ESTABLISHMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) METHOD FOR THE DETECTION OF VIRULENT NEWCASTLE DISEASE VIRUS

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Establishment Of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-Lamp) Method For The Detection Of Virulent Newcastle Disease Virus

By

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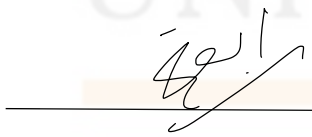

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ABSTRACT

Abstract from research papers submitted to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan to fulfil the requirements of the course DVT 55204 – Research Project.

Newcastle Disease Virus (NDV) is a highly contagious viral pathogen that affects various bird species, primarily domestic and wild birds, including poultry such as chickens and turkeys. It belongs to the Avian Paramyxovirus serotype 1 (APMV-1) within the *Paramyxoviridae* family. NDV have different pathotypes, ranging from asymptomatic to virulent strains. The virulent pathotype, termed velogenic and mesogenic, poses a significant threat due to its potential to cause severe illness and high mortality rates in poultry flocks. Birds infected with the virulent strain can exhibit a range of clinical signs, including respiratory distress, nervous system disorders, diarrhoea, and a sharp decline in egg production. Current methods to diagnose the pathotype of the virulent NDV are by Mean death time (MDT), intra-cerebral pathogenicity index (ICPI), intra-venous pathogenicity index (IVPI) and PCR-sequencing to determine the presence of multiple amino acid motif at the fusion protein cleavage site. However, these methods are time-consuming and required specialized laboratory facilities. The establishment of robust and reliable diagnostic methods is paramount for detecting virulent strains of the Newcastle Disease Virus (NDV). This study was aimed to establish Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) to detect pathogenic ND virus The results were further confirmed by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and PCR sequencing to determine the presence of polybasic amino acid sequences at the F protein cleavage site for virulent viruses. The RT-LAMP method offers a promising avenue for the rapid and sensitive identification of virulent NDV strains. The results obtained from this research using the archived samples in the laboratory showed that our designed RT-LAMP detected only the virulent NDV strains, in which confirmed with the RT-PCR using primers specific to virulent NDV. For further confirmation, amino acid sequencing was performed and the RT-LAMP and RT-PCR results yielded congruent findings with amino acid sequencing results. Both RT-LAMP and RT-PCR techniques exhibit high sensitivity and specificity, however, due to its simplicity and rapidity, RT-LAMP is more preferable for diagnosing virulent NDV strains especially in low resource settings. However, the RT-LAMP results need to be further confirmed by RT-PCR and amino acid sequencing as a gold standard molecular method. This is important for timely disease management and control strategies of ND infection.

Keywords : Virulent Newcastle Disease Virus (NDV), Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP), Reverse Transcription Polymerase Chain Reaction (RT-PCR)

PENUBUHAN AMPLIFIKASI ISOTERMA PERANTARA-LOOP TRANSKRIPSI TERBALIK (RT-LAMP) UNTUK PENGESANAN VIRUS VIRULEN PENYAKIT NEWCASTLE

ABSTRAK

Abstrak daripada kertas penyelidikan yang diserahkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan

Virus Penyakit Newcastle (NDV) merupakan patogen virus yang sangat berjangkit yang mempengaruhi pelbagai spesies burung, terutamanya burung ternakan dan liar, termasuk ayam dan ayam Belanda. Ia tergolong dalam Avian Paramyxovirus serotip 1 (APMV-1) dalam keluarga Paramyxoviridae. NDV mempunyai pelbagai patotip, dari tidak menunjukkan gejala hingga strain virulen. Patotip virulen, dikenali sebagai velogenik dan mesogenik, membawa ancaman yang besar kerana boleh menyebabkan penyakit yang teruk dan kadar kematian yang tinggi dalam kumpulan ternakan ayam. Burung yang dijangkiti dengan strain virulen boleh menunjukkan pelbagai tanda klinikal, termasuk kesukaran bernafas, gangguan sistem saraf, cirit-birit, dan penurunan mendadak dalam pengeluaran telur. Kaedah semasa untuk mendiagnos patotip NDV yang virulen adalah menggunakan 'Mean death time (MDT)', 'intra-cerebral pathogenicity index (ICPI)', 'intra-venous pathogenicity index (IVPI)' dan urutan tindakbalas berantai polimeras untuk menentukan kehadiran polibasik asid amino motif di tapak belahan protein fusion (F). Walaubagimanapun, kaedah ini boleh mengambil masa dan memerlukan kemudahan makmal yang khusus. Pembangunan kaedah diagnostik yang teguh dan boleh dipercayai adalah sangat penting untuk mengesan strain NDV yang virulen. Kajian ini bertujuan untuk menubuhkan Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) untuk mengesan virus ND pathogen. Keputusan yang terhasil telah disahkan lagi oleh transkripsi terbalik-tindakbalas berantai polimeras (RT-PCR) dan pengurutan RT-PCR untuk menentukan kehadiran urutan asid amino polibasik di tapak pemecahan protein F virus virulen. Kaedah RT-LAMP menjanjikan pengenalpastian cepat dan tepat bagi strain NDV yang virulen. Keputusan yang diperoleh dari kajian ini menggunakan sampel yang disimpan di makmal menunjukkan bahawa RT-LAMP yang direka oleh kami dapat mengesan strain NDV yang virulen, yang disahkan dengan RT-PCR menggunakan primer spesifik untuk virus NDV virulen. Untuk pengesahan lanjut, pengurutan asik amino telah dilakukan dan didapati kaedah diagnostik RT-LAMP dan RT-PCR memberikan penemuan yang konsisten dengan kaedah pengurutan asik amino. Kedua-dua teknik RT-LAMP dan RT-PCR menunjukkan sensitiviti dan spesifisiti yang tinggi, namun, disebabkan kaedah RT-LAMP lebih ringkas dan cepat, ia lebih disukai untuk mendiagnosa strain NDV yang virulen terutama di dalam persekitaran sumber yang terhad. Walaubagaimanapun, keputusan ujian RT-LAMP ini perlu disahkan melalui ujian lanjut RT-PCR dan pengurutan asik amino sebagai ujian molekular piawaian emas. Ini adalah penting untuk pengurusan penyakit tepat pada waktunya dan strategi kawalan jangkitan ND.

