in buffer.

A total volume of 500 µl of viral suspension was added to 1,000 µl of LB and the tube inverted to thoroughly mix the contents. Then, the entire volume of the tube was transferred to a second tube that had an O-ring seal to ensure that there was no untreated viral suspension in the screw cap. The outside of tube was sterilised by wiping with 70% alcohol and then the tube was sealed using parafilm. Afterwards, the tubes were fully labelled and transferred to a second container that conforms to IATA packaging instructions 650.

The inactivation of virus was also confirmed by inoculation into embryonated SPF eggs in Indonesia prior to being transported to JCU in Australia.

8.4.5 RNA extraction

A Corbett X-tractor Gene automated RNA/DNA Extraction system (Corbett Robotic, Brisbane, Australia) was used to extract viral RNA. The method of extraction was based on the nucleic extraction protocol from Corbett Robotics (Prototype kits) or Sigma chemicals catalogue Number XTRV as described in Chapter 3.6.1

8.4.6 Real time (quantitative) PCR

qPCR assay was performed to demonstrate viral RNA as described in Chapter 4 using primers which have been described in Table 3.3 Chapter 3.6.2. The primers can be seen in Table 8.1

Table 8.1. Primers for diagnosing AIV and NDV in samples collected and transported from Indonesia.

Primer Name	Primer Sequence (5'-3')	Target sequence	Product length
Wise-NDVM /M+ 4100 (Forward)	AGTGATGTGCTCGGACCTTC	NDV Class II Matrix	120 bp
Wise-NDVM / M- 4220 (Reverse)	CCTGAGGAGAGGCATTTGCTA		
IVA-D161M (Forward)	AGATGAGYCTTCTAACCAGAGGTCG	AI Matrix	101 bp
IVA-D162M (Reverse)	TGCAAANACATCYTCAAGTCTCTG		
IVA-Ma (FAM-Probe)	FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1		
Ward MF	AAGACCAATCCTGTACCTCTGA	AI Matrix	95 bp
Ward MR	CAAAGCGTCTACGCTGCAGTCC		
IVA-D148H5-F	AAACAGAGAGGAAATAAGTGGAGTAAAAAT	AI H5 short length	121 bp
IVA-D148H5-R	AAAGATAGACCAGCTACCATGATTGC		
IVA-H5a (Probe)	CalFluor Red-CAACAGTGGCGAGTTCCTAGCA-BHQ2		

To ensure the pathogenicity of Indonesian AI isolates, another pair of primers was performed (Payungporn *et al.*, 2006b). This pair of primers (H5F3+ and H5R2+) were used to discriminate the low and highly pathogenic of H5 AI viruses.

Full information of the primers and probes was outlined in Chapter 3.6.2

8.4.7 Reverse transcriptase PCR and purification of PCR products

Conventional RT-PCR was generated using a One Step RT-PCR kit (Qiagen Catalogue No. 210210). The amplification conditions are as outlined in Chapter 3.6.3. Then, the PCR products were examined by agarose gel electrophoresis utilising 2.5% w/v

Agarose Standard low EEO Applichem (CAS No. 9021-36-6) as described in Chapter 3.6.4.

Primers were used for sequencing in this study were designed using AlleleID (Premier Biosoft). The primers can be seen in Table 8.2

Table 8.2. Primers for sequencing NDV and H5 AIV

Primer Name	Primer Sequence (5'-3')	Target sequence	Product length
H5F-GB590	TATAGAGGGAGGATGGCAGGGAATG	H5	590 bp
H5R-GB590	GACCAGCTATCATGATTGCCAGTGC		
H5F-GB1307	CTGGAAAAGACACACAACGGGAAGC	H5	1307 bp
H5R-GB1307	AAACAACCGTTACCCAGCTCCTTTG		
NDV-F343-F	5'-CCCAAGGATAAAGAGGCGTCTGC-3'	Fusion	343 bp
NDV-F343-R	5'-GCTGCATCTTCCCAACTGCCAC-3'		343 bp

Purification of PCR products were performed using Real GenomicsTM (Real Biotech Corporation) kit, HiYieldTM Gel/PCR DNA Extraction kits (catalogue no. YDF100) as described in Chapter 3.6.5

8.4.8 Sequencing

Purified PCR products were sent to Macrogen, South Korea to attain the targeted sequences as described in Chapter 3.6.7.

ContiqExpress, Vector NTI Advanced 10 (Invitrogen), or Sequencher version 4.7 (Gene Codes corporation, USA) were used to assemble the sequences. Then, comparison between the sequences to the Gen Bank database was carried out using NCBI BLAST. Sequence alignment was carried out using either Vector NTI or GeneDoc using the Clustal algorithms. Phylogenetic trees were drawn using Mega 5.

8.5 Results

8.5.1. Screening of ND and AI viral RNA

AIV and NDV materials were successfully processed using commercial lysis buffer and transported without a cold chain.

Detection of AI viral RNA was successfully performed in RT-qPCR assay based on SYTO 9 using AI M primer (Ward *et al.*, 2004). The quantitation analysis of AI viral RNA can be seen in Figure 8.1

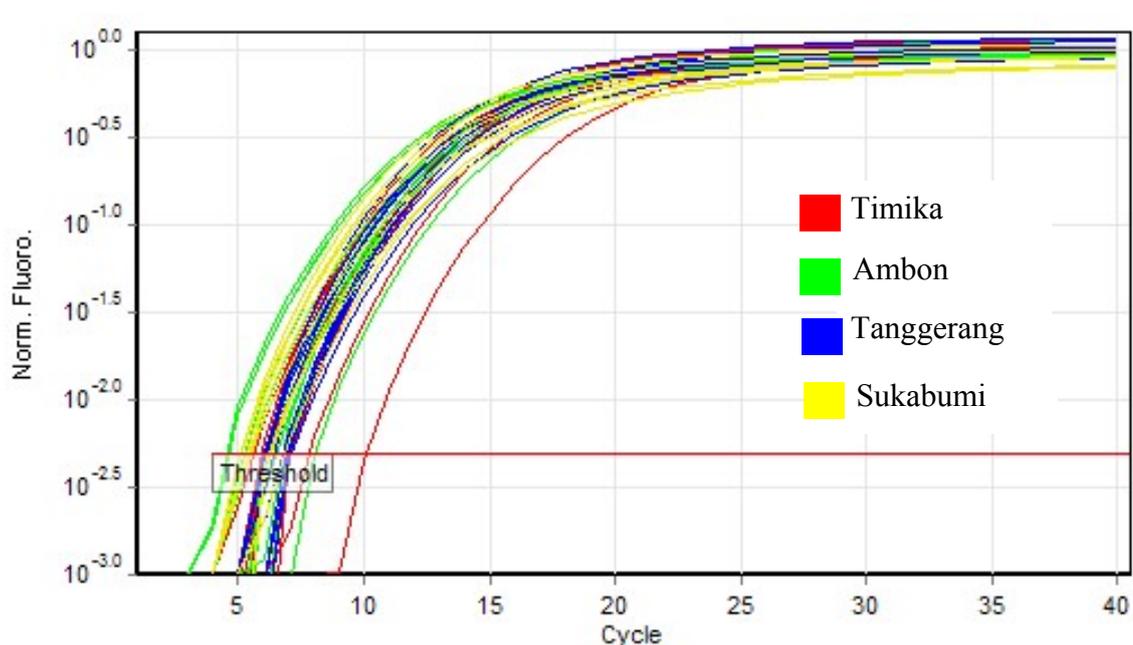


Figure 8.1 Quantitation analysis of AI viral RNA performed in SYTO 9 RT-qPCR assay using AI M Primers.

Melt curve analysis of the M gene of the AI viruses isolates shows the different melting points between the four different clusters of isolates namely Timika, Ambon, Tangerang and Sukabumi. Members of each of the groups of viruses had similar melt temperatures and there were consistent differences between the four groups of viruses (Figure 8.2).

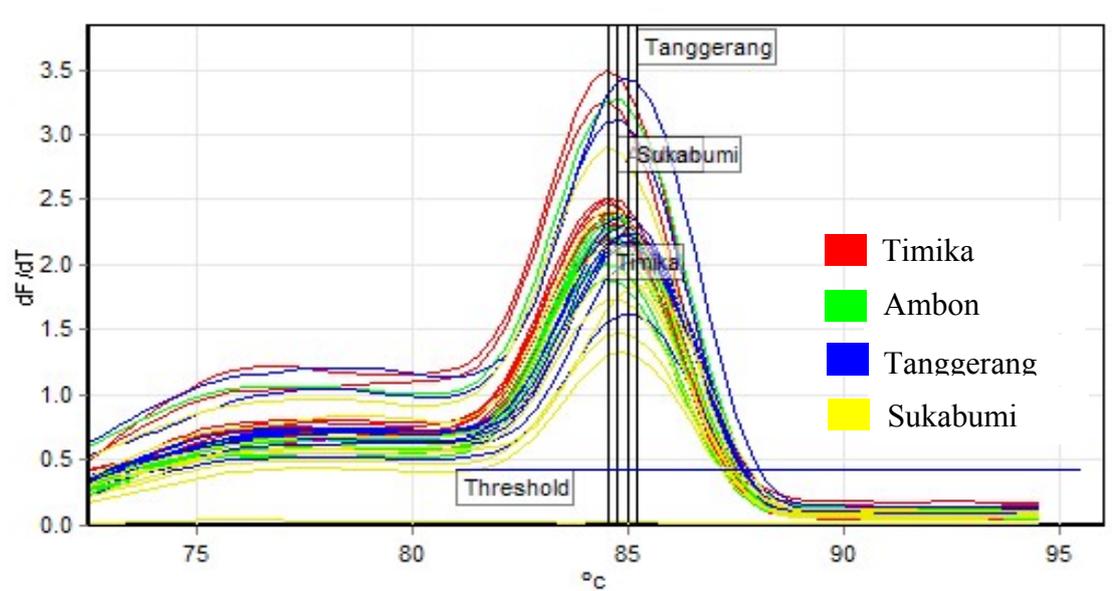


Figure 8.2. Melting Curve profile of SYTO 9 RT-qPCR of AI viral RNA's using AI M primers

Amplification of the AIV samples using the AIV M TaqMan IV dual labelled Probe assay (Heine *et al.*, 2005) indicates that all four groups of isolates were successfully detected (Figure 8.3)

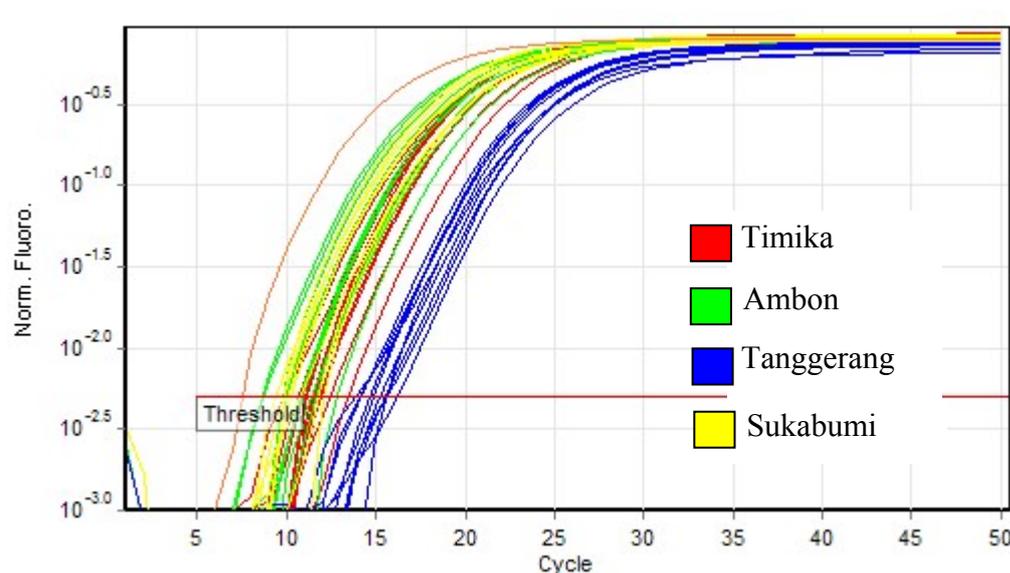


Figure 8.3 Quantitative analysis based on AI M TaqMan IV dual labelled Probe RT-qPCR assay. Ct-values were performed at 0.005

An AI H5 specific TaqMan assay was performed using IVA-D148H5 reagents (Heine *et al.*, 2005). Three groups of isolates (Timika, Ambon and Tangerang) reacted in this assay. However, this assay failed to detect the Sukabumi isolates (Figures 8.4 and 8.5).

