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DETECTION AND MOLECULAR CHARACTERISATION OF GENOTYPE VII NEWCASTLE DISEASE VIRUS ISOLATES FROM COMMERCIAL CHICKENS IN PENINSULAR MALAYSIA

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SUMMARY

Newcastle disease (ND) caused by Newcastle disease virus (NDV) is a significant threat to the global poultry industry, with recurring outbreaks of varying virulence. In Malaysia, NDV has been circulating since its first detection in 1934, with genotype VII being the predominant strain in recent years. This study aimed to characterise NDV isolates from commercial poultry in Peninsular Malaysia between 2021 and 2022, during the COVID-19 pandemic. Nineteen suspected ND cases from various states were investigated through virus isolation, RT-PCR, molecular characterisation, and pathotyping. The F gene was sequenced, revealing that all isolates contained virulent polybasic amino acid motifs at the fusion protein cleavage site. Phylogenetic analysis showed that the isolates belonged to genotype VII, with sub-genotypes VII.2/VIIh, VII.2/VIIi, and VII.1.1 identified. A selected isolate, UPM/NDV/IBS008/2021, exhibited virulence with an intracerebral pathogenicity index (ICPI) of 1.75 and a mean death time (MDT) of 88 hours, classifying it as a velogenic strain. This study underscores the persistent threat of genotype VII NDV, despite vaccination efforts, and highlights the need for continued surveillance and control measures in Malaysia and surrounding regions.

Keywords: F Gene Cleavage site, Genotype VII, Velogenic

INTRODUCTION

Newcastle disease virus (NDV) also known as Avian Orthoavulavirus 1 (AOAV-1) was classified under the genus Orthoavulavirus, subfamily Avulavirinae, and family Paramyxoviridae (International Committee on Taxonomy of Viruses (ICTV) 2019). NDV is recognised as a single serotype virus, but it can be further classified into various pathotypes according to the pathogenicity of NDV (velogenic, mesogenic and lentogenic) strain and the sequencing of the F gene cleavage site (Angeliya et al., 2022).

Malaysia witnessed its first case of Newcastle Disease (ND) in 1934. The virus was isolated from Parit Buntar, Perak (Awang & Ishak, 1993). Ever since the detection of NDV in Malaysia, virulent and avirulent NDV strains of various genotypes have been reported in poultry flocks. Several studies have identified NDV isolates circulating in commercial poultry farms as well as in backyard flocks, indicating continuous viral circulation within different poultry production systems (Berhanu et al., 2010; Shohaimi et al., 2015; Mahamud et al., 2021; Tan et al., 2010). The spread of NDV in poultry worldwide led to a high genetic diversity of NDV that was driven by factors such as viral mutation, host adaptation, and vaccination pressure and the stable emergence of new virus genotypes (Dimitrov et al., 2019).

Since 2000, repeated outbreaks of ND have been reported. For instance, in Peninsular Malaysia, a total of 84 outbreaks and 525,981 cases were reported in 2001 (Tan et al., 2010) and more recent reports indicate that NDV genotype VII continues to circulate within poultry populations in Malaysia affecting both commercial broiler and layer farms as well as backyard poultry (Mahamud et al., 2021). Despite there is a routine vaccine programme in commercial poultry production, outbreaks of Newcastle disease continue to occur (Mahamud et al., 2021; Angeliya et al., 2022). Recently, according to Malouki et al. (2021), the fifth panzootic that is associated with virulent strains of NDV genotype VII, specifically sub-genotypes VIIh and VIIi, is still circulating and being studied in Asia and the Middle East. In Malaysia, ND outbreaks reported from 2000 to 2009 are mainly associated with genotype VII (VIIa, VIIb, VIId, VIIe, and VIIh), which was found in a few bird species with the main isolation from poultry (Shohaimi et al., 2015; Tan et al., 2010). However, the detection of NDV in Malaysia is still being studied, especially NDV genotype VII, which has been circulating in Malaysia, Indonesia, Thailand, Vietnam, Cambodia, and China (Mahamud et al., 2021; Angeliya et al., 2022) for many years. Several of these outbreaks have been associated with genotype VII NDV strains, which are known to be highly virulent and widely distributed globally. Therefore, characterisation of genotype VII isolates is important for improving our understanding of

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NDV epidemiology and supporting effective disease control strategies. This study was conducted to characterise the molecular characteristics of NDV isolated from various commercial poultry flocks from different states in Peninsular Malaysia based on sequence analysis of the F gene and pathotyping one of the isolates based on MDT and ICPI. The samples were received from various farms between 2021 to 2022 during the COVID-19 pandemic.

MATERIALS AND METHODS

Isolation of NDV Isolates from Commercial Chickens

The Laboratory of Vaccines and Biomolecules (VacBio), Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) received tissue samples of suspected NDV cases from various states in Malaysia from 2021 to 2022 for the diagnosis of NDV during the COVID-19 pandemic. The tissue samples include the brain, trachea, cecal tonsil, spleen, proventriculus, intestine, bursa, and kidney from commercial broiler and layer farms. The samples were handled in the Biosafety Level 2 Laboratory (BSL 2) following OIE Guidelines 2017 in the Biosafety Chamber (ESCO, Singapore).

Molecular Characterisation

Virus isolation and Propagation

Each of the samples were propagated into five 10-day-old specific-pathogenic-free embryonated chicken eggs (SPF ECE) and the eggs were incubated for five days at 37 °C with daily candling to track the embryonic mortality. Any embryonic mortality occurring less than 24 hours after inoculation will be discarded and eggs that die after 24 hours were transferred to 4 °C for further analysis. The harvested allantoic fluids were tested for hemagglutination activity by haemagglutination (HA) test according to World Organisation for Animal Health (WOAH) (2021).

RNA Extraction

Viral RNA was extracted from allantoic fluids by using TRIzol reagent (Invitrogen, USA) according to manufacturer protocol and stored at –20℃ (ESCO, Singapore) for future use.