Kata Kunci: Virus Virulen Newcastle Disease (NDV), Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP), Reverse Transcription Polymerase Chain Reaction (RT-PCR)

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LIST OF SYMBOLS AND ABBREVIATIONS

NDV	Newcastle Disease Virus
AV	Avirulent
APMV	Avian Paramyxovirus
PCR	Polymerase Chain Reaction
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
F gene	Fusion gene
NCBI	National Centre for Biotechnology Information
WOAH	World of Animal Health organisation
RT-LAMP	Reverse-Transcription Loop-mediated isothermal amplification
LAMP	Loop-mediated isothermal amplification
˚C	Celsius
uL	Microliter
pmol	picomole
F3	Forward outer primer
R3	Reverse outer primer
LF	Forward loop primer
LB	Reverse loop primer
FIP	Forward inner primer
BIP	Backward inner primer

CHAPTER 1

INTRODUCTION

1.1 Introduction

Newcastle disease is a disease of many wild birds and poultry species that has causative agent of virulent strains of Avian Paramyxovirus serotype 1 (APMV-1), from the genus Orthoavulavirus and species avian orthoavulavirus 1. Description of the virulence of NDV strains were created by pathotypes of disease in which defined by clinical signs in birds after experimental inoculation. List in decreasing order of virulence: (1) velogenic, (2) mesogenic, (3) lentogenic and (4) asymptomatic carrier (Swayne et al., 2020). Velogenic form have high mortality rates and are divided into viscetrophic velogenic NDV (vvNDV) and neurotropic velogenic NDV (nvNDV), with the former having haemorrhagic intestinal lesions and latter, with high mortality following respiratory and neurological signs (Alexander & Senne, 2008b).

The gold standard method for diagnosing this disease is by virus isolation and followed with serological or molecular identification. For NDV isolation, processed samples are inoculated in Specific-Pathogens-free (SPF) chicken eggs and then hemagglutination test (HA) is used for virus presence in the allantoic fluid, followed by hemagglutination inhibition test (HI) using NDV specific antisera to identify the virus (Bello et al., 2018). Primary cell cultures can also be used to isolate NDV for instance chicken embryo fibroblasts (CEF), chicken embryo kidney (CEK) and chicken embryo liver cells (CEL), in which the presence of cytopathic effects (CPE) are observed (Bello et al., 2018). Although identified as a gold standard method, viral isolation method takes time and have shortcomings in term of epidemic situations where rapid diagnosis is important.

Molecular based assays can also be used for NDV for more rapid diagnosis compared to the virus isolation. Some of the molecular based assays that can be used are Reverse-Transcription Polymerase Chain Reaction (RT-PCR), Quantitative Polymerase Chain Reaction (qPCR) and Reverse-Transcription Loop Mediated Isothermal Amplification (RT-LAMP). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) targeting the nucleotide sequences of multiple basic amino acid motif at position 113-116 of the fusion (F) protein cleavage site is used to detect and identify the virus pathotype (Dimitrov et al., 2016). The test can rapidly and accurately detect viral genome in clinical samples with high sensitivity especially if appropriate samples are taken (Bello et al., 2018).

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method which can amplify Deoxyribonucleic Acid (DNA) or Ribonucleic Acid (RNA) using RT-LAMP under isothermal conditions rapidly and with high specificity and sensitivity (Kirunda et al., 2012). This method is highly applicable in diagnostic laboratories where sophisticated and advanced equipment are lacking and not readily available. Due to its rapidness and simplicity, LAMP assay is recommended to be used in the detection of virulent NDV in low resource settings especially in the underdeveloped countries and rural areas.

1.2 Research Problem Statement

The current practice NDV diagnosis is by virus inoculation in embryonated chicken egg (ECE) and confirmation using Hemagglutination Assay/ Hemagglutination Inhibition Assay tests (HA/HI), followed by MDT, ICPI, IVPI and sequencing of F protein cleavage site for pathotyping. However, these methods are time-consuming, labour intensive and tedious which are not efficient for outbreak detection. Thus, RT-LAMP that targeting the nucleotide

sequences for polybasic acid amino at F protein cleavage site needs to be established as a rapid, specific and sensitive method for the detection of pathogens for prompt control of the outbreak.

1.3 Research Questions

1.3.1 Can we establish Reverse-Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) to detect the virulent NDV strains (velogenic and mesogenic)?

1.3.2 Can we obtain the same result using RT-PCR and sequencing of amino acid at the F protein cleavage site?

1.4 Research Hypothesis

1.4.1 RT-LAMP can be established to detect the virulent strains of NDV.

1.4.2 Same result is obtained as RT-PCR result and sequencing of amino acid at the F protein cleavage site?

1.5 Research Objectives

1.5.1 To establish RT-LAMP to detect virulent NDV strains.

1.5.2 To compare the RT-LAMP result with RT-PCR result and sequencing of amino acid at the F protein cleavage site.

CHAPTER 2

LITERATURE REVIEW

2.1 Newcastle Disease Virus

Newcastle disease is a negative sense single- stranded and non-segmented RNA genomes belongs to order Mononegavirales, family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Avulavirus.* Newcastle disease virions are roughly spherical, 150 nm or more in diameter in length. There are densely packed fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins, 17 nm in length on the virion surface (Swayne *et al*., 2020). The nucleocapsid protein appears in herringbone type pattern and associated with phosphoprotein (P) and large polymerase protein (L) (Figure 1) (Mast & Demeestere, 2009).