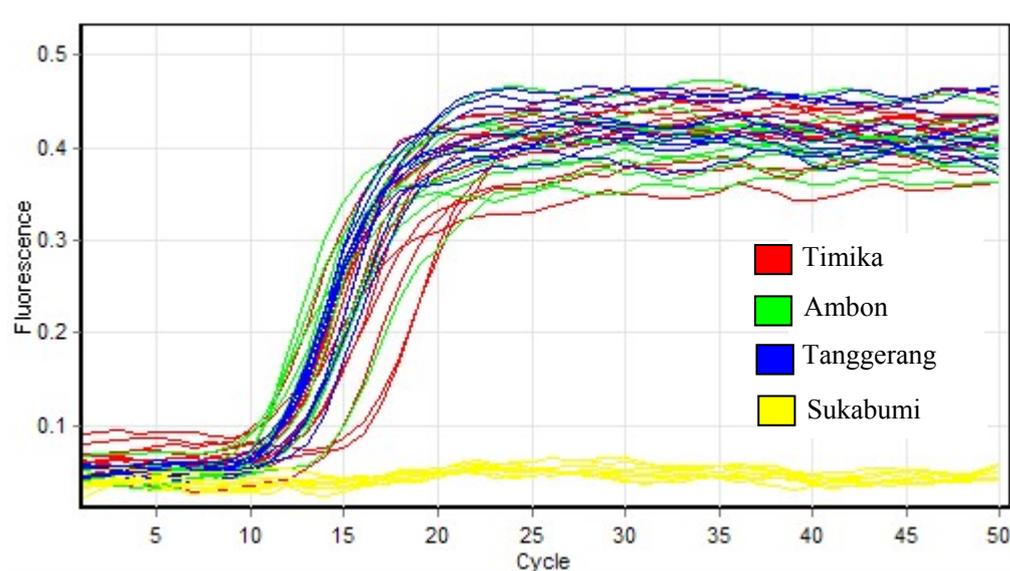


Figure 8.4 Amplification profiles of RT-qPCR four different batches of AI viral isolates from Indonesia using the IVA-Subtype H5 TaqMan dual labelled probe assay

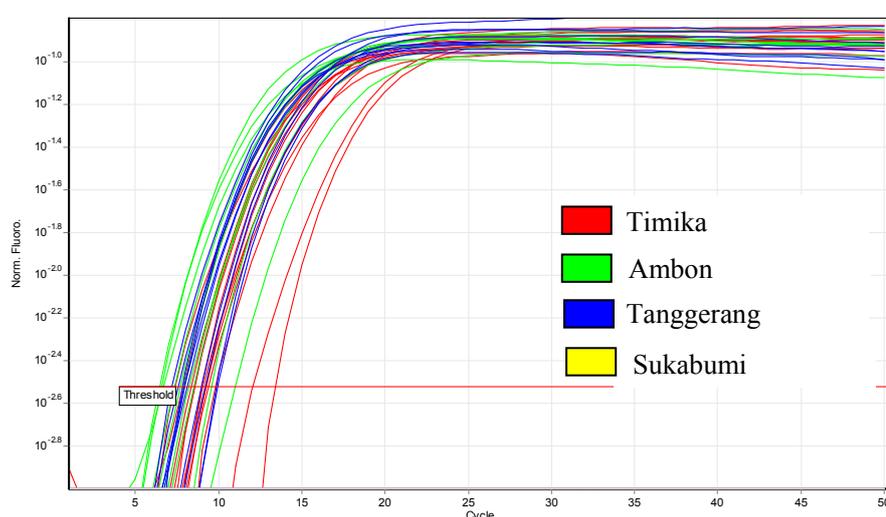


Figure 8.5 Quantitation analyses of RT-qPCR four different AI viral isolates from Indonesia using IVA-Subtype H5 TaqMan dual labelled probe assay (Heine *et al.*, 2005)

As the Sukabumi isolates failed to react in the TaqMan they were then tested using the corresponding primers and the interchelating dye SYTO9. All four groups of isolates were amplified in this assay (Figure 8.6).

These assays indicated that all four groups of viruses were AIV H5. There were differences in M sequence and the Sukabumi isolates were sufficiently different that they did not bind the H5 probe used for routine identification of H5 isolates in Australia (Heine *et al.*, 2005).

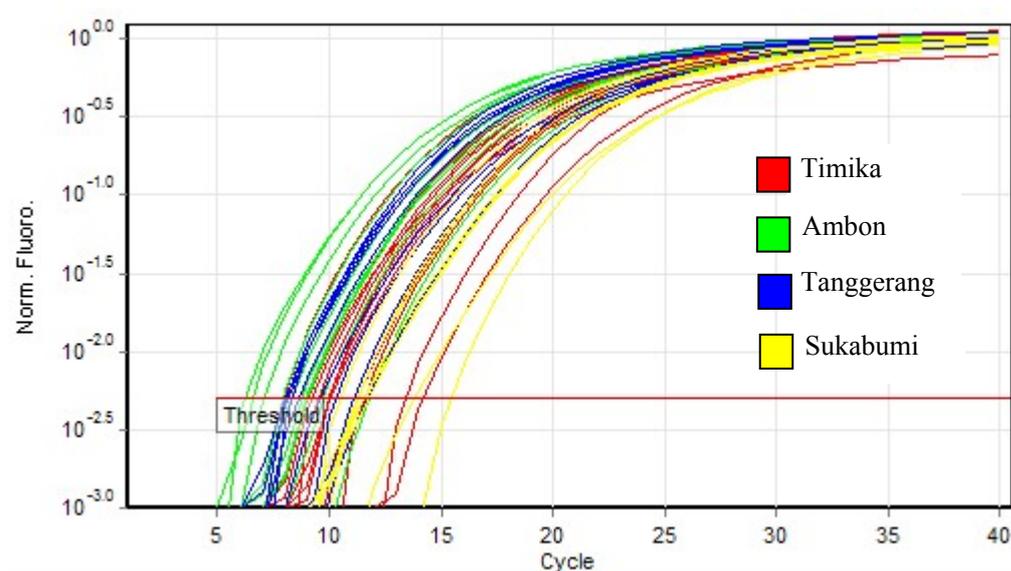


Figure 8.6 RT-qPCR assay based on SYTO 9 to the four groups of AI isolates from Indonesia. RT-qPCR were generated using H5 primers (Heine *et al.*, 2005)

Melt Curve analysis of the four different groups of AI viruses suggests that there may be some differences in sequence. When a high resolution melt curve analysis was carried out three different clusters of profiles were recognised representing Sukabumi, Tangerang, with Ambon or Timika having a similar profile. There appeared to be some variation in the profiles generated by the different groups. Up to three different profiles were detected in the Sukabumi group (Figure 8.7). This suggests that there may be some heterogeneity in the sequences of the H gene of these four groups of viruses.

However, the difference was better performed by running the PCR product in High Resolution Melt (HRM) assay in Rotor-GeneTM 6000. It can be seen from the graph that there were three different genotypes based on different melting point performed by Sukabumi isolates. The different genotypes were also shown by Tangerang isolates. However, Ambon isolates had two different melting points while melting point of Timika isolates was homologous.

This melting point analysis can be seen on Figure 8.7

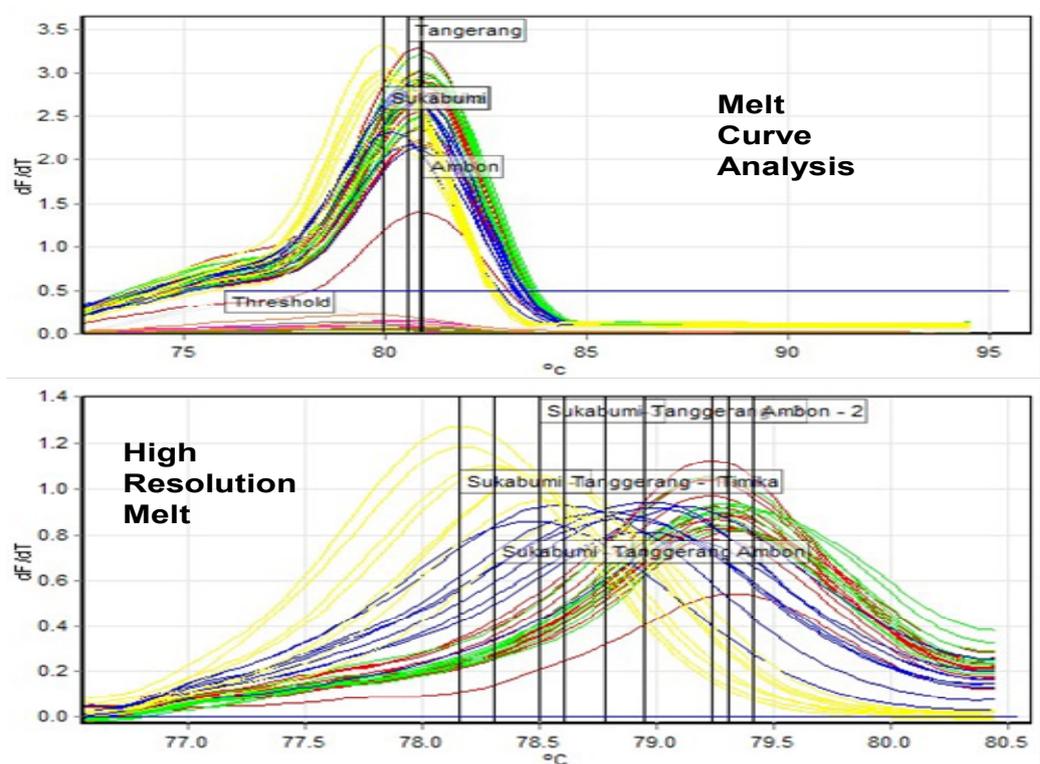


Figure 8.7 Melting curve profile of different locations processed isolates using Melting point analysis and HRM Analysis. The analysis was performed on Rotor-Gene™ 3000 and 6000 (Corbett Research, Australia)

Normalisation data of HRM has suggested three different genotypes. The profiles of the Timika and Ambon isolates were similar while the profiles for the Tangerang and Sukabumi isolates were clearly different. The most divergent were the Sukabumi isolates (Figures 8.8 and 8.9).