Primer Design

Amplification of the partial F gene with an expected size of 535 bp was performed using the forward primer 5'-ATGGGC(C/T)CCAGA(C/T)CTTCTAC- 3' and the reverse primer 5'-CTGCCACTGCTAGTTGTGATAATCC-3'. The set of primers was designated from a previous study that amplified the nucleotide sequence of the F gene in regions 47–581, including the F0 cleavage site (Berhanu et al., 2010).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

One-step RT-PCR was conducted using the One-Step RT-PCR kit (Biotechrabbit, Germany) with a total reaction volume of 20 µL. Specifically, 12 µL of 2× One-Step Mix was combined with 1 µL of RNAse-free water, 1 µL of RT-RI Blend 20×, 1 µL of forward primer (20 µM), 1 µL of reverse primer (20 µM), and 4 µL of RNA template. A non-template control (NTC) containing RNase-free water served as the negative control, while the NDV strain LaSota was used as the positive control to confirm the successful amplification of the target gene. The PCR mixture was placed in a thermocycler (C1000 Touch Thermal Cycler, Bio-Rad, USA) with the following settings: 10 min at 48 °C for reverse transcription, 3 min at 95 °C for polymerase activation, followed by 40 cycles of 20 s at 95 °C for denaturation, 30 s at 58 °C for annealing, and 20 s at 72 °C for extension, concluding with a final extension of 5 min at 72 °C.

Agarose Gel Electrophoresis

Agarose gel of 1.5% was prepared to resolve the amplified PCR products based on their molecular weight and visualised under ultraviolet light using the Gel-doc XR+ System (Bio-Rad, USA).

Fusion Gene Sequencing and Phylogenetic Analysis

The purified PCR products of the partial F gene from NDV isolates were sent for Sanger sequencing (Apical Scientific, Malaysia). The obtained chromatogram files were visually inspected for sequence quality, and low-quality regions at both ends were trimmed prior to analysis. Forward and reverse sequences were assembled to generate a consensus sequence using the BioEdit software (Hall, 1999). Ambiguous bases were manually checked against chromatogram peaks and resolved where possible; unresolved positions were retained using standard IUPAC ambiguity codes. The resulting consensus sequences were analysed using the BLAST program available through the National Center for Biotechnology Information (NCBI, USA) to identify the closely related NDV strains. Similarities and relationships of the partial F gene of Malaysian NDV isolates were compared with previously published NDV strains from GenBank using the ClustalW method in MEGA v7.0. A phylogenetic tree was constructed using the Maximum-Likelihood method based on the Kimura-2 parameter model (Tamura et al., 2011) which accounts for different rates of transition and transversion substitutions and is commonly used for nucleotide sequence comparisons of viral genes. The robustness of the phylogenetic tree was evaluated using 1000 bootstrap replicates.

Pathotyping of NDV Isolates

Based on the characterisation of molecular analysis of the NDV isolates, one NDV isolate (UPM/NDV/IBS008/2021) was selected to be further characterised in a pathotypic study that includes mean

death time (MDT) and intracerebral pathogenicity index (ICPI) and to further confirm the pathotype.

Biological Characterisation

Intracerebral Pathogenicity Index (ICPI)

The Intracerebral Pathogenicity Index (ICPI) is a method used to determine the pathotype of the UPM/NDV/IBS008/2021 isolates. The ICPI is calculated as the average score based on daily observations of chicks over an 8-day period. Fresh allantoic fluid from UPM/NDV/IBS008/2021 with a hemagglutination (HA) titer greater than 24 was filtered using a 0.45 µm syringe filter (Sartorius, Germany) and diluted 1:10 in sterile PBS. Ten SPF chicks aged between 24 and 40 hours were injected intracerebrally with 0.05 mL of the diluted virus. The chicks were monitored, and their daily scores were recorded every 24 hours for eight days. Chicks were scored as 0 for normal, 1 for unwell, and 2 for dead. The ICPI pathotype classifications are as follows: 1.50–2.0 indicates a virulent/velogenic strain, 0.5–1.5 indicates a mesogenic strain, and below 0.5 indicates an avirulent/lentogenic strain (Alexander & Senne, 2008). The ICPI study was approved by the Institutional Animal Care and Use Committee (IACUC) under AUP number UPM/IACUC/AUP- R040/2022.

Mean Death Time (MDT)

The mean death time (MDT) refers to the average duration required to kill all infected embryos using the minimum lethal dose. The minimum lethal dosage is defined as the highest virus dilution that causes 100% mortality in embryos. MDT categorises NDV isolates into three groups: velogenic (under 60 hours), mesogenic (60 to 90 hours), and lentogenic (over 90 hours) (Alexander 1988). To create a ten-fold serial dilution of UPM/NDV/IBS008/2021 allantoic fluid in 1× sterile PBS, 100 µL of allantoic fluid was combined with 900 µL of PBS, producing dilutions from 10–1 to 10–10. These dilutions were then filtered using a 0.45 µm syringe filter, and 0.1 mL of the inoculum was injected into the allantoic cavity of 10-day-old SPF eggs. The infected eggs were incubated at 37 °C and monitored daily for embryonic mortality over a period of 5 days.

RESULTS

Isolation of NDV from Commercial Chickens

Virus Isolation and RT-PCR

Nineteen ND-suspected cases from commercial chickens from different states in Peninsular Malaysia were diagnosed for NDV detection (Table 1). Tissue samples were homogenised and inoculated into 10-day-old SPF ECE and incubated until 5 dpi for virus propagation and identification. The harvested allantoic fluids were then tested with the HA spot test and RT-PCR. Twelve out of nineteen samples were detected positive NDV for the HA spot test (data not shown) and RT-PCR (Figure 1), respectively, and achieved a consistent 100% embryonic mortality at 3 dpi (Table 2). Many of the samples showed no mortality and were detected negative for HA spot test and RT-PCR (Table 2). Even though some of the samples showed embryonic mortality at 2, 3 and 4 dpi, however, it was detected as negative for the HA spot test and RT-PCR (Table 2).

Table 1. Direct detection of NDV from tissue samples by RT-PCR.