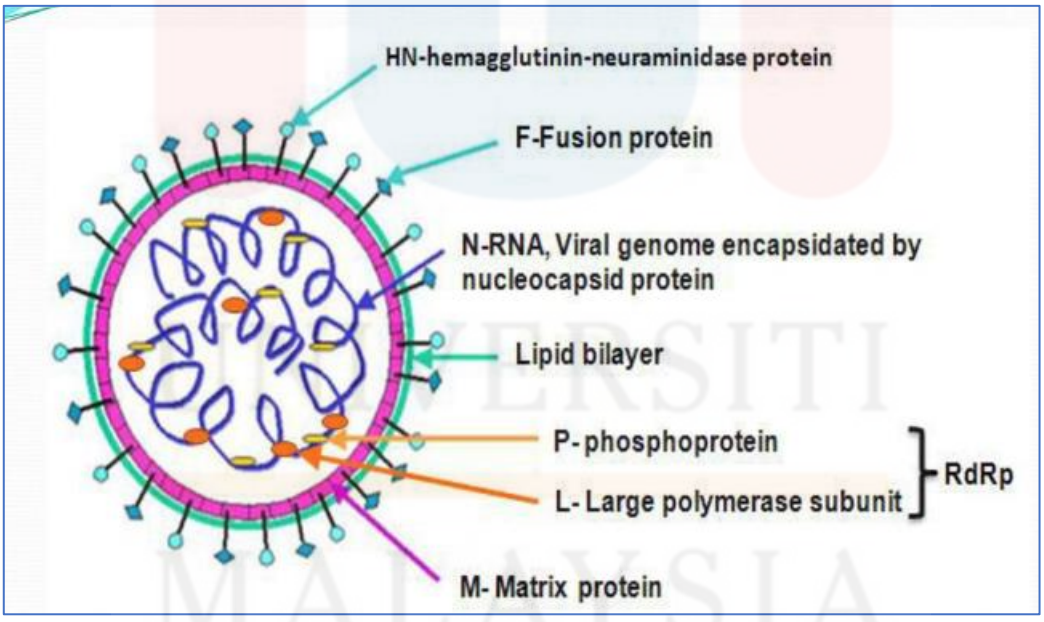

Figure 1 : Schematic structure of Newcastle Disease Virus (Abdisa & Tagesu, 2017*)*

2.2 Determinants Of Virulent NDV

For the pathogenicity of this virus, the virulence determinants depend on the F gene, HN gene, and replication complex (NP/P/L genes). NDV entry into the host cells requires the

activation of viral envelope fusion glycoprotein through the cleavage of precursor glycoprotein F0 into F1 and F2. Virulent strains have a multibasic amino acid sequence motif at the C-terminus of the F2 protein and a phenylalanine at the N-terminus of the F1 protein that are cleaved intracellularly by ubiquitous furin-like proteases found in most tissues (Swayne *et al*., 2020). The major determinant of systemic replication associated with VNDV is in the difference of protease activation. HN gene has the property to bind at the sialic acid receptors on the surface of red Blood Cells, agglutinating the RBCs (Bang & Foard, 1952). Ito *et al.,* (1999) has showed that the ability to agglutinate RBCs varies depending on the NDV strains, the species the RBCs is collected from and the species from which the virus isolated. The NP,P and L proteins form an active ribonucleoprotein (RNP) complex which is involved in this disease virulence. However, NDV virulence has a complex trait due to the determinants of multiple genes, with the minimum requirement of multiple basic amino acid cleavage motif at F0.

The mean death time (MDT) assessment in embryonated chicken eggs (Hanson et al., 1955), the intravenous pathogenicity index (IVPI) in six-week-old chickens (Alexander DJ, 1988), and the intracerebral pathogenicity index (ICPI) in one-day-old chickens (Alexander DJ, 2005) serve as valuable in vivo tests for evaluating virulence. MDT and IVPI are not deemed sufficiently dependable for NDV isolates characterizing in suspected outbreak situations due to imprecision especially when assessing viruses from hosts other than chickens (Alexander DJ,1988)(Pearson et al., 1987). MDT is the mean time in hours for the minimal lethal dose to kill inoculated embryos and different virulence have different MDT. Based on FAO, velogenic strains have MDT less than 60 hours, mesogenic strains have MDT in between 60 and 90 hours, lentogenic strains have MDT more than 90 hours and avirulent does not kill the embryo.

2.3 Diagnostic Methods for NDV Detection

Viral isolation method is considered the benchmark for confirming ND and is commonly used to validate findings from other detection techniques (Bello et al., 2005). The selection of samples necessary for virus isolation depends on where the virus replicates and how it spreads. For live birds, the required samples typically involve cloacal and oropharyngeal swabs taken in an isotonic solution, with or without antibiotics. If the birds are in a severely weakened state or have recently deceased, the samples should encompass lungs, kidneys, liver, intestines, spleen, and caecal tonsils collected separately or as a combined pool, alongside cloacal and oronasal swabs. NDV isolation can also be conducted in primary cell cultures like chicken embryo fibroblasts (CEF), DF1, chicken embryo kidney (CEK), chicken embryo liver (CEL) cells, and avian myeloblasts (QM5), all exhibiting a high susceptibility to the virus (McGinnes et al., 2006). These cells are exposed to clinical samples and observed for cytopathic effects (CPE) like cell rounding, syncytia formation, and cell demise (Ravindra et al., 2009).

Reverse transcription polymerase chain reaction (RT-PCR) is a widely used molecular technique for the detection of Newcastle Disease Virus (NDV). This method involves the conversion of viral RNA into complementary DNA (cDNA) through reverse transcription, followed by the amplification of specific NDV gene targets using polymerase chain reaction (PCR). RT-PCR has been extensively utilized in various studies for the detection and characterization of NDV due to its high sensitivity and specificity. The technique allows for the amplification of NDV RNA, enabling the differentiation of virulent and non-virulent strains. RT-PCR has been used to detect NDV in various sample types, including organ samples, tissue homogenates, and faecal samples, demonstrating its versatility in NDV detection. Additionally, RT-PCR has been used to differentiate between different pathotypes of NDV, such as velogenic, mesogenic, and lentogenic strains, through the development of

multiplex RT-PCR assays. The technique has also been applied for the rapid and accurate detection of NDV, providing a valuable tool for disease surveillance and control. Furthermore, RT-PCR has been utilized in the development of novel strategies for the molecular pathotyping of NDV, allowing for the simultaneous detection and discrimination of low-pathogenic and virulent strains. Overall, RT-PCR has proven to be an essential and effective method for the detection and differentiation of virulent NDV strains, contributing to the understanding and management of NDV infections (Qian et al., 2022) (Wise et al., 2004).