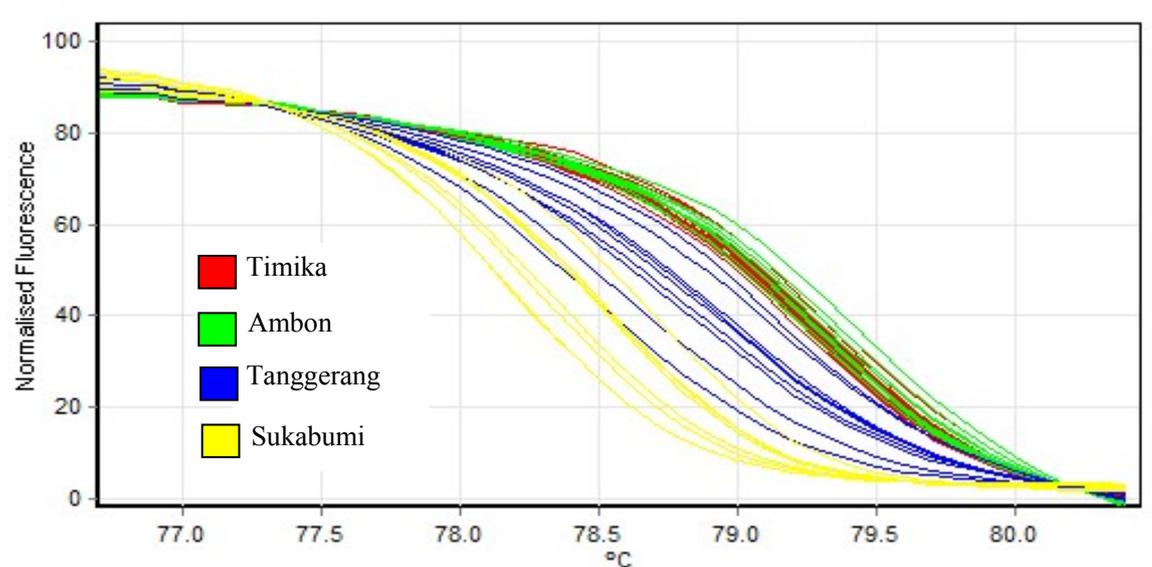


Figure 8.8 Normalised Melt Curve of different isolates performed by HRM Analysis assay. The assay was generated on Rotor-Gene 6000 (Corbett Research, Australia) using IVA-D148H5 primer

To view the difference plot, a Timika isolate was defined as a representative genotype. This confirms that the Timika and Ambon isolates had similar profiles while the Tangerang and Sukabumi isolates clearly stood out as being divergent (Figure 8.9).

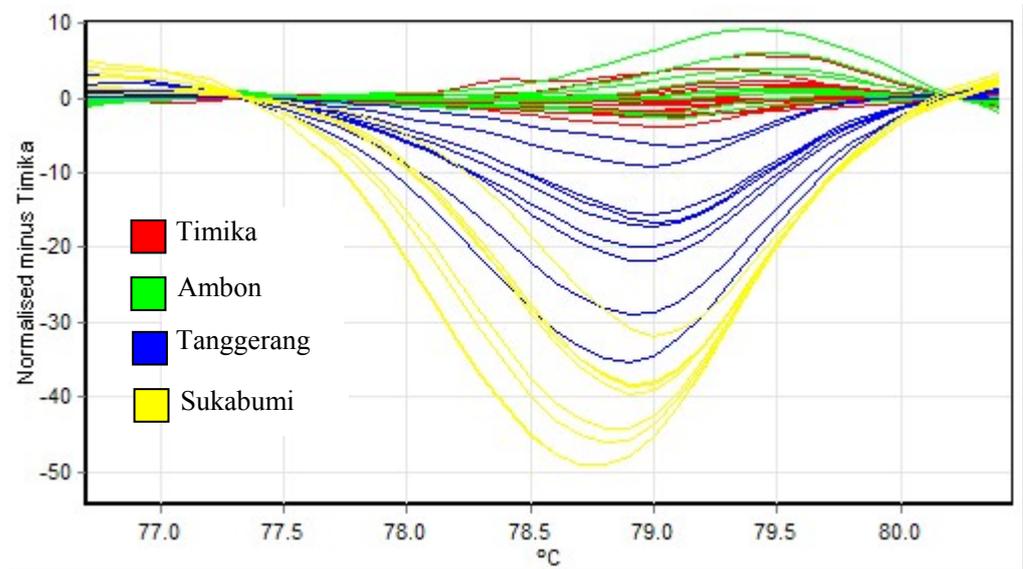


Figure 8.9 Genotype comparisons of four Indonesian AI viral isolates using Difference graph analysis on HRM Assay with isolates from Timika being the reference.

A real time RT-PCR assay based on SYTO 9 using H5F3+ and H5R2+ primers (Payungporn *et al.*, 2006b) also successfully detected AI H5 in four isolates from Indonesia. The detection performance can be seen in Figure 8.10

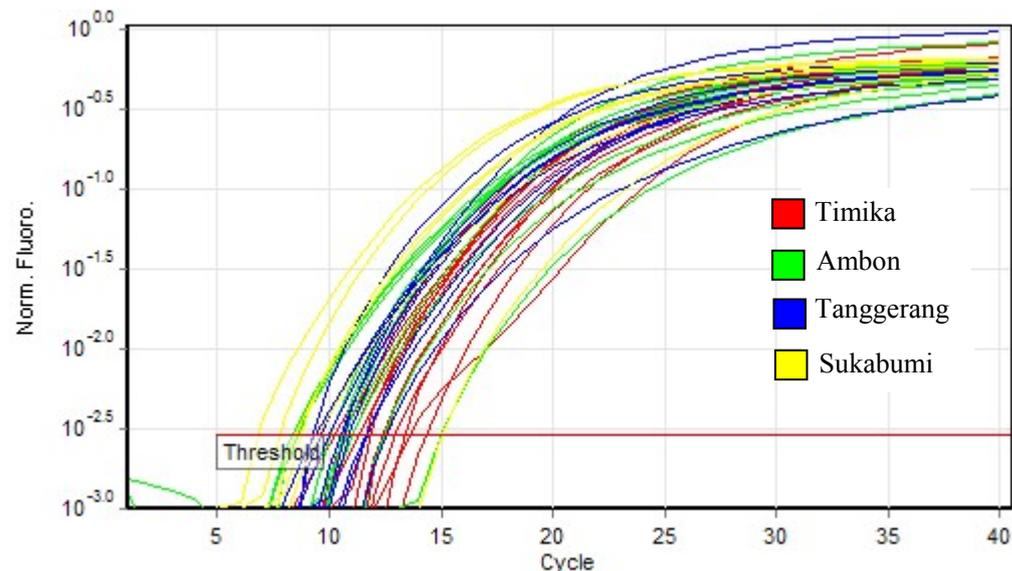


Figure 8.10 Fluorescence data of SYTO 9 RT-qPCR assay of AI viral isolates using H5F3+ and H5R2+ primers (Payungporn *et al.*, 2006b)

Analysis of melting curve was shown that there was a difference in melting point for isolates from all four different locations of Indonesian. These differences are outlined in Figure 8.11. The high resolution melt curves suggest that while there is significant sequence variation even within the groups of isolates there was further variation.

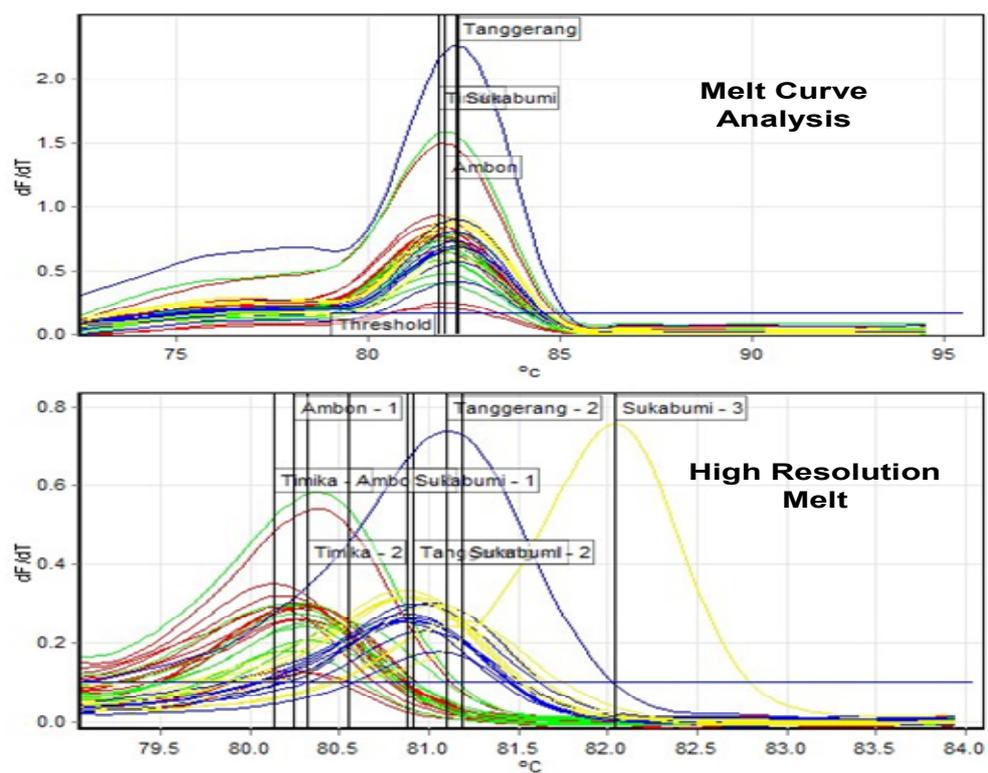


Figure 8.11 Melting Curve Analysis and HRM of the recent Indonesian AI isolates performed by SYTO 9 RT-qPCR assay using H5F3+ and H5R2+ primers (Payungporn *et al.*, 2006b)

Normalisation fluorescence data of HRM assay clearly demonstrates that the similarities in the Timika and Ambon isolates are similar to that seen with the previous set of primers. The Tangerang and Sukabumi isolates clearly diverge with some variation within the Sukabumi isolates (Figures 8.12 and 8.13).

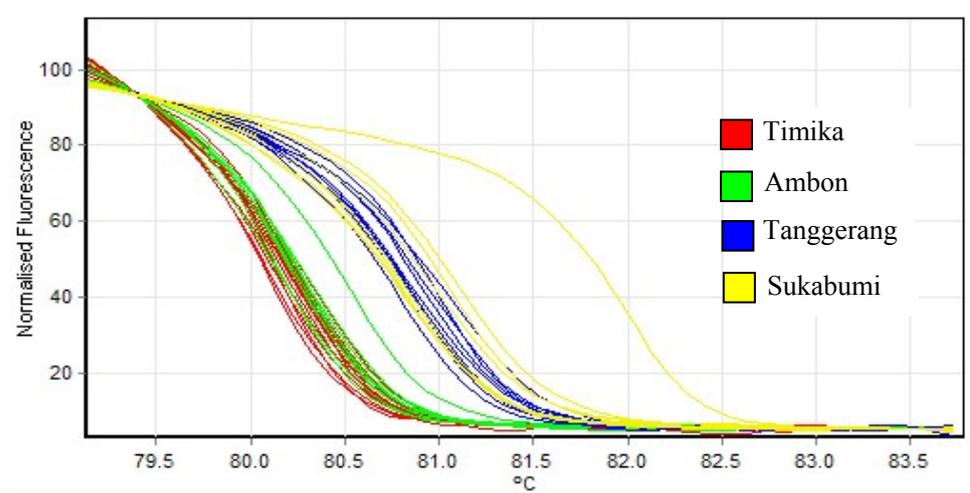


Figure 8.12 Melt plots of normalised graphs of Indonesian AI isolates performed by HRM Assay using H5F3+ and H5R2+ primers