ID	Origin	History	Organs	Year
UPM/NDV/111/2021	Pahang	Broiler	Intestine Trachea Cecal Tonsil	2021
UPM/NDV/112/2021	Johor	Broiler	Cecal Tonsil Trachea Proventriculus	2021
UPM/NDV/113/2021	Pulau Pinang	Broiler	Bursa	2021
UPM/NDV/114/2021	Selangor	Broiler	Cecal Tonsil Spleen	2021
UPM/NDV/115/2021	Johor	Broiler	Trachea Bursa Cecal Tonsil Intestine	2021
UPM/NDV/116/2021	Perak	Layer	Trachea Proventriculus	2021
UPM/NDV/117/2021	Johor	Broiler	Trachea	2021
UPM/NDV/118/2021	Selangor	Broiler	Trachea	2021
UPM/NDV/004/2021	Perak	Layer	Trachea Cecal Tonsil	2021
PM/NDV/007/2021	Pulau Pinang	Native broiler	Trachea	2021

UPM/NDV/008/2021	Selangor	Broiler	Kidney Trachea	2021
UPM/NDV/124/2021	Johor	Broiler	Kidney Cecal Tonsil	2021
UPM/NDV/125/2021	Melaka	Layer	Cecal Tonsil	2021
UPM/NDV/126/2021	Melaka	Layer	Trachea Trachea	2021
UPM/NDV/129/2021	Kedah	Colored broiler	Proventriculus Trachea	2021
UPM/NDV/130/2022	Johor	Broiler	Kidney Trachea	2022
UPM/NDV/131/2022	Selangor	Broiler	Trachea	2022
UPM/NDV/132/2022	Pulau Pinang	Broiler	Cecal Tonsil Cecal Tonsil	2022
UPM/NDV/133/2022	Johor	Broiler	Trachea Brain	2022

Table 2. Isolation of NDV isolates from commercial chickens.

ID	Embryonic mortality					HA Spot test	RT-PCR
	1dpi	2dpi	3dpi	4dpi	5dpi		
UPM/NDV/111/2021	0/5	5/5	N/A	N/A	N/A	+ve	+ve
UPM/NDV/112/2021	0/5	1/5	4/5	N/A	N/A	+ve	+ve
UPM/NDV/113/2021	0/5	5/5	N/A	N/A	N/A	+ve	+ve
UPM/NDV/114/2021	0/5	2/5	3/5	N/A	N/A	+ve	+ve
UPM/NDV/115/2021	0/5	4/5	1/5	N/A	N/A	+ve	+ve
UPM/NDV/116/2021	0/5	2/5	3/5	N/A	N/A	+ve	+ve
UPM/NDV/117/2021	0/5	0/5	0/5	0/5	0/5	-ve	-ve
UPM/NDV/118/2021	0/5	0/5	0/5	0/5	0/5	-ve	-ve
UPM/NDV/004/2021	0/5	0/5	0/5	0/5	0/5	-ve	-ve
UPM/NDV/007/2021	0/5	2/5	3/5	N/A	N/A	+ve	+ve
UPM/NDV/008/2021	0/5	4/5	1/5	N/A	N/A	+ve	+ve
UPM/NDV/124/2021	0/5	0/5	0/5	0/5	0/5	-ve	-ve
UPM/NDV/125/2021	0/5	0/5	0/5	0/5	0/5	-ve	-ve
UPM/NDV/126/2021	0/5	2/5	1/5	1/5	0/5	-ve	-ve
UPM/NDV/129/2021	0/5	3/5	3/5	N/A	N/A	+ve	+ve
UPM/NDV/130/2022	0/5	0/5	5/5	N/A	N/A	+ve	+ve
UPM/NDV/131/2022	0/5	4/5	1/5	N/A	N/A	+ve	+ve
UPM/NDV/132/2022	0/5	0/5	2/5	0/5	0/5	-ve	-ve
UPM/NDV/133/2022	0/5	5/5	N/A	N/A	N/A	+ve	+ve

Note. HA = Hemagglutination; RT-PCR = Reverse transcriptase-polymerase chain reaction

Molecular Characterisation of NDV Isolates

RT-PCR Amplification of Fusion Gene

All twelve NDV isolated from commercial chickens were amplified at 535 bp, the expected amplicon size of the partial F gene of NDV (Figure 1). The samples were then purified and sent for Sanger sequencing. The sequence analysis of all twelve NDV isolates was analysed using Mega v7.0 to determine the amino acid sequences of the F cleavage site. The results indicated that the isolates were detected as velogenic NDV pathotype with polybasic amino acid motifs at position 112-116 and phenylalanine motif at position 117, as shown in Table 3.

Phylogenetic Analysis of Fusion Gene

The positive NDV isolates showed high nucleotide similarity (>96.50%) to previously reported genotype VII NDV strains based on partial F gene sequence alignment. The highest sequence identity was observed with NDV isolate UPM/NDV/IBS007/2021 and UPM/NDV/IBS008/2021 when compared to NDV genotype VII isolate from Sabah (GenBank accession no. ON210972.1), indicating a close genetic relationship with

circulating genotype VII viruses. Phylogenetic analysis was performed using partial F gene nucleotide sequences from the twelve NDV isolates, including the F protein cleavage site region. The phylogenetic tree was constructed using the Maximum Likelihood method based on the Kimura two-parameter model. The analysed sequences were compared with representative NDV sequences of genotypes I–XXI obtained from GenBank (Figure 2), showed that all twelve Malaysian NDV isolates clustered within genotype VII, supported by strong bootstrap values ranging from 95% to 100%. Further analysis with reference genotype VII sequences (Figures 3A–B) revealed that the majority of isolates grouped within sub-genotype VIIh, forming a well-supported cluster with bootstrap values of 99–100%. Two isolates (UPM/NDV/IBS007/2021 and UPM/NDV/IBS008/2021) clustered within sub-genotype VIIi with strong bootstrap support (98%), while one isolate (UPM/NDV/IBS129/2021) grouped within sub-genotype VII.1.1, showing a close relationship to a strain from Iran (GenBank: MH481362.1) with a bootstrap value of 100%. These clustering patterns indicate the co-circulation of multiple genotype VII sub-genotypes in Malaysia and suggest ongoing viral evolution and regional connectivity of NDV strains.