Loop-mediated isothermal amplification (LAMP) principle is with a strand displacement reaction that forms a stem loop structure followed with sensitive and specific amplification at the target template. LAMP test can detect six independent regions during the amplification which increase the specificity (Bello et al., 2018). For visualisation of the result, a green dye is added to the LAMP mixture before the isothermal incubation. A study evaluating the LAMP assay for the detection of NDV directly from culture isolates and clinical samples showed that LAMP was as sensitive and specific as nested PCR (Pham et al., 2005). Another study developed a real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the rapid detection of genotype VII of Newcastle disease viruses. This method combines the advantages of real-time PCR and LAMP, providing a more rapid and sensitive detection method for NDV (Selim et al., 2022).

2.4 Impact Of NDV

The impact of NDV outbreaks have a significant economic value on human welfare, especially in rural places such as village or backyard chickens, which they are crucial food and income source. The disease causes up to 100% mortality in non-vaccinated birds, with transmission through infected saliva and faeces. Financial help was dispensed for countries with industrialized poultry production to prevent ND or prevent losses, to maintain a ND-free

status or eradicate ND after an outbreak (Swayne *et al*., 2020). ND is endemic in Malaysia with outbreaks occurred in backyard chickens and in commercial farms (Shohaimi *et al*., 2013). A study was conducted by Veterinary Research Institute (VRI) from 2004 until 2009 that out of 318 ND cases, chicken comprises 249 cases (78%); duck, 48 cases (15%) and other avian 21 cases (7%) (Leow *et al*., 2011). This supports the World Organization for Animal Health (WOAH) stated that chickens are the most susceptible to ND. A very high number of NDV cases with high mortality in broilers and lower prevalence in layers, breeders and native broilers were reported by field veterinarians towards the end of 2010 to 2011 in Malaysia (Jaganathan *et al*., 2015).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Samples Information

Six confirmed Newcastle Disease Virus samples were taken from the archives of the Virology Laboratory, Faculty of Veterinary Medicine (FPV), UMK.

3.2 RNA Extraction of NDV

The RNA of the NDV archived samples were extracted using GENEzol reagent (Geneaid Biotech Ltd, Taiwan) which contains phenol, chloroform, and guanidine isothiocyanate. The RNA extraction was done following the manufacturer's instructions. The extraction starts by adding 750 L of GENEzol reagent into a 250 L homogenate sample and incubated at room temperature for 5 minutes. A total of 200 L chloroform was added to the tube and shaken vigorously for 15 seconds using a vortex. Then, all tubes containing samples were placed into a chill centrifuge and centrifuged at 14000 g for 15 minutes at 4˚C and the precipitation of RNA could be observed at the bottom of the tubes as white or translucent pellets. A total of 600 L of supernatant (aqueous phase) was transferred into a new tube using a micropipette to be discarded. A total of 600 L of isopropanol was added to the pellet and kept at room temperature for 10 minutes. It was then continued for centrifugation at 14000g for 10 minutes at 4˚C. The RNA precipitate formed a white pellet at the bottom of the tubes. The supernatant was removed, and the pellet was air-dried for 15 minutes. A total of 30 L of nuclease free water was added into the pellet in each tube and it was mixed by pipetting gently up and down. The tubes were incubated at 60˚C for about 15 minutes in a dry water bath and were stored in -80˚C freezer until further use.

3.3 RT-LAMP

The reaction was carried out by making a 25 L reaction mixture using 2X IsoFast Bst Mix reagent kit (PCR Biosystems, UK). Prior to this mixture, 5 L of extracted RNA and 5 L of known sample of Avian Influenza virus and Infectious Bronchitis Virus was pre-heat at 90 ˚C for 5 minutes using Thermocycler (Biorad T100, USA). The master mix preparation was indicated as in Table 1. Forward and reverse primers (Table 2) were designed manually by aligning the reference sequences of S1 Fusion (F) genes that covering the F cleavage site of NDV velogenic and mesogenic strains from the National Center for Biotechnology Information GenBank (NCBI-GenBank) using ClustalW program (GenomeNet, Kyoto University Bioinformatic Center). The specificity of the primers was checked using NCBI-Basic Local Alignment Search Tool (NCBI-BLAST) against all the sequences from the NCBI-GenBank. Negative control was also included. The samples were heated at 63˚C in a dry water bath for 2 hours. The products of RT-LAMP were visualised using naked eyes by looking at the colour changes.

Table 1 : RT-LAMP mixture, Volume and Concentration of reagent

Component	**Volume (μL)**
2X Isofast Bst Mix	12.5
20X Fluorescent Dye	1.25
Rtase Go enzyme	2
Reverse Transcriptase	0.5
Bst polymerase enzymes	0.5
RNase Inhibitor	0.5
Complete Forward primers	1
Complete Reverse primers	1

Nuclease free-water	0.75
Total volume	**25 μL**

Table 2 : Designed RT-LAMP primers based on F1 gene that flanked F cleavage site

Primer	Primer Sequence	Source
NDF3	5'-GRA TTG TAR TAA CAG GAG A-3'	This study
NDFIP	5'-ATG GAR TCG CCA AGR GGA GTT TTT CAA GTT GCT YCC KAA TAT GC- 3'	
NDLF	5'-AGY ARN GTA GTC ART GTT C -3'	
NDBIP	5'-GAT MCA AGG GTC YGT GTC CTT TTT TGC ACC TAT AAA GCG TTT -3'	
NDLB	5'-CAC GTC CGG AGG AAG GAG AC-3'	
NDB3	5'-CTC CAA GAG CGA CAC TGC C-3'	

3.4 Specificity Of RT-LAMP Evaluation

The specificity of RT-LAMP assay was evaluated using two other RNA viruses that commonly affected poultry with similar clinical signs of respiratory signs which are Avian Influenza Virus and Infectious Bronchitis Virus.