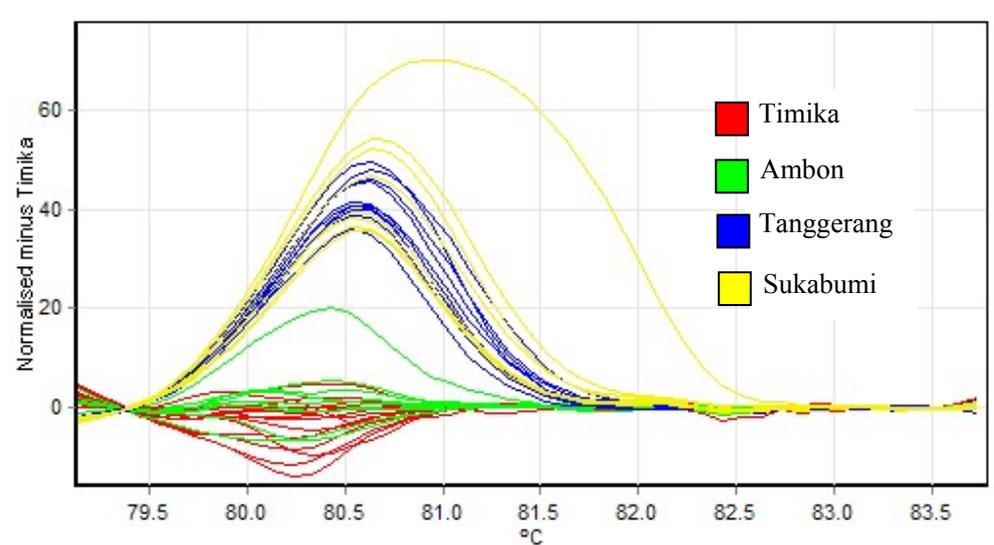


Figure 8.13 Genotype comparisons of four Indonesian AI viral isolates performed by Difference graph analysis on HRM Assay using H5F3+ and H5R2+ primers with a Timika isolate as the reference sequence.

Detection of processed NDV antigen using duplex primer in RT-qPCR based on SYTO 9 showed that the commercial lysis buffer not only preserved viral RNA from processed live viruses but also from viruses inactivated with beta propiolactone. The results of detection curves and melt curves for the ND RNA samples are shown in Figures 8.14 and 8.15

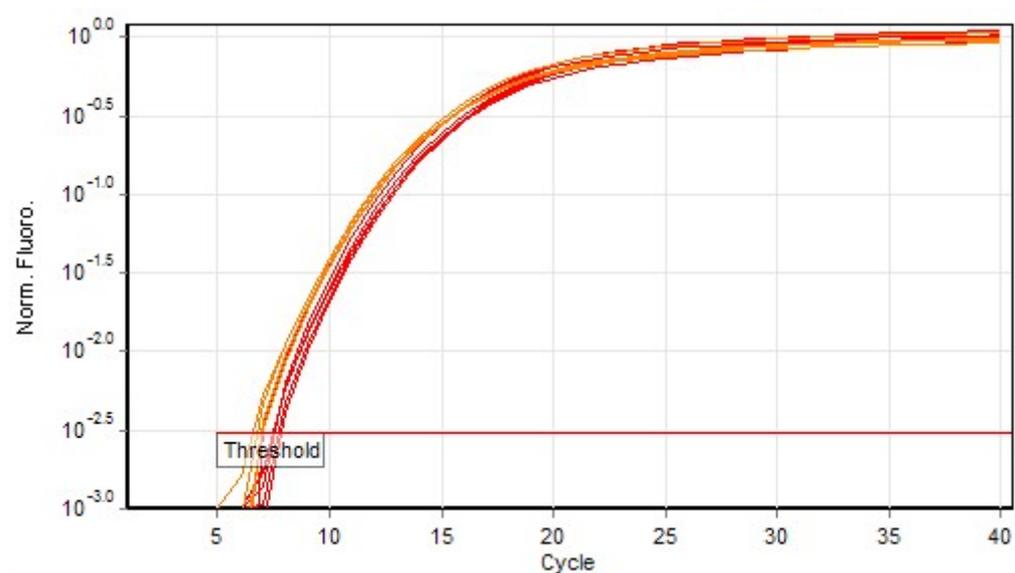


Figure 8.14. Quantification curves of NDV antigen transported from Indonesia in commercial lysis buffer. The assay was performed using RT-qPCR assay based on SYTO 9 and the duplex primers

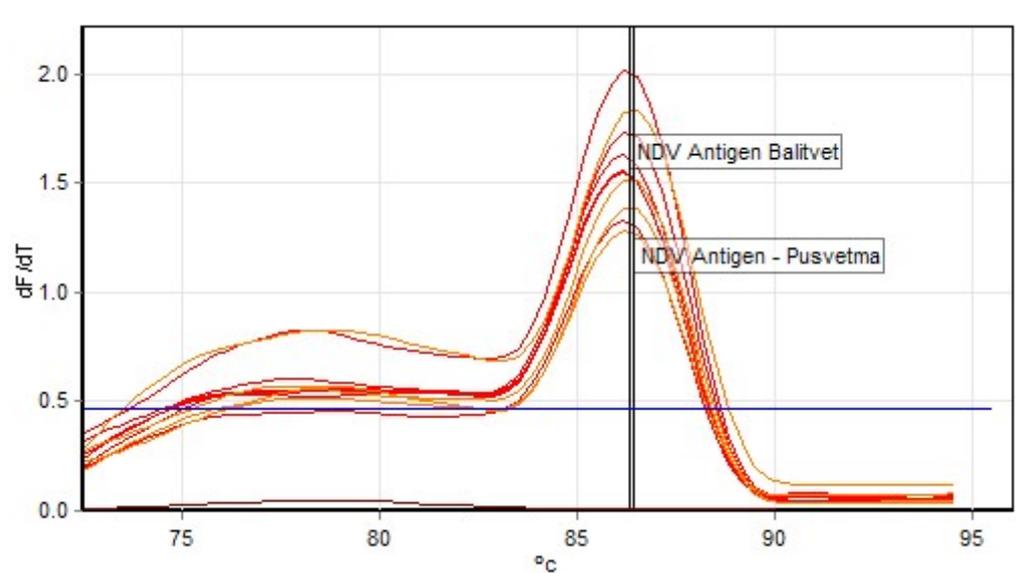


Figure 8.15 Melt curves of ND antigen transported from Indonesia in commercial lysis buffer. The assay was performed using RT-qPCR assay based on SYTO-9 and the duplex primers

8.5.2. Sequencing of Newcastle Disease and AI viral RNA

Amplification of NDV cDNA was successfully carried out using the templates from Indonesia and selected Australian isolates. A 343 bp product from the fusion gene was amplified using NDV-F343 forward and reverse primers (Figure 8.16)

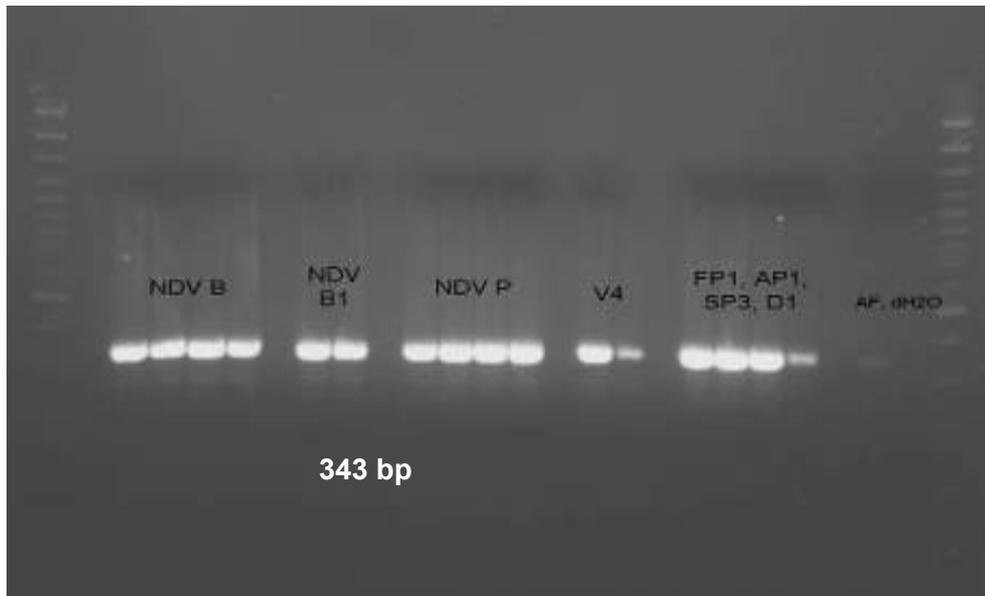


Figure 8.16 Amplification of a 343 bp product of the fusion gene of Indonesian and Australian isolates of NDV

Sequencing analysis of the PCR products from the NDV RNA transported from Indonesia showed that the isolates were similar to the Australian V4 NDV isolated from chicken isolates. The similarities and differences can be seen in Figure 8.17

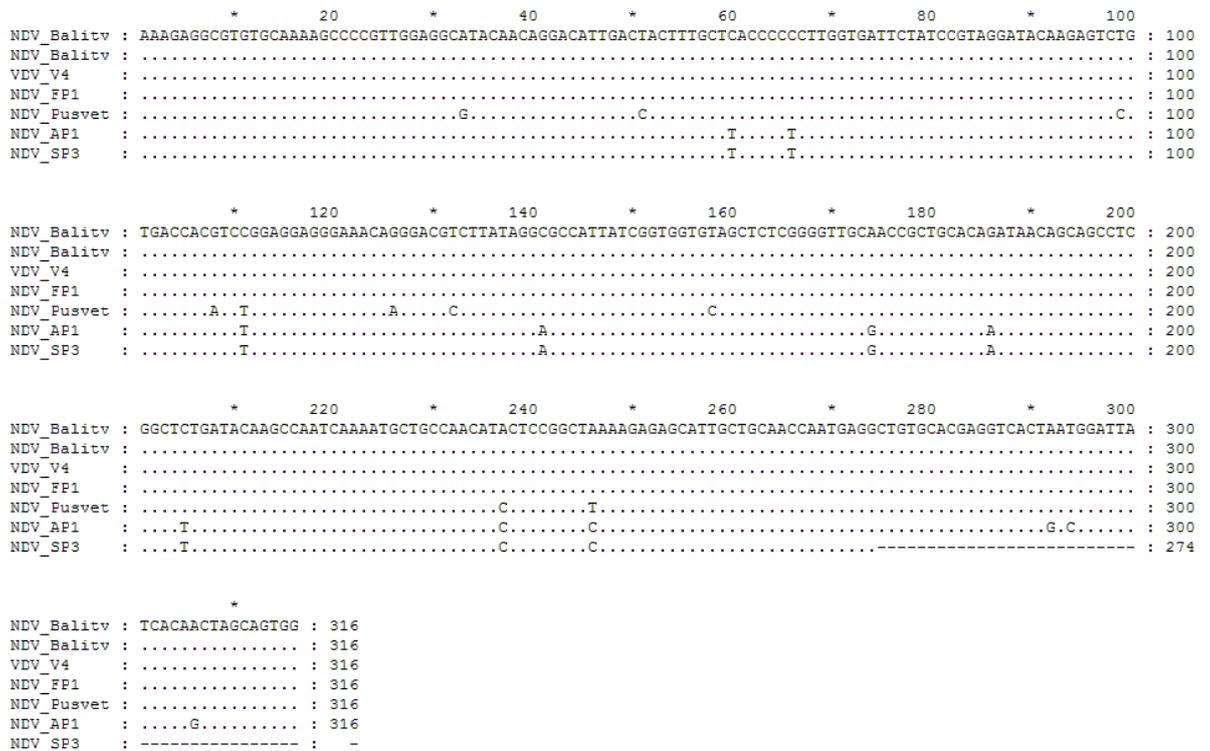


Figure 8.17 Alignment of partial sequence of the F gene of NDV isolates from Indonesian vaccine viruses as well as Australian reference NDV isolates.