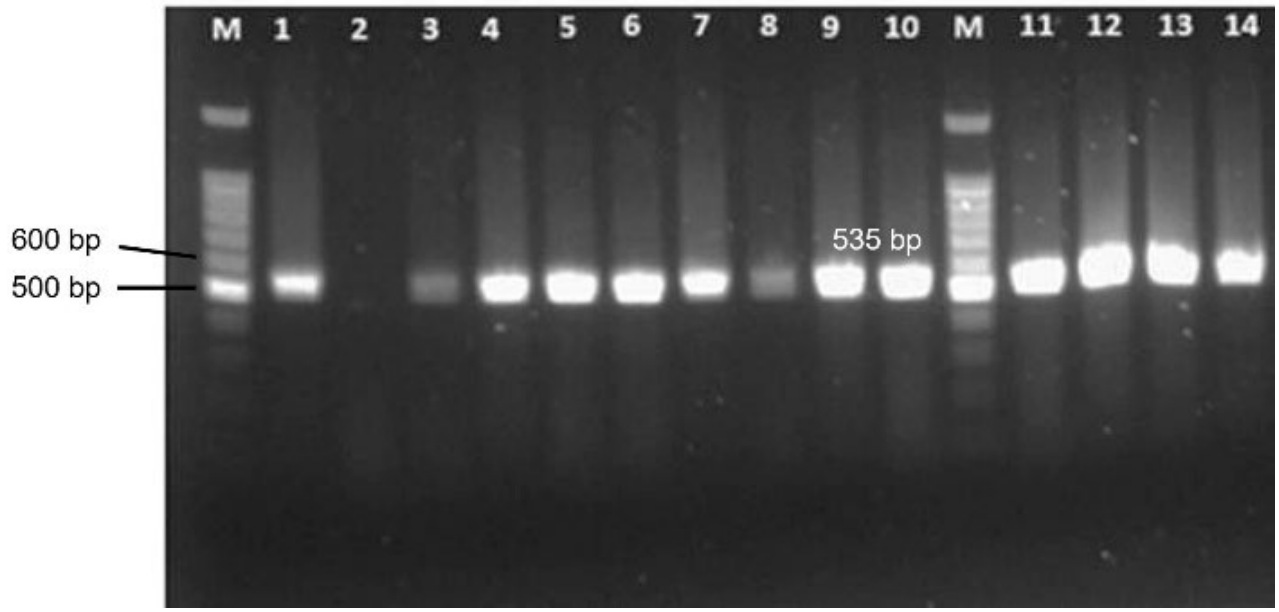

Figure 1. Agarose gel electrophoresis of NDV isolates; positive control LaSota (lane 1), non-template control NTC (lane 2), UPM/NDV/IBS111/2021 (lane 3), UPM/NDV/IBS112/2021 (lane 4), UPM/NDV/IBS113/2021 (lane 5), UPM/NDV/IBS114/2021 (lane 6), UPM/NDV/IBS115/2021 (lane 7), UPM/NDV/IBS116/2021 (lane 8), UPM/NDV/IBS007/2021 (lane 9), UPM/NDV/IBS008/2021 (lane 10), UPM/NDV/IBS129/2021 (lane 11), UPM/NDV/IBS130/2022 (lane 12), UPM/NDV/IBS131/2022 (lane 13), and UPM/NDV/IBS133/2022 (lane 14). Lane M is a molecular weight ladder.

Table 3. Sequence analysis of the F cleavage site

ID	Host	Genotype	F cleavage site	Virulence
UPM/NDV/111/2021	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/112/2021	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/113/2021	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/114/2021	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/115/2021	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/116/2021	Layer	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/007/2021	Native broiler	VII.2/VIIi	^{112}RRQKRF^{117}	Velogenic
UPM/NDV/008/2021	Broiler	VII.2/VIIi	^{112}RRQKRF^{117}	Velogenic
UPM/NDV/129/2021	Coloured broiler	VII.1.1/VIIl	^{112}RRQKRF^{117}	Velogenic
UPM/NDV/130/2022	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/131/2022	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic
UPM/NDV/133/2022	Broiler	VII.2/VIIh	^{112}RRRKRF^{117}	Velogenic

Pathotyping of UPM/NDV/IBS008/2021

Among all twelve positive NDV isolates that have been propagated, the pattern of achieving 100% embryonic mortality for all isolates was on the 2nd and 3rd days post-inoculation (2 dpi and 3 dpi), indicating all isolates showed their virulence properties. Therefore, one of the isolates, UPM/NDV/IBS008/2021, was selected as a representative isolate for further molecular and pathogenicity analysis due to its consistent pathogenic profile and successful propagation. This isolate has an ICPI index of 1.75 and the MDT was able to achieve 100% embryo mortality at 10^6 dilutions after 88 hours post-inoculation, with MDT value of 59.2 which are characteristic of velogenic NDV strains. These biological findings are consistent with the molecular analysis, where the isolate clustered within genotype VII, a genotype commonly associated with highly virulent NDV strains circulating in poultry populations.

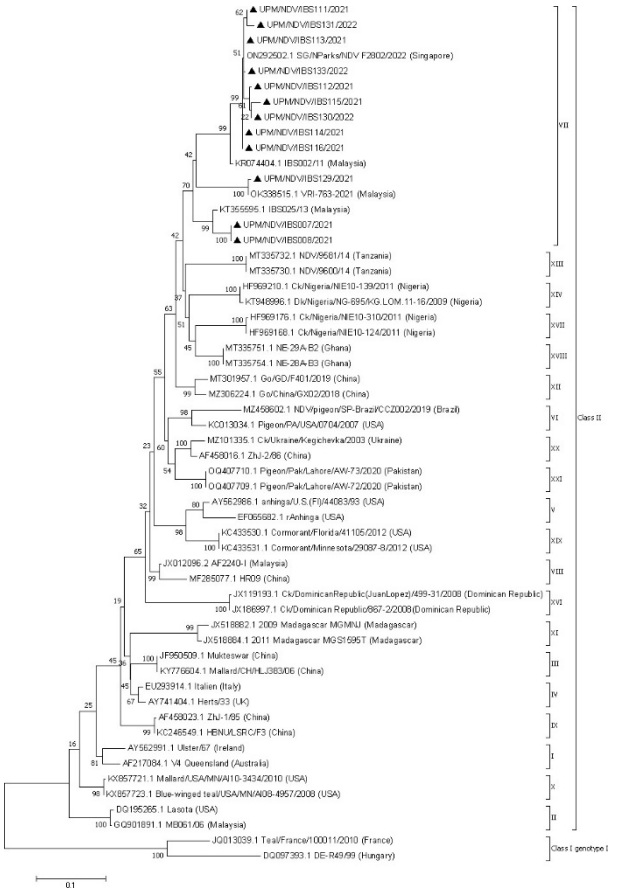

Figure 2. Phylogenetic analysis of 44 previously characterised NDV isolates of class I and class II and twelve isolates (marked with ▲) of NDV isolates belonging to genotype VII. The Roman numerals on the right represent the genotypes to which all isolates belong. The tree was deduced using the Maximum Likelihood method based on the Kimura-2 parameter model (1000 bootstrapping replicates) using MEGA v7.0 software.