3.5 RT-PCR

3.5.1 RT-PCR using NDV primers (MV1 and B2) and velogenic NDV primers (NDFIP and NDBIP)

The reaction was performed in a 25 μL reaction mixture using AccessQuick RT-PCR reagent kit (Promega, USA) from the extracted RNA products from the archived samples. In RT-PCR reaction, 5 μL of the extracted RNA product from the samples were preheated at 95˚C for 5 minutes in a Thermocycler (Biorad T100, USA). The samples were then immediately put

on ice. The preheated samples will be added into 20 μL of reaction mixture accordingly that contained 12.5 μL of 2x AccessQuick buffer, 0.5 μL of AMV reverse transcriptase enzyme, 1 μL of forward and reverse primer with 20 pmol concentration, 0.5 μL (8U) Taq Polymerase, 4.3 μL of Nuclease free Water following the protocol by the manufacturer (Promega, USA) with modification. RT-PCR reaction was carried out using the RT-PCR conditions as shown in Table 3 (common detection using MV1 and B2 primers) and Table 4 (NDV velogenic detection using NDFIP and NDBIP primers) using Thermocycler (Biorad T100, USA). The published MV1 and B2 primers (Table 5) were used in the common detection of NDV (Boucher et al., 2010). Designed FIP and BIP (Table 6) of the RT-LAMP primers were used in the RT-PCR for the detection of velogenic NDV.

Table 3 : RT-PCR conditions and cycles using MV1 and B2 primers for common NDV detection

Reaction	**Temperature (˚ C)**	**Duration (Min)**	**Number of Cycles**
Pre-heat of RNA sample	90	5	-
cDNA Synthesis	45	60	1
RT Inactivation	65	5	1
Denaturation	90	0.5	35
Annealing	50	0.5	35
Extension	72	0.5	35
Post-extension	72	7	1
Hold	15	∞	

Table 4 : RT-PCR conditions and cycles using FIP and BIP primers for velogenic NDV detection

Reaction	Temperature (˚C)	Duration (Min)	Number of Cycles
Pre-heat of RNA sample	90	5	-
cDNA Synthesis	45	60	1
RT Inactivation	65	5	1
Denaturation	90	0.5	35
Annealing	56	0.5	35
Extension	72	0.5	35
Post-extension	72	7	1
Hold	15	∞	

Table 5: RT-PCR primers for common NDV detection using MV1 and B2 primers

Primer	Sequence	Volume and Concentration	Product size (bp)	Reference
MV1 (Reverse)	5'- CCY RAA TCA YYR YGR YRC YRG ATA A-3'	1 μL, 20 pmol	572	Boucher et al, 2010
B2 (Forward)	5'- KCR GCR TTY TGK KTG KCT KGT AT-3'	1 μL, 20 pmol		

Table 6 : RT-PCR primers for velogenic NDV detection using NDFIP and NDBIP primers

Primer	Sequence	Volume and Concentration	Product size (bp)
NDFIP (Forward)	5'- ATG GAR TCG CCA AGR GGA GTT TTT CAA GTT GCT YCC KAA TAT GC -3'	1 μL, 20 pmol	223
NDBIP (Reverse)	5'- GAT MCA AGG GTC YGT GTC CTT TTT TGC ACC TAT AAA GCG TTT -3'	1 μL, 20 pmol	

3.6 Gel Electrophoresis

RT-PCR products were run in 1.5% agarose gel to observe the presence of expected size of the bands. Approximately around 1.2g of agarose powder and 80ml of 1 x Tris-acetate-EDTA buffer was mixed inside the Schott glass bottle and microwaved for 3 minutes using medium heat until fully dissolved. The bottle was taken out and temporarily cooled down under running tap water. One μL of Midori Green DNA stain (NIPPON Genetics, Japan) was pipetted to the warm agarose gel and the bottle was swirled to mix the solution. The gel solution was poured into the gel casting tray with 25 wells-comb and was allowed to solidify in 20 minutes. The solidified agarose gel was loaded into the gel box that was filled with 1 x TAE buffer which was enough to cover the solidified gel. Five μL of 100 bp DNA ladder was loaded into the first lane of the well. Five μL of individual RT-PCR product was mixed with 1 μL of 6X loading dye before pipetted into respective lane. Negative control was pipetted to the last lane. The gel was run at 100V for 40 minutes from negative to positive charge until the dye covered 2/3 of the gel. The gel was then visualized with Gel Doc Imager (Bio-Rad, USA).

3.7 Purification By Column Elution Of RT-PCR For Sequencing

Column extraction of RT-PCR product was done with Geneaid extraction kit following the protocol described by manufacturer. Sample preparation was conducted by adding 200 μL of DF Buffer into 40 μL of RT-PCR samples product in 1.5 microcentrifuge tube.

3.7.1 DNA Binding

The 240 μL sample mixture was transferred into the DF Column that was already been attached to 2 ml Collection tube. The mixture was centrifuged at 14 000g for 30 seconds. The flow-through was discarded and the DF Column was placed back to the same 2 ml Collection tube.

3.7.2 Wash

The 600 μL Wash Buffer with ethanol was added to the centre of the DF Column and left for 1 minute at room temperature. The DF Columns and Collection tubes were centrifuged at 14 000g for 30 seconds and flow-through was discarded. The DF Column and Collection tubes were centrifuged again at 14 000g for 3 minutes.

3.7.3 DNA Elution

The dried DF Columns were transferred to new 2 ml Collection tubes respectively. 20 μL of pre-heated Elution Buffer was added into the centre of column matrix and left for 2 minutes. The DF Columns and Collection tubes were centrifuged at 14 000g for 2 minutes to elute purified DNA. The DF Columns were discarded and 2 ml Collection tubes was for sequencing.

3.8 DNA Sequencing

DNA sequencing was done by sending the purified RT-PCR products to the Apical Scientific Sdn. Bhd. The obtained nucleotide sequences were analyzed using Bioedit ver 7.2.5

(Informer Technologies, Inc.) and translated to amino acid sequences using Generunner ver 6.5 (Informer Technologies, Inc.)and the presence polybasic amino acid motifs were analysed for the sequences of virulent NDV strains.