However, it showed in Figure 8.17 that Balitvet NDV antigens have a closer relationship to the Australian NDV V4 than Pusvetma antigens. Also, there were some nucleotide mismatches between NDV antigens Balitvet and Pusvetma.

Comparison of Balitvet and Pusvetma NDV antigen sequences with the available GenBank sequences showed that NDV Pusvetma aligned closely with a Japanese NDV isolate: Japan/Ishii/62 while the NDV Balitvet was closely related to V4/AUS/(QLD)/66. Both of these isolates are Class 2 genotype 1 viruses. This alignment can be seen in the phylogenetic tree in Figure 8.18.

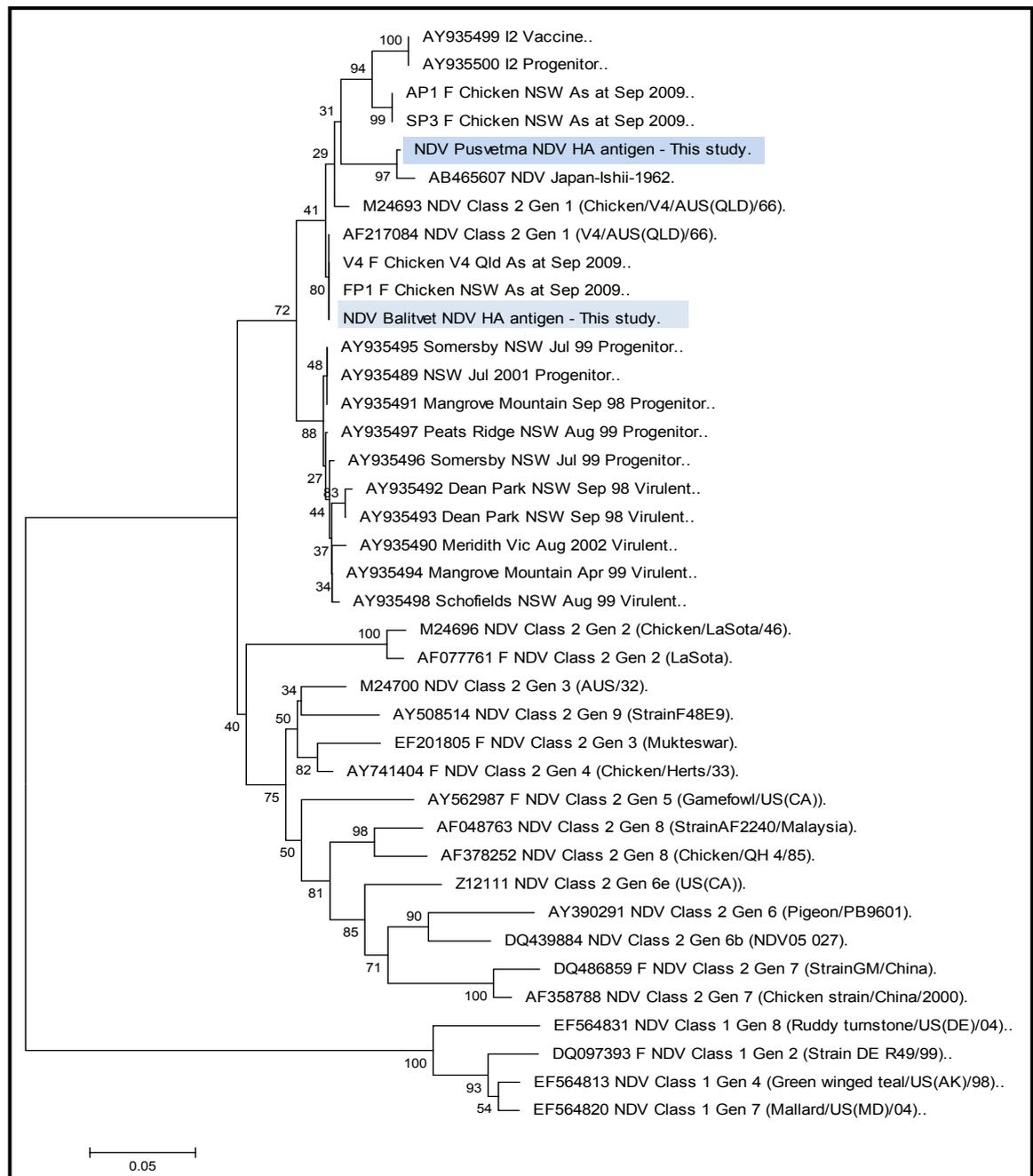


Figure 8.18. Phylogenetic trees of the F gene of Inactivated ND samples transported from Indonesia. The analysis was conducted in MEGA5 involved 39 nucleotide sequences. There were a total of 307 positions in the final dataset.

The RNA's from the AIV samples from Indonesia were extracted and a 590 bp sequence from each of the haemagglutinin genes was amplified using the H5-GB590 forward and reverse set of primers primer (Figure 8.19)

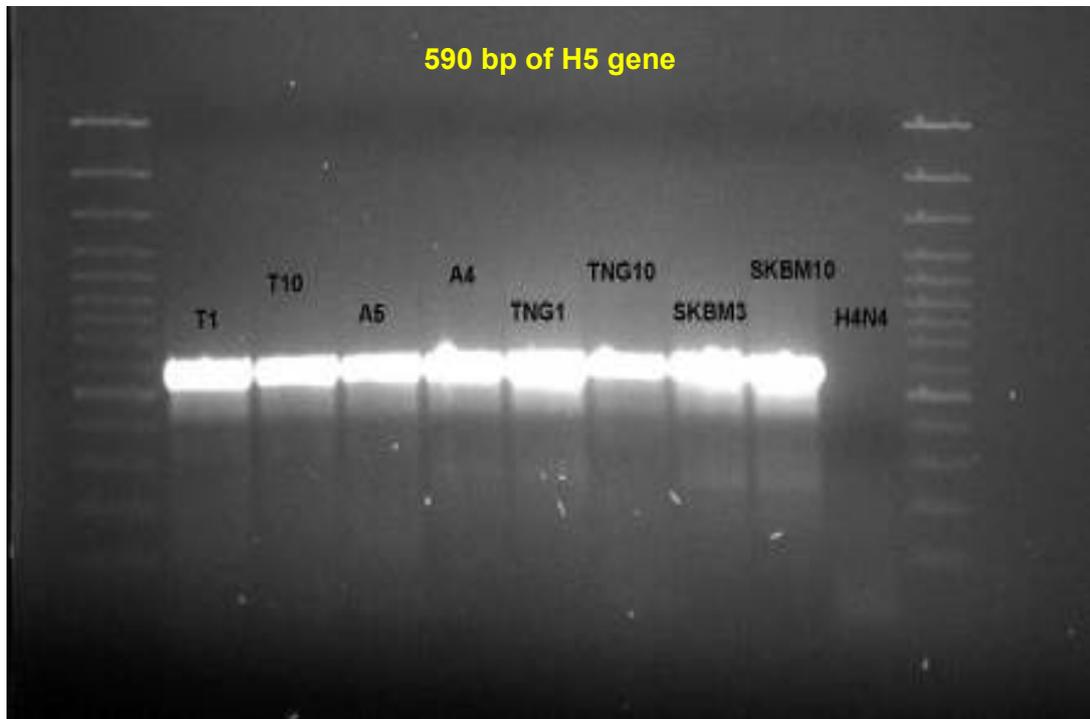


Figure 8.19 Amplification of 590bp of the H5 genes of AI viral RNA transported from Indonesia.

Amplification of 590bp and 1307bp fragments from the transported AI viral RNA not only indicates that the RNA can be used for diagnostic purposes but that it is not fragmented. These sequences could then be used to pathotype the isolates.

Sequences of amplified 590 bp H5 gene of processed AI viral RNA were analysed and compared to and other isolates accessed from GenBank. This analysis showed that H5 sequences of the transported viral RNA's have a similarity with other H5 sequences submitted from Indonesian isolates.

Three different clusters were demonstrated. Tangerang and Sukabumi isolates were separated in two different clusters while Timika and Ambon grouped in one cluster. All of these isolates (Timika, Ambon, Tangerang and Sukabumi) were aligned in the clade 2.1.3 of H5N1 HPAI. The phylogenetic analysis can be seen in Figure 8.20

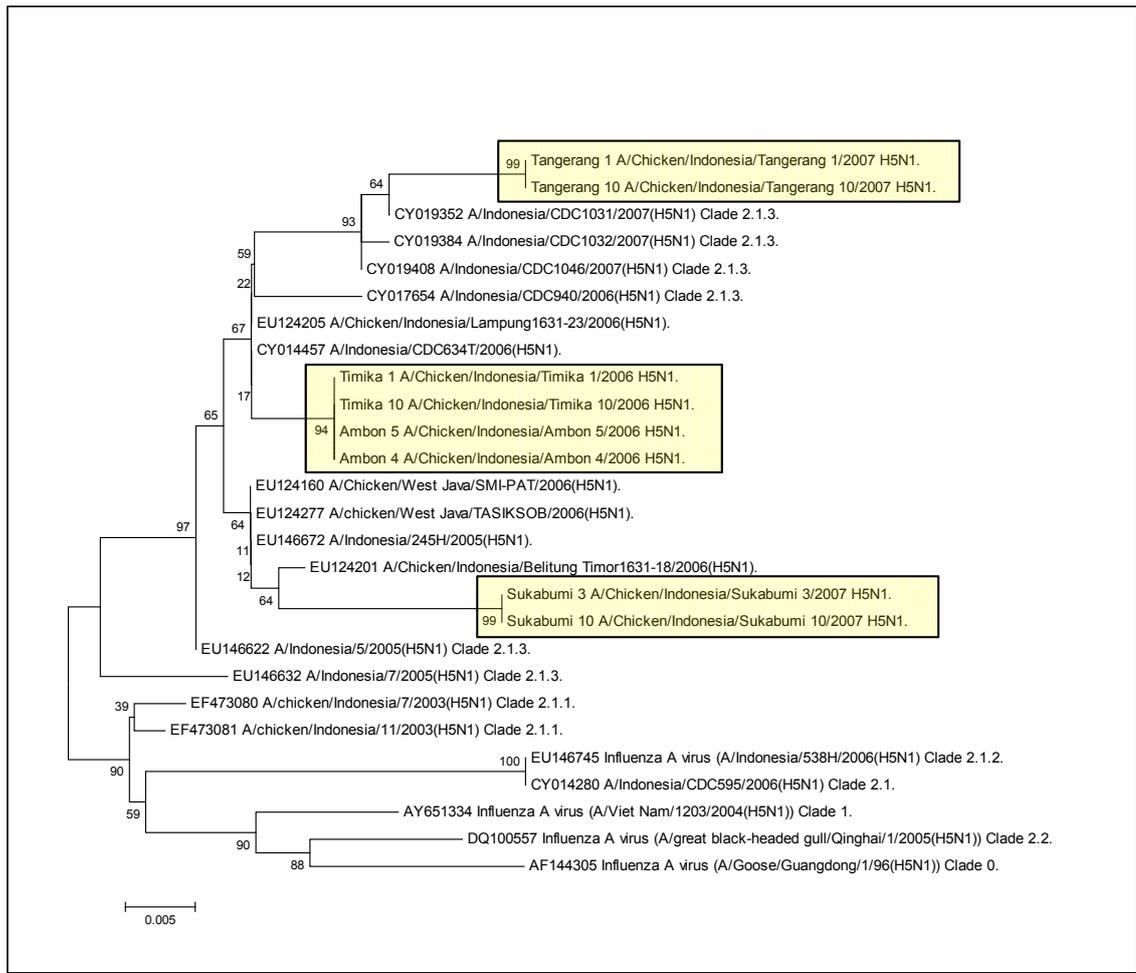


Figure 8.20 Phylogenetic trees of 590 bp of H5 of AI viral RNA transported from Indonesia The analysis involved 27 nucleotide sequences. A total of 516 positions in the final dataset were analysed using MEGA5

Alignment of the diagnostic primers on the 590bp sequences of AI viruses in this study showed that there were two mismatches identified in the Sukabumi isolates to the forward primer (IVA-DA148H5-F) and probe (IVA-H5a) (Heine *et al.*, 2005) These isolates failed to react in the TaqMan PCR (Figure 8.4). However, when the primers were used in RTPCR with SYTO 9, all of the isolates including the Sukabumi isolates reacted. It was concluded that the mismatches under the probe were critical for detecting these viruses (Figure 8.21).