DISCUSSION

Newcastle disease is one of the leading diseases of economic concern that threatens the global poultry industry. In Asia, the disease is currently endemic in many countries despite the use of vaccination and other control measures (Shohaimi et al., 2015). Some studies suggest that genotype VII is responsible for the fourth and fifth ND panzootics, where the fifth panzootic is highly related with mostly sub-genotypes VII.2 and VII.1.1 that are currently circulating in Asia, Africa and the Middle East (Dimitrov et al., 2019; Mahamud et al., 2021; Malouki et al., 2021; Miller et al., 2015). Given the widespread circulation of genotype VII strains in these regions, similar genotypes have also been reported in Southeast Asia, including Malaysia. A recent study by Mahamud (2021) detected genotype VIIi isolates from vaccinated commercial broiler chickens in Peninsular Malaysia between 2016 and 2017, suggesting the continued circulation of genotype VII strains in local poultry populations.

The COVID-19 pandemic has devastated all industries, including the agricultural and animal-producing industries, which affect the entire food production chain, from animal husbandry and production to disease control and prevention and processing and marketing of the final products (Allahbaksh & Jagannath, 2022). In Malaysia, pandemic-related restrictions may have led to reduced NDV surveillance, delays in routine vaccination, and lapses in biosecurity measures, potentially facilitating continued viral circulation and evolution in poultry populations. A study was done to characterise NDV isolates from commercial broiler and layer flocks from different states in Peninsular Malaysia from 2021 to 2022.

A total of nineteen suspected ND cases in year 2021-2022 from the states in Peninsular Malaysia were investigated. According to WOAH (2021), the gold standard approach for NDV isolation and identification is virus isolation via inoculation of the virus into SPF ECE, followed by a hemagglutination (HA) test. Using this approach, it allows NDVs to replicate in an embryonated chicken egg with a higher titter. In the current study, twelve out of nineteen isolates showed a consistent 100% embryonic mortality at 3 dpi (Table 2), followed by positive NDV for the HA test (data not shown) and RT-PCR (Figure 1).

To determine the pathotype of NDV strains from recently isolated Malaysian NDV isolates based on molecular pathogenicity, a partial F gene with a length of 535 bp was amplified from all twelve isolates. The amplified F gene was then sequenced and compared with previously published sequences through GenBank. Based on Table 3, the partial F gene cleavage site sequences of the twelve Malaysian NDV isolates revealed polybasic motifs 112R-R-Q-K-R116 or 112R-R-R-K-R116, which are characteristic of virulent strains according to WOAH criteria (2021). The presence of multiple basic amino acids at positions 112–116 and phenylalanine at position 117 is known to facilitate efficient cleavage of the F protein by host proteases, a key determinant of systemic viral spread and high pathogenicity in chickens. These molecular

findings are consistent with the biological pathotyping results, where the isolates exhibited rapid embryonic mortality in SPF embryonated chicken eggs and a high ICPI value (1.75), confirming their classification as velogenic NDV strains. The concordance between F gene cleavage site motifs and in vivo virulence highlights the value of molecular characterisation as a reliable indicator of NDV pathogenicity. Therefore, the detection of these motifs in recent Malaysian isolates highlights the continued circulation of highly virulent genotype VII NDV strains in local poultry populations.

In phylogenetic analysis (Figure 2), twelve Malaysian NDV isolates were characterised based on the partial F gene and were analysed with the other 44 published sequences of all genotypes (Class I; genotype I) and (Class II; genotypes I–XXI). The data showed all twelve isolates in this study were clustered in genotype VII. The twelve isolates were then further analysed with 18 previously characterised sequences of NDV genotype VII that were retrieved from GenBank under classification by Diel et al. (2012) (Figure 3a) and Dimitrov et al. (2019) (Figure 3b). Further comparison with previously reported genotype VII sequences from GenBank indicated that the isolates clustered into three sub-genotypes: the majority grouped within sub-genotype VII.2/VIIh, two isolates clustered within VII.2/VIIi, and one isolate grouped within VII.1.1/VIIl, which is closely related to strains previously reported in Iran (GenBank: MH481362.1) (Figure 3). These findings indicate the circulation of multiple velogenic genotype VII sub-genotypes in Peninsular Malaysia, suggesting local diversification likely driven by nucleotide substitutions (Tan et al., 2010). Thus, this indicate the highly virulent NDV genotypes VII.2 and VII.1.1 in broiler and layer chickens, respectively, were responsible for outbreaks of ND in chicken flocks in recent years. These findings were in line with the other previously reported ND outbreaks. In Asia countries, including Malaysia, Indonesia, Singapore, India, and Vietnam, is home to the majority of NDV genotype VII cases (Angeliya et al., 2022; Mahamud et al., 2021; Maqbool et al., 2023; Tran et al., 2023) and expands across the Middle East and Africa as well (Amoia et al., 2024; Elbestawy et al., 2024; Kalonda et al., 2024; Khabiri et al., 2023; Tsaxra et al., 2023). NDV genotype VII.2 has recently become highly prevalent in Asia, Zambia and Tanzania (Amoia et al., 2024; Kalonda et al., 2024; Mahamud et al., 2022; Tsaxra et al., 2023). Interestingly, NDV genotype VII.1.1 was found in Sabah, Malaysia that is closely related to NDV strains from Iran (Figure 3) (Syamsiah et al., 2022). The recent research has also revealed the presence of NDV genotype VII.1.1 in Egypt and Iran (Elbestawy et al., 2024; Khabiri et al., 2023), suggests possible transboundary movement or shared ancestry of virulent strains across regions.