CHAPTER 4

RESULTS

4.1 RT-LAMP Result

Among six archived samples, three samples (ND2, ND4, ND6) were positive by RT-LAMP by discolouration of the orange dye to transparent colour (Figure 2).

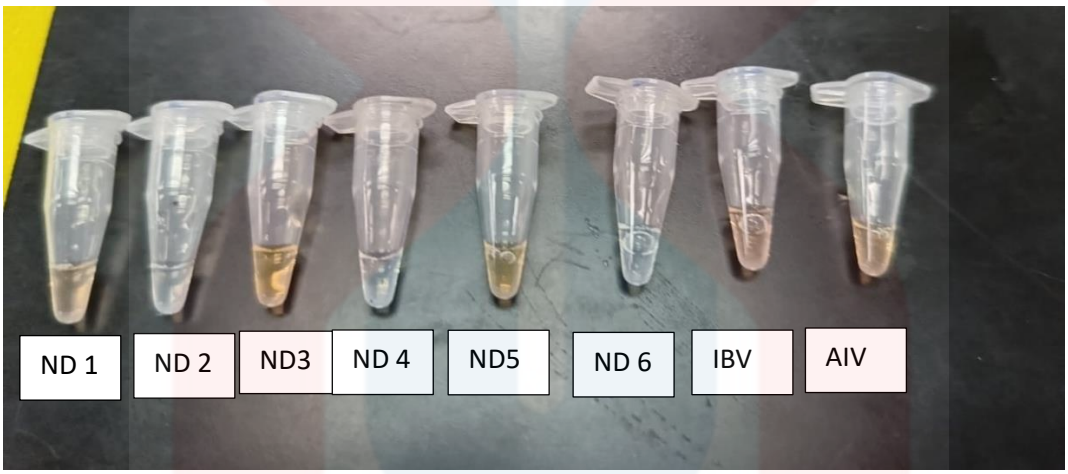

Figure 2 :Detection of RT-LAMP using fluorescent dye by naked eye observation. Samples ND2, ND4 and ND6 showed the transparent discolouration from normal orange dye which means positive for virulent NDV strains, while other samples (ND1, ND3, ND5) and other viruses (Infectious Bronchitis Virus-IBV and Avian Influenza Viru-AIV) showed negative results with retained orange dye colour

4.2 RT-LAMP Specificity

Both Avian Influenza Virus (AIV) and Infectious Bronchitis Virus (IBV) showed negative results by having orange dye colouration without changing to transparent colour. (Figure 2).

4.3 RT-PCR RESULT

Based on the RT-PCR with the 1.5% gel electrophoresis, all samples were positive for NDV as expected using primers MV1 and B2 as shown in Table 7 and all isolates shown expected bands of 572 bp (Figure 3), with no band observed in the negative control. Based on the RT-PCR using primers NDFIP and NDBIP for detection of virulent NDV strains, samples ND2, ND4 and ND6 were positive (Table 8) with the expected band of 236 bp (Figure 4).

Table 3 : RT-PCR result using MV1 and B2 primers

Sample	Result
ND1	Positive
ND2	Positive
ND3	Positive
ND4	Positive
ND5	Positive
ND6	Positive

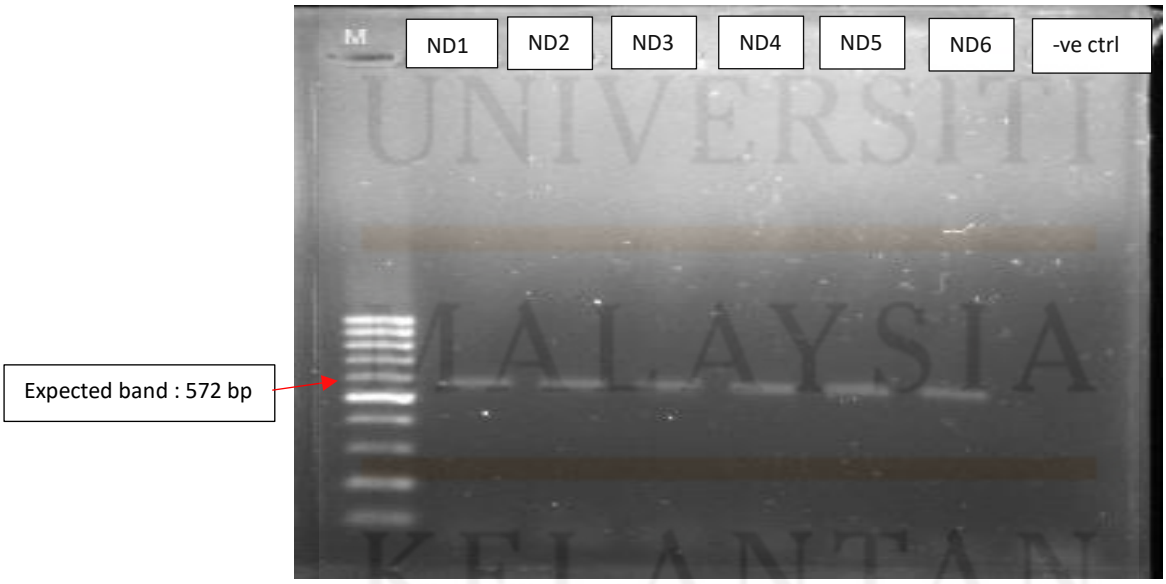

Figure 3 : RT-PCR of NDV archived samples using MV1 and B2 primers

Table 4 : RT-PCR result of virulent NDV strains using NDFIP and NDBIP primers

Sample	Result
ND1	Negative
ND2	Positive
ND3	Negative
ND4	Positive
ND5	Negative
ND6	Positive