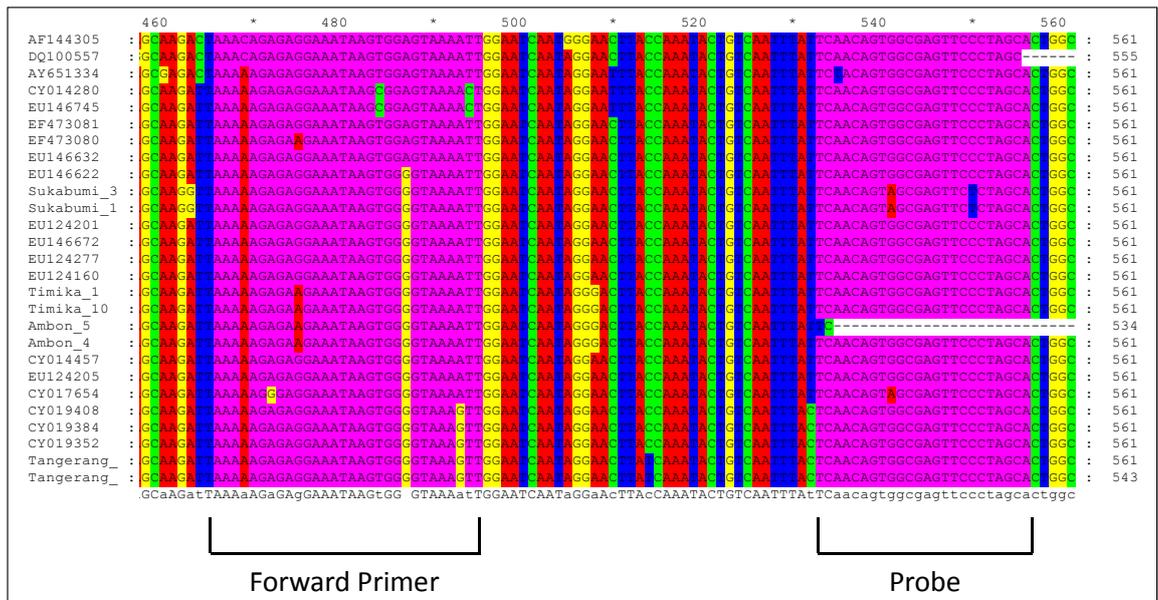


Figure 8.21 Mismatches in Indonesian sequences and IVA-DA148H5 forward primer and probe. At least two mismatches were identified under the IVA-DA148H5 forward primer and with the Sukabumi isolates there were mismatches under the probe.

Also 1307 bp PCR products were successfully amplified and sequenced from the Sukabumi AI viral RNA transported from Indonesia. The amplified DNA can be seen in Figure 8.22

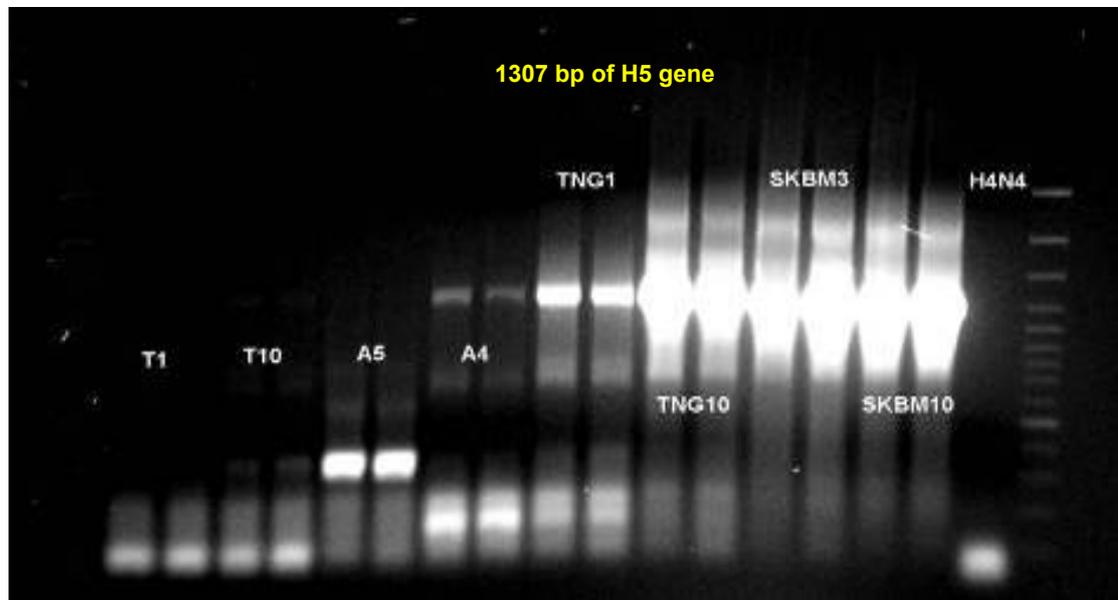


Figure 8.22 Amplification of a 1307 of H5 gene of AI viral RNA's processed and transported from Indonesia

Sequencing a full length sequence (1307 bp) H5 of transported viral RNA confirmed that RNA fragmentation was not a problem. Near full length HA sequencing was only attempted for the Sukabumi isolates.

The phylogenetic analysis indicated the similarity of Sukabumi 3 H5 sequence to the other Indonesian H5 isolates submitted to GenBank (EU124150, EU124276, EU124160, EU124148). This accession confirmed the similarity of H5 gene of viral RNA Sukabumi 3 with other H5 genes submitted from H5 genes from West Java isolates in 2006. The phylogenetic tree of H5 genes of Sukabumi 3 can be seen in Figure 8.23

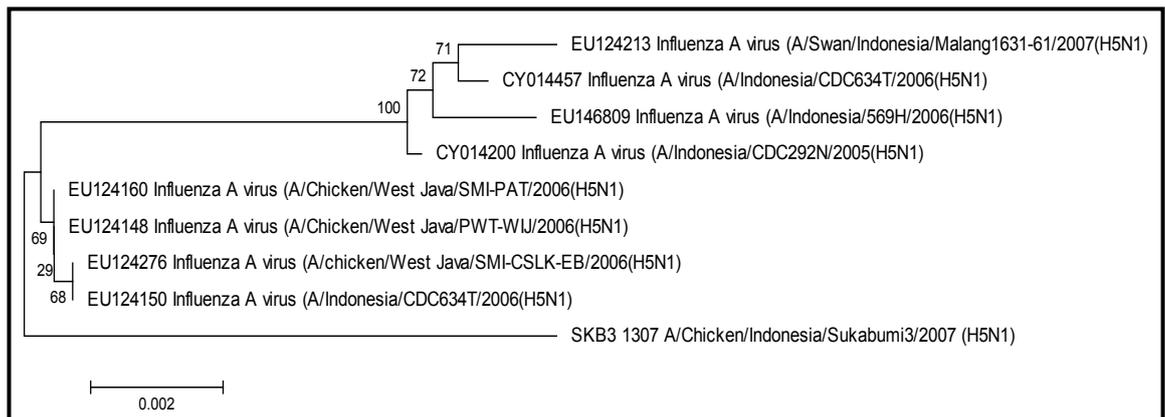


Figure 8.23. Phylogenetic analysis of 1307 bp of H5 of Sukabumi 3 viral RNA. The analysis involved 9 nucleotide sequences. There were a total of 1456 positions in the final dataset conducted in MEGA5

Moreover, the translation protein of H5 sequences of Sukabumi 3 viral RNA demonstrated that Sukabumi isolate were likely to be HPAI with the protein of cleavage site R-R-K-K-R-G-L-F (Figure 8.24)

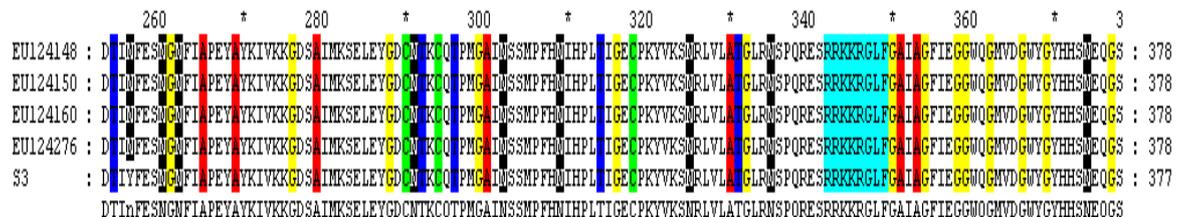


Figure 8.24 Amino acid translations of H5 sequences of Sukabumi 3 viral RNA

8.6 Discussion

Viral isolates processed using a commercial lysis buffer were successfully consigned from Indonesia to Australia without a cold chain. It is interesting to note that this not only applied to the AI viral isolates but also to the NDV HA antigens that were inactivated with Beta propiolactone

Beta propiolactone is an efficient inactivating agent for viruses such as rabies virus, NDV virus and AI virus. This reagent is utilised for vaccine production. As a viral inactivating agent, it was anticipated that the Beta propiolactone would have at least

fragmented the nucleic acid (Kubinski and Szybalski, 1975; King, 1991; Refaie *et al.*, 2004). However, this study indicated that the RNA could still be very effective in the screening assay and that a PCR product of more than 300 bp could be produced for sequencing.

Analysis of the sequences demonstrated that these two antigens were derived from isolates that were similar to the Australian NDV chicken isolates (V4, AP1, FP1, and SP3) and a not similar to the Australian NDV isolates WA Isolate 3245 and Ibis Queensland-06

Avian Influenza viral isolates from four different locations in Indonesian were successfully detected using RT-qPCR assays that utilised AI M primers (Ward *et al.*, 2004). This RT-qPCR assay was performed using DNA binding dye SYTO9 based on a protocol developed and described in Chapter 4 and Chapter 6. Another RT-qPCR assay based on a dual labelled probe for the M gene (Heine *et al.*, 2005) also successfully detected all transported Indonesian AI viral isolates.

An influenza A H5 assay based on an H5 dual labelled probe (Heine *et al.*, 2005) successfully detected the Timika, Ambon and Tangerang isolates but failed to detect the samples from Sukabumi.

Mismatches under the primers especially in the 5' portion of the primer can be tolerated. However mismatches under the probe may result in the samples failing to react. In this study the H5 primers in an RT-qPCR assay based on SYTO 9 detected all four groups of isolates. The two base mismatch under the probe resulted in failure to detect the Sukabumi isolates.