In addition, the current circulation of virulent NDV in vaccinated broilers and layer flocks may have originated from non-poultry flocks, especially wild birds that have been suspected as a mechanical intermediary of spreading of NDV to commercial poultry flocks (Angeliya et al., 2022; Xiang et al., 2017). Some studies in Malaysia have reported positive NDV cases of genotype II in parrots (Berhanu et al., 2010; Mahamud et al., 2021), genotype III in peacocks, genotype VI in pigeons, and genotype VII in peacocks, owls, and egrets (Shohaimi et al., 2015; Mahamud et al., 2021). In a recent study by Mahamud, a sample of black swan that was collected from Wetland, Putrajaya, showed consistent embryonic mortality after successive third passages and was shown to be positive for NDV genotype VII with RT-PCR. However, further characterisation of the sample is yet to be studied (Mahamud et al., 2021). Similarly, in West Java, Indonesia, NDV genotype VII.2 showed some clinical signs of NDV such as weakness, anorexia, and diarrhoea in Brontok Eagles (Angeliya et al., 2022). Meanwhile, in Egypt, positive NDV genotype VII.1.1 was reported in house sparrows and wild cattle egrets trapped in different farms and showed clinical symptoms of ruffled feathers and whitish green diarrhoea (Elfatah et al., 2021). These findings show that in recently reported NDV cases, wild birds could harbour virulent NDV and spread it to commercially vaccinated flocks from one farm to another. On the other hand, the introduction of virulent genotype VII in these vaccinated flocks by non-poultry birds is also probably facilitated by the poor vaccine-induced immune in the vaccinated flocks due to various factors such as improper use of vaccine and the presence of other infections, especially immunosuppressive diseases such as Infectious Bursal Disease (IBD) and Chicken Anaemia Virus (CAV) (Kamdi et al., 2020). Therefore, continuous surveillance and vaccination studies should be done to decrease the possible threats of circulating virulent NDV to commercial poultry flocks.

Molecular characterisation and biological pathotyping confirmed that all twelve Malaysian NDV isolates, including the representative strain UPM/NDV/IBS008/2021, are velogenic. The concordance between the polybasic F gene cleavage site motifs and high ICPI (1.75) and short MDT (59.5 hours) highlights the reliability of molecular markers as predictors of pathogenicity. The persistence of these highly virulent strains in vaccinated commercial flocks suggests that current vaccination strategies, such as use of the LaSota vaccine, may not provide complete protection against circulating genotype VII sub-genotypes. This could be due to antigenic differences between vaccine strains and field isolates hence poor protection against virus shedding or poor vaccine efficacy in the field due to other confounding factors such as concurrent infection and immunosuppression. The continued circulation of velogenic genotype VII strains poses a significant risk to poultry production in Malaysia, highlighting the need for ongoing surveillance, evaluation of vaccine efficacy, and potential consideration of genotype-matched vaccines to better control outbreaks.

CONCLUSION

In conclusion, the current responsible NDV sub-genotypes isolated from broiler and layer chickens in Peninsular Malaysia are VII.2 and VII.1.1 (the year 2021–2022). Twelve isolates from the study were detected positive for NDV via HA test and RT-PCR. Moreover, the isolates exhibited polybasic amino acid patterns at positions 112RRRKRF117 and 112RRQKRF117,

indicating that they are velogenic strains. As a result, ongoing surveillance is necessary to control genotype VII from spreading throughout Malaysia.

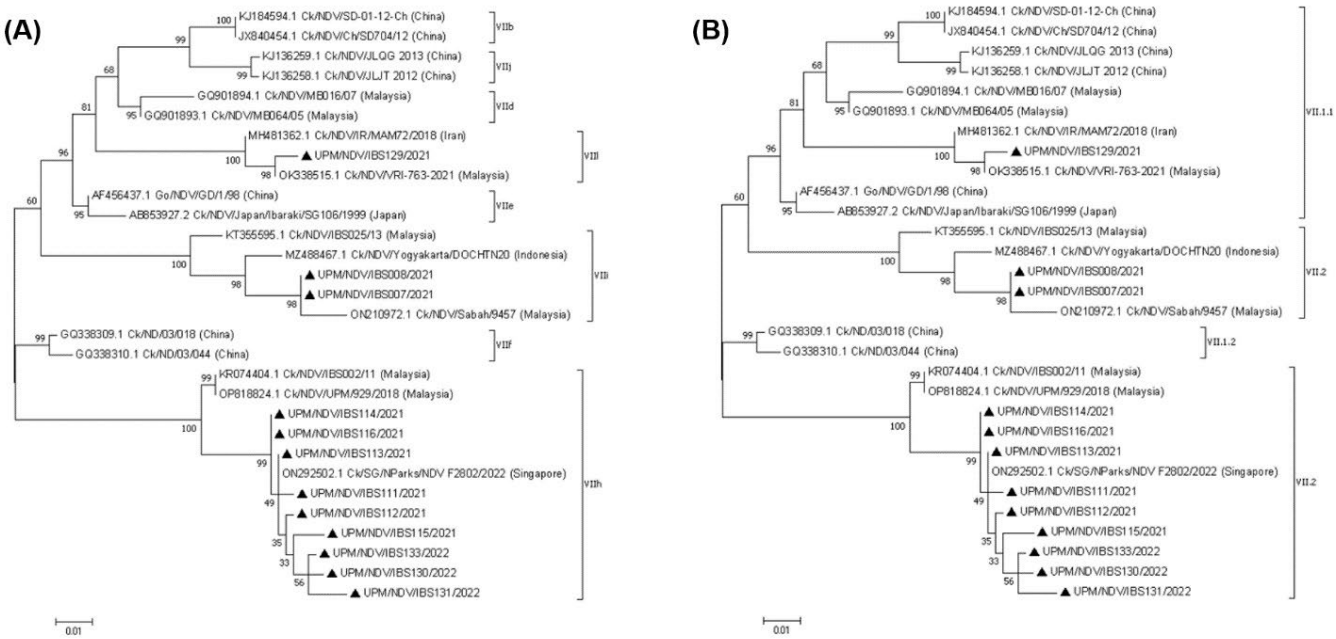

Figure 3. Phylogenetic analysis of 18 previously characterised genotype VII NDV and twelve NDV isolates (marked with ▲) isolated from infected chickens in Malaysia (year 2021–2022). The isolates were placed in their respective sub-genotypes using two classification systems. A) Diel et al. (2012) and B) Dimitrov et al. (2019). The Roman numerals on the right represent the sub-genotypes to which all isolates belong. The tree was deduced using the Maximum Likelihood method based on the Kimura-2 parameter model (1000 bootstrap replicates) using MEGA v7.0 software.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Elfatah, K.S.A., Elabasy, M.A., El-khyate, F., Elmahallawy, E.K., Mosad, S.M., El-Gohary, F.A., Abdo, W., Al-Brakati, A., Seadawy, M.G., Tahoon, A.E. and El-Gohary, A. E. (2021). Molecular characterization of velogenic Newcastle disease virus (Sub-Genotype VII.1.1) from wild birds, with assessment of its pathogenicity in susceptible chickens. *Animals*, 11(2): 505.