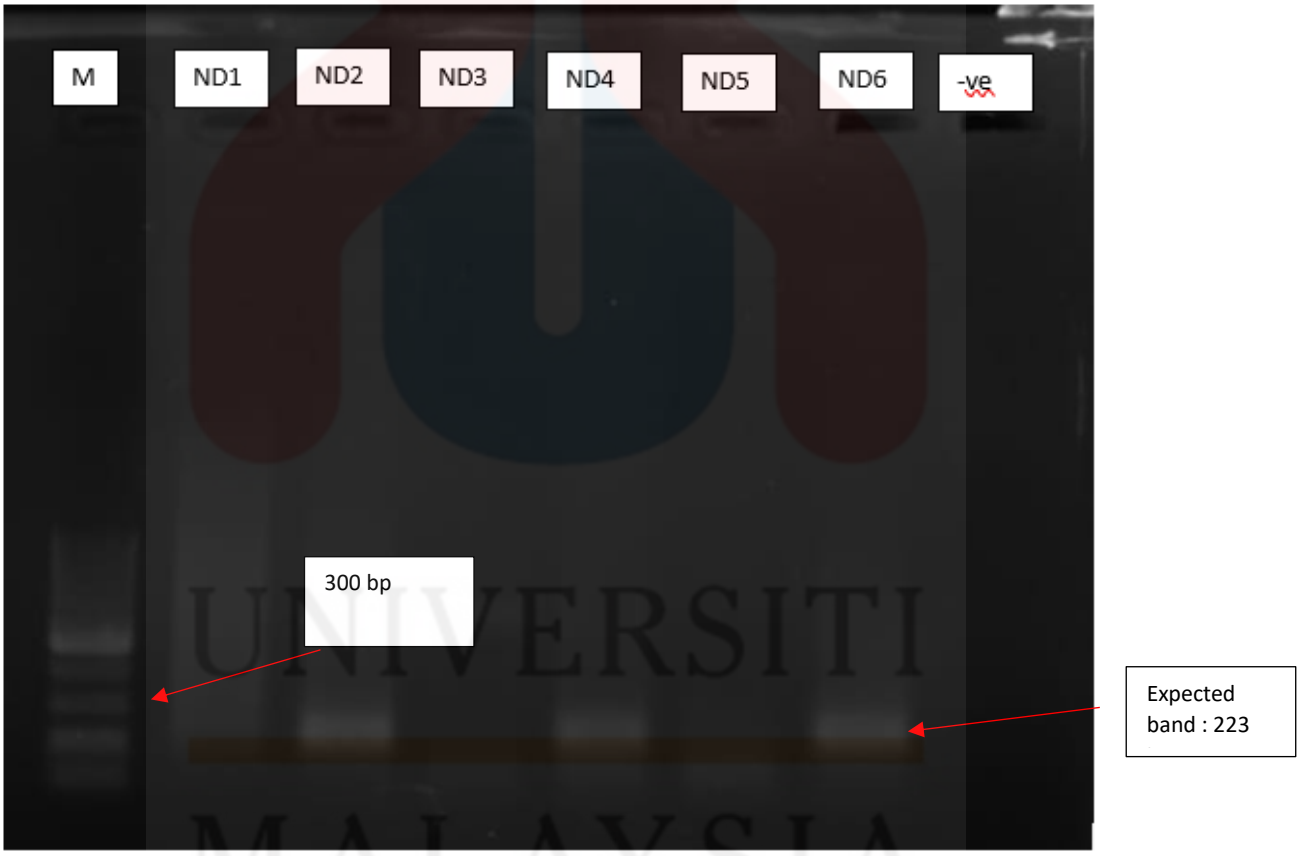

Figure 4 : RT-PCR of archived samples using NDFIP and NDBIP primers

4.4 Nucleotide And Acid Amino Sequences At F Cleavage

The nucleotide and amino acid sequences at the F cleavage site were observed as below (Figure 5).

Sample	DNA Sequence (5' to 3')	Amino Acid Sequence
ND1	TCTATCCGTAGGATACAAGAGTCTGTG ACTACATCTGGAGGGGGGAGACAGGG GCGCCTTATAGGCGCCATTATTGGCGGT GTGGCTCTTGGGGTT	NRTLTTLLTPLGDSIRRIQES VTTSGGGRQGRLIGAIIGGV ALGV AVIRULENT
ND2	ATTCCATCCGCAAGATCCAAGGGTCCG TGTCCACGTCTGGAGGAAGGAGACGG AAACGCTTTATAGGTGCAAAAAAGGAC ACAGACCCTTGTATCA	SIRKIQGSVSTSGGRRRKRFI GAKKDTDPCI VIRULENT
ND3	TCTATCCGTAGGATACAAGAGTCTGTG ACTACATCTGGAGGGGGGAGACAGGG GCGCCTTATAGGCGCCATTATTGGCGGT GTGGCTCTTGGGGTT	NRTLTTLLTPLGDSIRRIQES VTTSGGGRQGRLIGAIIGGV ALGV AVIRULENT
ND4	TATAACAGAACACTGACTACTTTGCTC ACTCCTCTTGGCGACTCCATCCGCAAG ATCCAAGGGTCTGTGTCCACGTCTGGA GGAAGGAGACAAAAACGCTTTATAGGT GCTGTTATTGGCAGTGTCGCTCTTGGA GA	YNRTLTTLLTPLGDSIRKIQG SVSTSGGRRQKRFIGAVIGS VALGY VIRULENT
ND5	CAACAGGACATTGACTACTTTGCTCAC CCCCCTTGGTGATTCTATCCGTAGGAT ACAAGAGTCTGTGACCACGTCCGGAG GAGGGAAACAGGGACGTCTTATAGGC GCCATTATCGGTGGTGTAGCTCTCGGG GTT	NRTLTTLLTPLGDSIRRIQES VTTSGGGKQGRLIGAIIGGV ALGV AVIRULENT
ND6	TATAATAGAACATTGACTACATTGCTCA CTCCCCTTGGCGATTCCATCCGCAAGAT CCAAGGGTCCGTGTCCACGTCTGGAGG AAGGAGACGGAAACGCTTTATAGGTGC CATTATTGGCAGTGTCGCTCTTGGAGT	YNRTLTTLLTPLGDSIRKIQG SVSTSGGRRRKRFIGAIIGSV ALGV VIRULENT

Figure 5 : DNA and amino acid sequences at fusion protein cleavage site of NDV isolates

CHAPTER 5

DISCUSSION

NDV is classified as a list of 'A' disease with immediate report needs to be done to World Organisation for Animal Health (WOAH) for the outbreak of mesogenic or velogenic ND, which are characterised as virulent strains (OIE, 2012). It is endemic in Malaysia, the last reported case of ND was in 2023 which was caused by virulent strains of the sub-genotype VII.1.1 at Sabah and the strain is genetically close to Iran isolates (Syamsiah et al., 2022). The source for the new sub-genotype VII.1.1 introduction in Malaysia remained unknown. Proper disease control and prevention with continuous surveillance are recommended to be performed. Here, we established RT-LAMP assay for accurate and rapid detection of virulent NDV pathotypes, mesogenic and velogenic strains.

