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Pathological and Molecular Characterization of Newcastle Disease Virus Isolated from *Gallus gallus* in Java, Indonesia

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ABSTRACT

Background: Known as the vital avian disease in Indonesia, Newcastle disease (ND) has surged all over Indonesia which cause an immense drop in poultry industry. This investigation intended to examine the pathological and molecular characteristics of the latest Newcastle disease virus (NDV) derived from *Gallus gallus* in Java, Indonesia.

Methods: The samples were inoculated on the embryonic chicken eggs (ECEs), then identified using the hemagglutination test (HA test), insulated isothermal PCR (iiPCR) and hemagglutination inhibition test (HI test). It conducted the reverse transcription-polymerase chain reaction (RT-PCR) using MSF1 and MSF2 primers and followed by nucleotide sequencing for the fusion protein gene. B-cells epitope prediction analysis was applied using the Immune Epitope Database and Analysis Resource (IEDB) and VaxiJen v2.0 to predict its immunogenicity. In addition, it also performed the histopathological examination by applying hematoxylin and eosin staining.

Result: The results identified two viruses, NDV-1 and NDV-2, based on the fusion protein gene. Significantly, the cleavage site motif of NDV1 is ^{112}GRQGRL^{117} (avirulent) and whereas for NDV2 is ^{112}RRRKRF^{117} (virulent). Furthermore, it also predicted the "CKMGSRPSTKNPAP" peptide from NDV1 could be an immunogenic epitope candidate with a BepiPred linear epitope prediction score of 17.08. In summary, it suggests that NDV1 isolate can be used as a reference for vaccine design in Indonesia.

Key words: Avian paramyxovirus type-1, Fusion protein gene, Molecular characterization, Newcastle disease, Pathological.

INTRODUCTION

To meet the required amount and quality of animal protein, eggs and chicken can be the primary source for this need. Consequently, people would like to fulfill their protein needs with those sources which brings a large impact on poultry farming like in Indonesia. As its meat and eggs are inexpensive supply of animal protein widely available globally, thus, the poultry industry is a fundamental provider to global food supply. In 2013, the United States was ranked first as a producer of poultry meat, whereas in Asia, Indonesia was in the fourth position after China, India and Iran (Wahyono and Utami, 2018).

Avian paramyxovirus serotype-1 (APMV-1), also commonly known as NDV caused ND. ND has the possibility to cause catastrophic losses, due to the fact that it is an extremely contagious and deadly disease in poultry industry and is an A listed disease categorized by the Office International des Epizooties (OIE) (Ganar *et al*., 2014; Chowdhary *et al*., 2020). Affliction by a virulent NDV is associated with paralysis, neurological, respiratory and high mortality in poultry (Mohanambal *et al*., 2018). Furthermore, ND also can determine any economic losses due to the death, growth retardation and decreased egg production, as well as other losses connected with post-vaccination responses (Cornax *et al*., 2012). One of the attempts to fight against NDV is through a vaccination program (Ganar *et al*., 2014).

Moreover, NDV has a single serotype (APMV-1) and a genome size of about 15.2 kb which is single stranded, not segmented, negative-sense RNA genomes that encode nucleocapsid protein (NP), fusion protein (F), phosphoproteins (P), RNA-dependent RNA polymerases or RdRps (L), matrix protein (M), hemagglutinin-neuraminidase protein (HN) and two collateral nonstructural proteins (V and W) (Ganar *et al*., 2014). Among the aforementioned proteins, F protein is the main target of the immune response and has the high immunogenic properties (Putri *et al*., 2021). NDV can infect almost all species in poultry industry, both wild and domesticated species. In addition, ND outbreaks have occurred in almost all countries in the world (Ewies *et al*., 2017; Nath and Kumar, 2017), including Indonesia (Putri *et al*., 2021).

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Therefore, this research intended to study the pathological and molecular characteristics of the latest NDV isolated from *Gallus gallus* in Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

The approval of this study was obtained from the Professor Nidom Foundation, Surabaya, Indonesia (approval number: 18-02-5/6). This study conducted in the Vaccinology Laboratory, Professor Nidom Foundation, Surabaya, Indonesia from January 2018 to September 2019.

Clinical symptoms

Clinical symptoms were obtained from anamnesis referring to Etriwati *et al*. (2017).