Alazawy, A.K. and Ajeeli, K. S.A. (2020). Isolation and molecular identification of wild Newcastle disease virus isolated from broiler farms of Diyala Province, Iraq. *Veterinary World*, 13(1): 33–39.

Aldous, E.W., Mynn, J.K., Banks, J. and Alexander, D. J. (2003). A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathology*, 32(3): 239–257.

Alexander, D.J. (1998). Newcastle disease diagnosis. In D. J. Alexander (Ed.), Newcastle disease: Developments in veterinary virology. Springer. pp.147-161.

Alexander, D.J. and Chettle, N.J. (1977). Procedures for the haemagglutination and the haemagglutination inhibition tests for avian infectious bronchitis virus. *Avian Pathology*, 6(1): 9–17.

Alexander D.J. and Senne D.A. (2008). Newcastle Disease, Other Avian Paramyxoviruses, and Pneumovirus Infections. In: Diseases of Poultry, Twelfth Edition, Saif Y.M., Fadly A.M., Glisson J.R., McDougald L.R., Nolan L.K. and Swayne D.E., eds. Iowa State University Press, Ames, Iowa, USA, 75–116.

Aljumaili, O.A., Yeap, S.K., Omar, A.R. and Aini, I. (2017). Isolation and characterization of genotype VII Newcastle disease virus from NDV vaccinated farms in Malaysia. *Pertanika Journal of Tropical Agricultural Science*, 40(4): 677-690.

Allahbaksh, S.J. and Jagannath, N.D. (2022). Impact of covid-19 pandemic on agricultural production and food system in India. *International Journal of Advanced Research Trends in Science*, 1(1): 30-33.

Amoia, C.F., Hakizimana, J.N., Chengula, A.A., Makange, M.R., Weger-Lucarelli, J. and Misinzo, G. (2024). Phylogenetic analysis of virulent strains of the Newcastle disease virus isolated from deceased chickens in Tanzania's Morogoro and Iringa regions. *Discover Animals*, 1(1): 10.

Angeliya, L., Kristianingrum, Y.P., Asmara, W. and Wibowo, M.H. (2022). Molecular characterization and pathogenesis of Newcastle disease virus isolated from brontok eagle in West Java, Indonesia. *HAYATI Journal of Biosciences*, 29(5): 648–657.

Ballagi-Pordány, A., Wehmann, E., Herczeg, J., Sándor Bélak and Lomniczi, B. (1996). Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. *Archives of Virology*, 141(2): 243–261.

Bello, M.B., Mahamud, S.N.A., Yusoff, K., Ideris, A., Hair-Bejo, M., Peeters, B.P.H. and Omar, A.R. (2020). Development of an effective and stable genotype-matched live attenuated Newcastle disease virus vaccine based on a novel naturally recombinant Malaysian isolate using reverse genetics. *Vaccines*, 8(2): 270.

Berhanu, A., Ideris, A., Omar, A.R. and Bejo, M.H. (2010). Molecular characterization of partial fusion gene and C-terminus extension length of haemagglutinin-neuraminidase gene of recently isolated Newcastle disease virus isolates in Malaysia. *Virology Journal*, 7(1).

Chambers, P., Millar, N.S., Platt, S.G. and Emmerson, P.T. (1986). Nucleotide sequence of the gene encoding the matrix protein of Newcastle disease virus. *Nucleic Acids Research*, 14(22): 9051–9061.

Czeglédi, A., Herczeg, J., Hadjiev, G., Doumanova, L., Wehmann, E. and Lomniczi, B. (2002). The occurrence of five major Newcastle disease virus genotypes (II, IV, V, VI and VIIb) in Bulgaria between 1959 and 1996. *Epidemiology and Infection*, 129(3): 679–688.

Czeglédi, A., Ujvári, D., Somogyi, E., Wehmann, E., Werner, O. and Lomniczi, B. (2006). Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus Research*, 120(1): 36–48.

Diel, D.G., da Silva, L.H., Liu, H., Wang, Z., Miller, P.J. and Afonso, C.L. (2012). Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection, Genetics and Evolution*, 12(8): 1770-1779.

Dimitrov, K.M., Abolnik, C., Afonso, C. L., Albina, E., Bahl, J., Berg, M., Briand, F.-X., Brown, I.H., Choi, K.-S., Chvala, I., Diel, D.G., Durr, P.A., Ferreira, H.L., Fusaro, A., Gil, P., Goujgoulova, G.V., Grund, C., Hicks, J.T., Joannis, T.M. and Torchetti, M. K. (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, Genetics and Evolution*, 74: 103917.

Dortmans, J.C., Koch, G., Rottier, P.J. and Peeters, B.P. (2011). Virulence of newcastle disease virus: what is known so far? *Veterinary Research*, 42(1): 122.

Dzogbema, K.F.-X., Talaki, E., Batawui, K.B. and Dao, B.B. (2021). Review on Newcastle disease in poultry. *International Journal of Biological and Chemical Sciences*, 15(2): 773–789.

Elbestawy, A., Elnagar, A., Saad, A. and Khalifa, E. (2024). Isolation and molecular characterization of velogenic Newcastle disease virus genotype VII.1.1 from commercial broilers and wild birds in Matrouh governorate, Egypt. *Journal of Veterinary Medicine*, 4(1): 16-25.

Hall, T. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41: 95-98.

Herczeg, J., Wehmann, E., Bragg, R.R., Travassos, M.G., Hadjiev, Werner, O. and B. Lomniczi. (1999). Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. *Archives of Virology*, 144(11): 2087–2099.

International Committee on Taxonomy of Viruses. (2019). Virus taxonomy. https://talk.ictvonline.org/taxonomy/

Kalonda, A., Saasa, N., Kajihara, M., Nao, N., Moonga, L., Ndebe, J., Mori-Kajihara, A., Mukubesa, A.N., Sakoda, Y., Sawa, H., Takada, A. and Simulundu, E. (2024). Phylogenetic analysis of Newcastle disease virus isolated from poultry in live bird markets and wild waterfowl in Zambia. *Microorganisms*, 12(2): 354.