RT-LAMP method is more rapid and simple compared to RT-PCR. A simple instrument for amplification of genomic RNA such as water bath and heat block can be carried out for RT-LAMP test within an hour or two which were more rapid and less time consuming as compared to RT-PCR. Meanwhile, RT-PCR requires thermocycler, a more expensive and complex machine with longer duration of hours for it to be done. RT-LAMP result also can be visually detected by colour change, without usage of agarose gel electrophoresis (Song et al., 2018). To confirm the virulent strains of NDV that were detected using the one-step RT-LAMP using designed primers for virulent ND strains, RT-PCR with NDFIP and NDBIP primers was done. The results of both tests were in agreement with each other. The detection of virulent NDV strains using specific primers in RT-PCR involves the targeted amplification of unique genetic sequences associated with virulence (F fusion cleavage site). Putri et al. utilized RT-PCR with specific primers targeting the M and F genes to detect and differentiate virulence NDV strains,

which importance of primer specificity is emphasized in this. Kim et al. also designed primers that target the large polymerase protein (L) gene and the fusion protein (F) gene of NDV for detection of virulent NDV. The RT-LAMP test with six primers targets the HN and F genes which responsible for encoding the surface glycoproteins of the viral envelope, which responsible for attachment to cell surface receptors and fusion between the cellular and viral membranes (Roohani et al., 2015)(Glickman et al., 1988).

Amino acid motifs at the F cleavage sites were analyzed for further confirmation as WOAH requires these sequences for the gold standard technique for virulent NDV. Viral F protein (Fcs) sequences of natural isolates are classified into virulent Fcs (VFcs) with the motif "^{112}G/R/K-R-Q/R/K-R/K-R↓F^{117}" (R, arginine; K, lysine; Q, glutamine; G, glycine; F, phenylalanine; Arrow, cleavage position) and the avirulent Fcs (AFcs) with the motif "^{112}G/R/E-R/K/Q-Q-G/E-R↓L^{117}" from 112 to 117 position (Wang et al., 2017). The change from phenylalanine (F) to lysine (L) at position 117 of the fusion protein, which has the polybasic amino acid Fcs motif "^{112}RRQKR↓F^{117}", has been shown to block syncytium formation, indicating its significance in virulence (Wang et al., 2017). In this study, three samples ND2, ND4 and ND6 are virulent with the virulent acid amino motifs (R-R-R-K-R-F, R-R-Q-K-R-F and R-R-R-K-R-F, respectively) present at position 112 and 117, while the other three samples ND1, ND3 and ND5 were avirulent NDV strains (G-R-Q-G-R-L, G-R-Q-G-R-L and G-K-Q-G-R-L, respectively).

The specificity of this research NDV RT-LAMP against other viruses which are Avian Influenza Virus and Avian Infectious Bronchitis Virus yields good result of negative towards these avian respiratory pathogens. This means that this RT-LAMP primers only specific to virulent NDV. Other avian viruses with similar respiratory clinical signs such as avian reovirus

and infectious laryngotracheitis virus were not included in this study due to the unavailability of these viruses in our archives.

During the course of our study, one of the limitations is the inadequacy of the samples in our achieves which associated with the sample size. It is widely known that a larger sample size facilitates a more comprehensive dataset. Another limitation is that the sensitivity of RT-LAMP was not performed due to the time constraint of this research. To assess the sensitivity of our RT-LAMP, this method needs to be conducted on different concentrations of the viruses to observe the detection limit by using 10-fold serial dilutions of known Hemagglutination assay test (HA) titre of NDV to check the detection limit. For instance, a study published in ACS Sensors reported that RT-LAMP has been used for Newcastle Disease Virus (NDV) detection with a sensitivity of 96.8%. The study used a 10-fold serial dilution of the NDV RNA standard from 10^6 copies/μL to 1 copy/μL to determine the sensitivity of the RT-LAMP assay (Tian et al., 2016). Another study published in the same journal used a 10-fold serially diluted RNA standard from 10^6 copies/μL to 1 copy/μL to determine the sensitivity of a multiplex, real-time, point-of-care RT-LAMP assay for SARS-CoV-2 detection. Therefore, serial fold dilution is a reliable method to check the sensitivity of RT-LAMP in virus detection, including NDV. The application of RT-LAMP in the field is highly suggested as it offers simplicity, rapidity, and high sensitivity which make it a promising tool for the field screening of virulent NDV strains, thus, contributing to a timely disease management and control.

CHAPTER 6

CONCLUSION

In conclusion, RT-LAMP was successfully established in this study that can be used to detect virulent NDV. The results were further confirmed by RT-PCR and sequencing of the F cleavage site for the presence of polybasic amino acid motifs for virulent NDV strains which is required by WOAH for NDV disease reporting. It shows 100% sensitivity in comparison to these two methods. This RT-LAMP offers rapid and simple diagnostic methods which is important for prompt disease management and surveillance strategies in veterinary medicine.

CHAPTER 7

RECOMMENDATION

For a better understanding and input of data, I recommend using more samples for future project and to have different virus concentrations for sensitivity test. More viruses affected avian should also be used to check the specificity of RT-LAMP for virulent NDV detection such as laryngotracheitis virus (ILV) and low-pathogenicity avian influenza virus (LP-AIV).

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