A second pair of H5 primers was used in an attempt to detect the isolates. These primers were successfully used to detect H5 influenza A viruses isolates from several avian species including chicken, duck, quail, pigeon, and open bill stork during outbreak in Thailand in 2004-2005 (Thontiravong *et al.*, 2007). However, this pair of primers failed to detect Indonesian AI isolates from four different locations. This may be related to differences between the Thai and Indonesian isolates.

The rapid evolution requires the application of a range of detection and scanning assays. The HRM assay has considerable potential for identifying changes in the genome. The principal of high resolution melt assay is measuring subtle differences in T_m that are the result of base changes. The assay uses relatively small amplicons (Ikuta *et al.*, 1987; Zhou *et al.*, 2004; Zhou *et al.*, 2005; Gundry *et al.*, 2008). It is a simple assay that can be applied to large numbers of samples to highlight differences that can warrant further investigation.

High resolution analysis was applied to the isolates derived from four different locations in Indonesia. Differences were subsequently reflected in the differences in the sequences and it highlights the continued evolution of these viruses (Smith *et al.*, 2006). While this assay is effective in recognising changes in the isolates it is still necessary to follow this with sequencing to determine the phylogenetic relationships and to carry out pathotyping of the Indonesian isolates.

Sequencing of the transported AI and ND viral RNA was carried out. Sequencing of the NDV F and AI H5 genes has indicated that fragmentations of the RNA was not a problem when the lysis buffer was used.

Genetic analysis of the F gene the ND viral antigens showed that both NDV Balitvet and Pusvetma fitted into Class II genotype I. The NDV Balitvet had a close relationship to the Australian V4 and FP1 isolates while the Pusvetma sequence was similar to the Japanese Ishii 1962 isolate.

Phylogenetic studies of the AI viral RNA demonstrated that all of the samples from Indonesia fitted into clade 2.1.3 of H5N1 HPAI. The four groups of isolates were placed into three different clusters. Timika and Ambon were in one cluster while Tangerang and Sukabumi formed two separate clusters.

Analysis of sequences of these AI viruses showed the heterogeneity of H5 sequences between three clusters. This analysis confirmed the different sequences between AI viruses collected in 2006 (Timika and Ambon) and in 2007 (Tangerang and Sukabumi). This also corresponded to an apparent change in virulence with a reduction in clinical signs and mortality rate based on information from the field.

8.7 Conclusion

Collection and transportation without a cold chain was successfully achieved by delivering ND and AI viral RNA to Australia. These isolates could be successfully detected in the appropriate diagnostic assays. In addition the RNA did not appear to be fragmented and could be used for generating sequences that allowed phylogenetic studies and an estimation of the pathogenicity of the isolates. This was achieved without the use of a cold chain.

Use of this technology could substantially enhance the effectiveness of molecular epidemiological studies by facilitating the transfer of representative samples without the problems associated with quarantine. There may also be a very substantial improvement in the quality of the end results.

CHAPTER 9

GENERAL DISCUSSION

Sample collection and transportation without a cold chain is an important method in AI and ND diagnosis. This method can facilitate the limited infrastructure facilities in developing countries and also overcome the quarantine restriction between countries. Indeed, this method can be also facilitate a rapid and reliable diagnosis as a response of an outbreak in different areas or countries. Thus, this method can support epidemiological surveillance programs for the early detection and warning systems for poultry diseases.

However, collection and transportation without cold chain is not enough to support AI and ND diagnosis. Therefore in this study investigated several steps to address the molecular diagnosis and typing of AI and ND using samples collected and transported without a cold chain.

The first step was reviewing or developing a set of diagnostic assay for AI and ND and integrating them into the collection and transportation protocols.

Diagnostic assays based on real time RT PCR have been developed and published. SYBR Green and dual labelled TaqMan probes have been widely used to diagnose AI and ND (van Elden *et al.*, 2001; Spackman *et al.*, 2002; Tan *et al.*, 2004; Ward *et al.*, 2004; Pham *et al.*, 2005; Payungporn *et al.*, 2006a; Ong *et al.*, 2007). However, SYBR Green and dual labelled probe have limitations. SYBR green can inhibit PCR reactions in a dependent concentration manner caused by the degradation products of the dyes. Whereas dual labelled probes with mismatches may fail to bind resulting in false negative results (Wittwer *et al.*, 2001; Monis *et al.*, 2005; Kim *et al.*, 2006). In this study assays based on the interchelating dye SYTO 9 were used to augment the probe based assays for ND and AI.

RT-qPCR assays to diagnose both Class I and Class II NDV were successfully developed using SYTO 9. This dye has low toxicity and bright fluorescence and it can be used in a high resolution melt configuration to differentiate primer dimer from specific products and to type the isolates based on variations in their melting points

(Wittwer *et al.*, 2001). However, assay based on SYTO 9 RT-qPCR must utilise appropriate pairs of primers. This study designed and evaluated primers for both the M and F genes of NDV. The M gene is relatively conserved and two pairs of M gene primers were developed into a duplex configuration. A set of published primers (M+4100 forward and M-4220 reverse) based on the M gene of Class II NDV was evaluated (Wise *et al.*, 2004). A second set (NDV MGB1) based on the GenBank submission AY626266M was designed and evaluated for the detection of Class I NDV isolates. The controls for these primers were a WA Duck Isolate 3245 (Alexander *et al.*, 1986) and Ibis Queensland. The controls for the Class II primers were V4 Queensland and a set of similar isolates.

A SYTO 9 RT-qPCR was also successfully applied to AI viruses. A few pairs of primers (Ward *et al.*, 2004; Heine *et al.*, 2005) were also evaluated by another student using both TaqMan and SYBR Green formats (Nguyen, 2007). Therefore, in this study, those primers were applied using SYTO 9 in RT-qPCR.

The diagnostic assays for ND were followed by sequencing of the viral isolates. This sequencing was crucial to determine viral pathotyping and phylogenetic relationships of ND isolates. Sequencing was based on both the M and F genes.

The F gene of NDV is an important determinant of pathogenicity. Sequencing and estimating the amino acid sequence can determine the F₀ cleavage site. The presence of two pairs of basic amino acids is indicative of a furin cleavage site. Virulent isolates of Class II NDV have a motif ¹¹²R/K-R-Q-K/R-R¹¹⁶ at the C terminus of the F2 protein and Phenylalanine at the N terminus of the F1 protein whereas low virulence viruses have the amino acid sequence motif ¹¹²G/E-K/R-Q-G/E-R¹¹⁶ and Leucine at residue 117 (Alexander, 1990; Alexander, 1995; Collins *et al.*, 1996; Aldous and Alexander, 2001; Gould *et al.*, 2003; OIE, 2004).

In this study, a pair of F primers (F302f and F596r) (Stanislawek *et al.*, 2002), was used to produce a 274 bp product from Class I viruses. A published pair of F primers (Creelan and McCullough, 2006) failed to reliably produce a suitable product from the Class II viruses. Therefore, a pair of primers was designed to produce a 343 bp product for sequencing. These primers were designed using an aligned set of NDV Class II

Fusion genes and it was successfully used to sequence the V4, AP1, FP1, and SP3 isolates.

Analyses of F gene sequences of six ND isolates confirmed that the isolates represented both classes. It was noted that V4 and FP1 clustered together while FP1 and SP3 were more closely aligned with the I2 vaccine virus (Wang *et al.*, 1992; Gould *et al.*, 2003; Kattenbelt *et al.*, 2006a). All Class II isolates could be differentiated from the Australian viruses responsible for recent outbreaks in chickens. However, they were all genotype 1 viruses.

The WA duck isolate 3245 had a cleavage site S-G-G-E-R-Q-E-R-L-V. The Ibis isolate had a S-G-G-E-Q-Q-G-R-L-I while the Class II isolates V4, AP1, FP1 and SP3 had a sequence of S-G-G-G-K-Q-G-R-L-I at the cleavage site. Therefore it was concluded that all of the isolates used in this study had a cleavage site consistent with low virulence.

A total of four buffers STDB, LB, VBB, and LSDB were initially evaluated in a pilot study. These buffers contained various different chemicals including SDS, EDTA, Proteinase K, Guanidine HCL, Guanidine Isothiocyanate, Dithiothreitol. Two buffers were chosen for the definitive study. The solid tissue digest buffer was based on SDS and Proteinase K while the lysis buffer contained Guanidine Isothiocyanate and Dithiothreitol. Carrier RNA was added to both buffers.

The SDS and Proteinase K in STDB successfully preserved RNA and gave the best performance. As a detergent, sodium dodecyl sulphate solubilises and denatures proteins by disrupting the cell membrane. Moreover, Proteinase K in the STDB behaves as a proteolytic enzyme which has the ability to degrade ribonuclease and purify the nucleic acid from a cell (Brown, 1995; Albert *et al.*, 2002; Albert *et al.*, 2004; Clark, 2005; Turner *et al.*, 2005; Privé, 2007). However, this buffer failed to completely inactivate the V4 strain of NDV. Therefore, lysis buffer was chosen for further study as samples needed to be completely inactivated to be imported under an AQIS permit.

GuSCN is used in many application of nucleic acid extraction (Chirgwin *et al.*, 1979; Chomczynski and Sacchi, 1987; Boom *et al.*, 1990; Chomczynski and Mackey, 1995; Chomczynski and Sacchi, 2006). This chaotropic agent has a powerful ability to disrupt

and dissolve molecular structures, destabilize proteins, lyse cells and inhibit nuclease and protease activities (Chirgwin *et al.*, 1979; Boom *et al.*, 1990; Ness and Chen, 1991; Sambrook and Russel, 2001; Clark, 2005). The addition of Dithiothreitol and carrier RNA allowed the buffer to preserve RNA for at least one month at 37°C with minimal loss. This is sufficient for the transportation in the absence of a cold chain.

Both of the buffers could be readily integrated with the RNA extraction protocols using a robotic device (Corbett Research, Brisbane).

However, extracted and purified RNA can potentially be degraded during storage or when it is frozen and thawed. Two buffers (TCB and mRSB) containing carrier RNA were evaluated for the storage of extracted RNA. They were evaluated for long term storage at -20°C for nine months and for a repeated freeze and experiment with 10 successive cycles of thawing and freezing. Both buffers successfully preserved the RNA. However, it was speculated that the mRSB which contained sodium azide would be more likely to provide added protection for the RNA if there was aerosol bacterial contamination during the freeze thaw steps.

The definitive study was carried out using samples collected in Indonesia. A total of four groups of isolates of AI from outbreaks from different regions in Indonesia were processed at the Quarantine Agency laboratory of Indonesia. In addition two samples of NDV HA antigen was purchased from two suppliers (Balitvet and Pusvetma).in Indonesia. The allantoic fluids and NDV antigens were added to lysis buffer and transported to JCU, Townsville.

The AIV and NDV samples were confirmed using SYTO 9 real time RT-PCR screening assays. In order to confirm that the RNA was not fragmented sequencing was carried out using PCR products.

The AIV isolates were initially screened using a SYTO 9 RT-qPCR and TaqMan dual labelled probe in RT-qPCR. Both of these assays targeted the M gene (Ward *et al.*, 2004; Heine *et al.*, 2005). As these isolates were suspected to be H5N1 they were tested with H5 primers in a TaqMan format (Heine *et al.*, 2005) and with SYTO 9 in RT-PCR. All of the isolates reacted in the SYTO 9 assay while the isolates from Sukabumi failed to react in the TaqMan assay. All of the AI samples were amplified with a set of H5

primers that produced a 590 bp product. Selected isolates were amplified with a set of H5 primers specifically aimed at the Sukabumi isolates to produce a 1370 bp product.