Kamdi, B.P., Kolhe, R.P., Dhaygude, V.S. and Mote, C.S. (2020). Chicken infectious anemia: An emerging immunosuppressive viral threat to the poultry industry. *Journal Poultry Sciences Technology*, 8: 16-22.

Khabiri, A., Toroghi, R., Mohammadabadi, M. and Tabatabaeizadeh, S. (2023). Introduction of a Newcastle disease virus challenge strain (sub-genotype VII.1.1) isolated in Iran. *Veterinary Research Forum*, 14(4): 221–228.

Miller, P.J., Haddas, R., Simanov, L., Lublin, A., Rehmani, S.F., Wajid, A., Bibi, T., Khan, T. A., Yaqub, T., Setiyaningsih, S., and Afonso, C. L. (2015b). Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Infection, Genetics and Evolution*, 29: 216–229.

Mahamud, S.N., Tan, S.W., Youn, S.-Y., Lee, H.-J., Lee, J., Kwon, Y.-K., Ideris, A., and Omar, A.R. (2021). Isolation and characterization of Newcastle disease virus subgenotype VII.2/VIIi from commercial chicken and swan in Malaysia. *Pertanika Journal of Tropical Agricultural Science*, 44(4).

Maqbool, R., Gul, I., Rasool, A., Wani, S., Kashoo, Z., Gul, N., Hassan, A., Ahmad, W., Wali, A. and Qureshi, S. (2023). Molecular characterization and dynamics of the fusion protein of an emerging genotype VIII of Newcastle disease virus. *Agricultural Research*, 1-12.

Orakpoghenor, O., Oladele, S. B. and Abdu, P. A. (2020). Infectious bursal disease: transmission, pathogenesis, pathology and control - An overview. *World's Poultry Science Journal*, 76(2): 292–303.

Pedersen, J.C., Senne, D.A., Woolcock, P.R., Kinde, H., King, D.J., Wise, M.G., Panigrahy, B. and Seal, B. S. (2004). Phylogenetic Relationships among Virulent Newcastle Disease Virus Isolates from the 2002-2003 Outbreak in California and Other Recent Outbreaks in North America. *Journal of Clinical Microbiology*, 42(5): 2329–2334.

Peeters, B.P.H., Gruijthuijsen, Y.K., De Leeuw, O.S. and Gielkens, A. L. J. (2000). Genome replication of Newcastle disease virus: involvement of the rule-of-six. *Archives of Virology*, 145: 1829-1845.

Reed, L.J. and Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology*, 27(3): 493-497.

Roohani, K., Tan, S.W., Yeap, S.K., Ideris, A., Bejo, M.H. and Omar, A. R. (2015). Characterisation of genotype VII Newcastle disease virus (NDV) isolated from NDV vaccinated chickens, and the efficacy of LaSota and recombinant genotype VII vaccines against challenge with velogenic NDV. *Journal of Veterinary Science*, 16(4): 447.

Shohaimi, S.A., Raus, R.A., Huai, O.G., Asma Yatim, B.M., Nayan, N. and Yusuf, A.M. (2015). Sequence and phylogenetic analysis of Newcastle disease virus genotype VII isolated in Malaysia during 1999-2012. *Jurnal Teknologi*, 77(25).

Syamsiah A.S., Leow, B.L., Faizul Fikri, M.Y., Muhammad Redzwan, S. and Faizah Hanim, M.S. (2022). Identification of Newcastle Disease Virus sub-genotype VII 1.1 isolated from chickens in Sabah, Malaysia. *Tropical Biomedicine*, 39(4): 579-586.

Steward, M., Vipond, I.B., Millar, N.S. and Emmerson, P.T. (1993). RNA editing in Newcastle disease virus. *Journal of General Virology*, 74(12): 2539-2547.

Suarez, D.L., Miller, P.J., Koch, G., Mundt, E. and Rautenschlein, S. (2020). Newcastle disease, other avian paramyxoviruses, and avian metapneumovirus infections. *Diseases of Poultry*, 109-166.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28(10): 2731–2739.

Tan, S.W., Ideris, A., Omar, A.R., Yusoff, K. and HairBejo, M. (2010). Sequence and phylogenetic analysis of Newcastle disease virus genotypes isolated in Malaysia between 2004 and 2005. *Archives of Virology*, 155(1): 63-70.

Tran, T.H.G., Dong, V.H., Le, V.T., Vu, T.N., Dang, H.A. and Huynh, T.M.L. (2023). Detection and molecular characterization of virulent Newcastle disease virus (subgenotype VII.2) in broiler chickens in Northern Vietnam. *Veterinary World*, 2086–2095.

Tsaxra, J.B., Abolnik, C., Kelly, T.R., Chengula, A.A., Mushi, J.R., Msoffe, P.L.M., Muhairwa, A.P., Phiri, T., Jude, R., Chouicha, N., Mollel, E.L., Zhou, H. and Gallardo, R.A. (2023). Molecular characterization of Newcastle disease virus obtained from Mawenzi

live bird market in Morogoro, Tanzania in 2020–2021. *Brazilian Journal of Microbiology*, 54(4): 3265–3273.

Toyoda, T., Sakaguchi, T., Hirota, H., Gotoh, B., Kuma, K., Miyataj, T. and Nagai, Y. (1989). Newcastle disease virus evolution: II. Lack of gene recombination in generating virulent and avirulent strains. *Virology*, 169(2): 273-282.

Thayer, S.G. and Beard, C.W. (2008). Serologic Procedures. In: A Laboratory Manual for the Identification and Characterization of Avian Pathogens, Fifth Edition, Dufour-Zavala L., ed. American Association of Avian Pathologists. pp. 222–229.

World Organisation for Animal Health (WOAH). (2021). Newcastle disease (infection with Newcastle disease virus). https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.03.14_NEWCASTLE_DIS.pdf.

Xiang, B., Han, L., Gao, P., You, R., Wang, F., Xiao, J., Liao, M., Kang, Y. and Ren, T. (2017). Spillover of Newcastle disease viruses from poultry to wild birds in Guangdong province, southern China. *Infection, Genetics and Evolution*, 55: 199–204.

Zhang, D., Ding, Z., and Xu, X. (2023). Pathologic mechanisms of the Newcastle disease virus. *Viruses*, 15(4): 864–864.