Macroscopic and microscopic observations

We observed the macroscopic and microscopic changes from the internal organs of *Gallus gallus* according to Etriwati *et al*. (2017). The histopathological examination was completed descriptively and conducted by Nikon H550S using 400× magnification.

NDV isolates

The collection of two positive NDV isolates was conducted from *Gallus gallus* in East Java and West Java, Indonesia, in 2018 which were used in this study (Fig 1). Table 2 presents the origins and the years of isolation of NDV isolates (NDV1 and NDV2). Before conducting the testing in RT-PCR, the samples were propagated into ECEs (purchased from PT. IPB Shigeta Animal Pharmaceuticals, Surabaya, Indonesia) and then tested using the iiPCR (POCKIT™ Central Nucleic Acid Analyzer), HA test and HI test.

RNA isolation and amplification

In obtaining RNA of the NDV isolates, an extraction from allantoic fluids using QIAamp® Viral RNA Mini Kit (Qiagen, Germany) complying with the company's instruction was conducted. One-step RT-PCR was accomplished using Superscript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, USA) referring the company's instruction accordingly. Amplification for F gene was setup followed by Farooq *et al*. (2014). A set of primers was utilized for the amplification (targeting F gene cleavage site), MSF1 and MSF2, referring to Aldous *et al*. (2010) (Table 1).

Sequencing of PCR product

BigDye® Terminator v3.1 cycle Sequencing Kit (Thermo Fisher Scientific, USA) was employed to sequence the positive results of PCR products based on the company's instruction. Furthermore, the sequencing of the purified PCR products was completed with the primers which were MSF1 and MSF2 by First Base Company (Singapore).

Analysis of nucleotide sequence, homology and pathotyping of NDV

The editing of the obtained sequence was managed by applying BioEdit Sequence Alignment Editor Version 7. MEGA version X was administered to make an alignment of the sequences (Kumar, 2018). Furthermore, the analysis of nucleotide homology was carried out using the platform from NCBI, Needleman-Wunsch Global Align Nucleotide Sequences. The analysis of pathotype was performed on the cleavage site motif of NDV that appears at the 112th to 117th amino acid (Putri *et al*., 2021).

Prediction of B-cells epitope and protective antigens

This investigation predicted the epitope of B-cells and immunogenicity. Epitope and immunogenicity predictions were analyzed *via* the IEDB online webserver and VaxiJen v2.0 (Adianingsih and Kharisma, 2019; Nayan *et al*., 2021).

Phylogenetic tree analysis

The data was carried out using the maximum likelihood to generate a phylogenetic tree. Then, the phylogenetic tree was established by applying a bootstrap test on 1000 replications and the Tamura-Nei substitution model (Nayan *et al*., 2013; Ansori *et al*., 2020; Chowdhary *et al*., 2020).

RESULTS AND DISCUSSION

Clinical symptoms, macroscopic and microscopic observations

Despite the initiation of vaccines for governing the spreading of ND more than 50 years ago, ND still exists as one of the

Table 1: The sequence of fusion protein (F) primers.

Gene	Primers	Sequence	Product	Reference
Fusion protein (F)	MSF1	5′ -GACCGCTGACCACGAGGTTA-3′	700 bp	Aldous *et al*. (2010)
	MSF2	5′ -AGTCGGAGGATGTTGGCAGC-3′		

Table 2: Data and characteristics of NDV isolates.

Isolate	Origin	Year	Result				Cleavage site sequence	Pathotype
			HA test	HI test	iiPCR (POCKIT™)	RT-PCR		
NDV1	Tangerang, Indonesia	2018	+	+	+	+	^{112}GRQGRL^{117}	Avirulent
NDV2	Surabaya, Indonesia	2018	+	+	+	+	^{112}RRRKRF^{117}	Virulent

most substantial poultry diseases in many countries (Roohani *et al*., 2015; Kannaki *et al*., 2019), including in Indonesia (Putri *et al*., 2021). This study employed the samples obtained through tracheal and cloaca swabs from chickens, because these methods are relatively easier and there is no difficulty in obtaining permission from the farmer. On the other hand, samples can also be generated from dead birds in poultry by conducting oro-nasal swabs, isolation of lung organs, kidneys, intestines, tonsils, lymph, brain, liver and heart tissue (Etriwati *et al*., 2017). The observed symptoms were lethargy, greenish-white diarrhea, weight loss and decreased appetite in all chickens examined. Meanwhile, microscopically, the trachea showed mononuclear inflammatory cell infiltration, congestion and hemorrhage at almost every trachea sample. An investigation in the lungs expressed the suffered pneumonia with microscopic lesion of mononuclear inflammatory infiltration and congestion. A microscopic observation of the kidney exhibited the inflammatory cell infiltration, hemorrhage and tubular epithelial cell necrosis. In addition, a microscopic examination of the liver also revealed the multifocal inflammatory cell infiltration, necrosis and hemorrhage. Perivascular cuffing, microscopic lesion showing gliosis and hyperemia were examined from the brain (Fig 2).