The sequencing indicated that all of the isolates were H5 in the clade 2.1.3. The Timika and Ambon isolates formed a cluster that was separate from the Tangerang and Sukabumi isolates. It was noted that there were mismatches under the H5 forward diagnostic primer. This did not prevent the SYTO-9 assay from recognising the isolates. An additional two mismatches were noted under the probe for the Sukabumi isolates and it was concluded that these were sufficient to prevent the annealing of the probe. The sequencing did not include the binding site for the reverse primer.

The NDV isolates were also sequenced and it was clear that they were both Class II genotype 1 isolates. The Balitvet antigen was similar to V4-Queensland-66 while the NDV Pusvetma was almost identical to the NDV Japan-Ishii-1962. It was anticipated that the β propiolactone used to inactivate the antigens may degrade the RNA. A set of F primers successfully produced a 343 bp product that allowed sequencing to be carried out.

This component of the study demonstrated that samples could be collected in Indonesia, transported for up to a month at room temperature and they are suitable for both use in diagnostic assays and for sequencing studies. A product that spanned most of the H gene of influenza was amplified.

This molecular epidemiology study of AIV and NDV was successfully carried out using sample collection and transportation without a cold chain. However, at this stage, the method collection and transportation has not been applied to clinical and field samples. This is the logical extension of this work. If this can be achieved clinicians in developing countries could collect samples and place them into suitable transport media. They could be sent to the laboratories without a cold chain. This may decrease the urgency of the transportation. The samples as they arrive would be compatible with the extraction process.

The samples could be forwarded to reference laboratories that do not have to use high security facilities to handle the imported reagents. This would facilitate the use of a

much wider the range of reference and collaborating laboratories and there could be very significant cost savings.

CHAPTER 10

CONCLUSION

Molecular epidemiology of AIV and NDV has been successfully addressed using sample collection and transportation without a cold chain. In this method, lysis buffer was the preferred buffer to transport samples from one to another country.

In addition a set of diagnostic assays and sequencing tools was evaluated and buffers for the preservation of RNA were investigated.

The end result is a safe integrated system of sample collection, processing and transportation that does not require a cold chain. A proof of concept study was carried out in which AI and ND viral samples were sourced from Indonesia and transported to Australia at ambient temperatures. The extracted RNA was of high titre and fragmentation of the RNA did not impede sequencing. It is likely that the RNA was not fragmented.

This demonstrates the utility of this protocol. If this can be extrapolated to field samples it has the potential to make a very valuable contribution to disease diagnosis in both developing and developed countries

APPENDIX

1. Distilled water

Water used in the preparation of solutions and solvents was distilled three times and was autoclaved at 68 KPa for ten minutes before use.

2. Elsevier's Solution

8.16 g Tri-sodium citrate
0.55 g Citric acid
20.50 g Dextrose
4.20 g Sodium chloride, NaCl
Dissolved in 1,000 mL distilled water
and sterilized by autoclaving at 68 kPa for ten minutes.

3. Phosphate buffered saline (PBS).

a) PBS-A

8.00 g NaCl
0.2 g KCl
1.15 g Na₂HPO₄
0.2 g KH₂PO₄
1,000 mL Distilled water

b) PBS-B

1.0 g MgCl₂ 6H₂O
1.0 g CaCl₂ 6H₂O
100 mL Distilled water
Autoclaved each at 100 KPa for ten minutes.

c) To make PBS:

5 mL PBS-B
(500 mL) PBS-A added up to 500 mL
and adjusted to pH 7.2.

4. Profile of RT-qPCR assay of V4 and D1 using three different primers (Chapter 4)

No	Primers	Isolates	Mean of Ct-Value	Melting Point
1	Wise-NDVM /M+ 4100(F) and M-4220R (Wise et al., 2004)	V4	4.69	87.5
		D1 (WA 3245)	22.15	-
2	Cr-NDV-Ch-F and R (Creelan et al., 2002a)	V4	17.71	88
		D1 (WA 3245)	21.345	-
3	NDV-MGB1-F and R (GenBank ID. AY626266 M)	V4	24.68	-
		D1 (WA 3245)	6.41	86.5

5. Evaluation NDV chicken and duck isolates using chicken M Primer (Chapter 4)

Primers	Isolates	Mean of Ct-Value	Mean of Melting Point
Wise-NDVM /M+ 4100(F) and M-4220R (Wise et al., 2004)	V4	4.695	87.5
	FP1	4.585	87.56
	AP1	4.985	88.35
	SP3	7.26	82.25
	D1	22.355	-
	dNTC	22.15	-

6. Diagnosis of NDV using Class I M Primer (Chapter 4)

Primers	Isolates	Mean of Ct-Value
NDV-MGB1-F and R (GenBank ID. AY626266 M)	V4	23.913
	FP1	23.835
	AP1	24.28
	SP3	Neg
	D1	22.345
	dNTC	6.41

7. Diagnosis of NDV using Duplex primers (Chapter 4)

Primers	Isolates	Mean of Ct-Value	Mean of Melting Point
Wise-NDVM /M+ 4100(F) and M-4220R (Wise et al., 2004) and NDV-MGB1-F and R (GenBank ID. AY626266 M)	V4	4.8267	86.67
	D1	7.35	85
	Ibis	6.3934	85
	NTC	22.18	

8. Quantitation analysis of the ability of four different buffers to preserve RNA NDV V4 and H4N4 (Chapter 6)

Virus	Buffer	The amount of viral RNA NDV V4 (CT Value – RT-qPCR)											
		With RNA carrier						Without RNA carrier					
		Temperature 25 ⁰ C			Temperature 37 ⁰ C			Temperature 25 ⁰ C			Temperature 37 ⁰ C		
		1 day	1 week	2 weeks	1 day	1 week	2 weeks	1 day	1 week	2 weeks	1 day	1 week	2 weeks
NDV	STDB + 1% DBA	7.95	8.01	7.13	6.25	7.17	7.1	8.02	8	8.77	7.96	8	8.81
	VBB	9.73	10.61	0	10.59	8.95	8.05	0	8.79	9.73	0	8.85	8.86
	LB	7.98	7.93	7.95	8.01	7.23	8.75	8.81	7.05	7.92	7.95	7.97	8.78
	LSDB + 10% Digestion buffer additive	8.81	8.87	9.65	10.51	8.85	11.51	8.82	7.94	7.99	8.75	8.87	11.76
H4N4	STDB + 1% DBA	10.67	10.02	10.03	8.15	10.63	10.61	10.72	10.55	10.62	10.68	10.84	10.81
	VBB	12.57	12.43	12.58	12.69	11.46	10.82	11.62	11.57	10.86	11.7	10.93	11.66
	LB	9.66	7.93	10.68	10.8	11.62	12.32	9.03	10.64	10.73	10.77	10.92	13.32
	Liquid sample Digest Buffer + 10% Digestion buffer additive	10.82	8.87	10.87	13.32	14.41	17.21	11.51	10.9	11.5	12.73	14.47	19.24

9. Ct-Values of the PCR product of NDV V4 and AIV H4N4 processed in two transport buffers (Chapter 6)

Viruses	Buffer	The amount of viral RNA (Mean of Ct Values – RT-qPCR).							
		Temperature 25°C				Temperature 37°C			
		1 day	1 week	2 weeks	4 weeks	1 day	1 week	2 weeks	4 weeks
V4	STDB	6.69	6.68	6.84	6.42	6.38	7.17	5.99	6.26
	LB	7.44	6.54	6.64	6.6	6.76	6.23	6.18	6.26
H4N4	STDB	8.12	9.11	8.08	9.06	8.48	8.78	8.87	9.04
	LB	8.61	9.187	8.97	9.23	8.92	10.01	11.28	11.20

10. Mean of Ct-Values of viral RNA in thawing treatment using RNA storage buffers (Chapter 7)

Virus	Buffer	No thawing				5× thawing				10× thawing			
		No dilution	10× dilution	1000 × dilution	NTC	No dilution	10× dilution	1000 × dilution	NTC	No dilution	10× dilution	1000 × dilution	NTC
V4	TE	5.9	12.2	21.3	20.9	5.7	9.1	14.9	22.4	5.7	8.9	14.8	22.4
	Hoffman n	5.9	10.3	18.9	21.3	5.7	8.5	14.3	22.6	5.7	8.5	14.9	22.6
H4N4	TE	7.8	11.8	18.8	20.1	7.5	11.0	17.7	25.2	7.5	11.4	19.4	25.2
	Hoffman n	7.8	12.0	17.7	20.2	7.5	11.1	17.0	25.4	7.5	11.0	17.3	25.4

11. The mean of Ct-Values of viral RNA in times treatment using RNA storage buffers

Viruses	Buffer	The amount of viral RNA (CT Value – RT-qPCR)- V4											
		0 month				3 month				9 month			
		No dilution	10× dilution	1000 × dilution	NTC	No dilution	10× dilution	1000 × dilution	NTC	No dilution	10× dilution	1000 × dilution	NTC
V4	TE	5.9	12.2	21.3	20.9	5.1	9.5	21.9	24.5	5.7	9.1	24.8	22.4
	Hoffmann	5.9	10.3	18.9	20.9	5.1	9.1	15.7	26.4	5.7	8.8	15.3	23.4
H4N4	TE	7.8	11.8	18.8	20.1	7.7	11.4	19.8	27.0	8.9	11.4	22.0	28.9
	Hoffmann	7.8	12.0	17.7	20.2	7.7	11.6	18.0	27.5	8.9	11.2	18.0	28.8

12. The mean of Ct-Values, Melting point and HRM of qRT-PCR assay to the AI samples from Indonesia (Chapter 8)

No	Target Sequences	Primers / Probes	Isolates	Mean of Ct- Values	Melting Points	HRM
1	M AI	Ward primers + SYTO 9, modified from (Ward et al., 2004a; Munster et al., 2005)	Timika (T)	6.7	84.5	Not measured
			Ambon (A)	5.86	84.71	
			Sukabumi (SKBM)	5.69	85	
			Tanggerang (TNG)	6.5	85.2	
		TaqMan AAHL primers and probes (Heine et al., 2005b)	Timika (T)	11.3	Not available	Not available
			Ambon (A)	10.5		
			Sukabumi (SKBM)	10.4		
			Tanggerang (TNG)	15.0		
2	H5	TaqMan H5 AAHL primers and Fluor red and BHQ probes (Heine et al., 2005b)	Timika (T)	9.36	Not available	Not available
			Ambon (A)	7.99		
			Sukabumi (SKBM)	14.64		
			Tanggerang (TNG)	8.44		
		H5 AAHL primers + SYTO 9 modified from (Heine et al., 2005b)	Timika (T)	10.30	80.8	79.23
			Ambon (A)	8.39	81	79.3
			Sukabumi (SKBM)	12.13	80.5	79.29
			Tanggerang (TNG)	10.63	80	78.85
		H5a Pay primers H5F3 and H5R2 + SYTO 9 modified from (Payungporn et al., 2006b)	Timika (T)	12.13	81.84	80.24
			Ambon (A)	10.56	81.99	80.55
			Sukabumi (SKBM)	9.33	82.36	80.88
			Tanggerang (TNG)	10.58	82.3	81.19

13. The mean of Ct-Values, Melting point of qRT-PCR assay to the antigen NDV samples from Indonesia (Chapter 8)

No	Target Sequences	Primers / Probes	Processed Antigen	Mean of Ct-Values	Melting Points
1	M NDV	Duplex Wise-NDVM /M+ 4100F and M-4220 R (Wise <i>et al.</i> , 2004) and NDV-MGB1 F and R (GenBank ID. AY626266 M)	NDV Antigen - Balitvet	7.42	86.30
			NDV Antigen - Pusvetma	6.72	86.44

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