Polymerase chain reaction (PCR)

PCR has high specificity and sensitivity to determine pathogens and is able to determine pathogens to the species level. By applying this method, researchers can find out the nucleotide sequences that tell the difference or substitution and even the mutations in the amino acid sequence (Putri *et al*., 2021). This technique is often utilized in the molecular epidemiological studies (Aldous *et al*., 2010; Chowdhary *et al*., 2020). The primers used for one-step RT-PCR were MSF1 and MSF2. This one set primer refers to the research conducted by Aldous *et al*. (2010) for the investigation of the molecular epidemiology of APMV-1 or NDV isolated from the order Galliformes. Furthermore, electrophoresis was carried out to determine that the PCR product obtained at 700 bp (Fig 3). In addition, rapid tests such as iiPCR technology are now a breakthrough besides the other molecular examinations with RT-PCR and nucleotide sequencing. In addition, iiPCR is a qualitative PCR amplification of nucleic acid targets employing a special iiPCR reagent (specific to the NDV) based on fluorescence and a detection system originated from iiPCR technology that applies the Rayleigh-Benard convection concept to run PCR with a singular heating resource at the base of the capillary vessel (Lung *et al*., 2015). Moreover, this study is the first study which used iiPCR from POCKIT™ for NDV detection.

Nucleotide homology

Nucleotide homology values are obtained through the Needleman-Wunsch Global Align Nucleotide Sequences from the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, USA). In this study, NDV1 isolates had an interval of homology values of 79 to 81% when compared to NDV isolates from Indonesia stored in the GenBank® database. As for NDV2 isolates, the homology value interval is between 88 to 96% when compared with NDV isolates from Indonesia stored in the GenBank® database. In the development of bioinformatics, the Needleman-Wunsch algorithm was the first method found to indicate similarities between two DNA sequences. This algorithm is an extension of string matching which is one of the dynamic programming techniques.

Molecular phylogenetic analysis

The molecular phylogenetic tree was constructed according to the nucleotide sequence of the F protein gene from the NDV. The maximum likelihood method is the method used in the construction of phylogenetic tree in this study referring to the study by Roohani *et al*. (2015). The results of molecular phylogenetic tree analysis exhibited that NDV2 isolates originating from Surabaya (East Java) had a close kinship with isolates in Indonesia, compared to NDV1 isolates originating from Tangerang (Fig 4). Furthermore, NDV2 isolates have a close relationship with NDV isolates from Sukorejo/2010 (HQ697255.1), Indramayu/2009 (KF767114.1), Cianjur/2010 (KF767115.1), Sukabumi/2010 (KF767117.1), and Bogor/2011 (KF767119.1). Based on the results of molecular phylogenetic tree analysis, this study found that

Fig 1: Tangerang, West Java (A) and Surabaya, East Java (B) in Indonesia where surveillance for NDV in chickens was conducted in 2018.

NDV2 isolate was included in class II genotype VII and NDV1 isolate was included in class II but in other genotypes. Roohani *et al*. (2015) stated that NDV strains are categorized into genotypes I through XI based on the F protein gene.

Pathotyping of NDV

NDV can be divided into two, which are virulent and avirulent viruses (Ganar *et al*., 2014). The results of molecular pathotype determination are further strengthened by the ECEs mortality data. In addition, the cleavage site of F protein motif analysis identified NDV1 isolate as avirulent with the cleavage site ^{112}GRQGRL^{117} amino acid motif while NDV2 isolate was considered as virulent with the cleavage site amino acid motif ^{112}RRRKRF^{117}. NDV1 isolates which are avirulent, do not cause ECEs death for up to 72 hours, whereas NDV2 isolates cause the death in ECEs. On the other hand, the ten NDV isolates circulating in Indonesia from the GenBank® database were identified as viruses with virulent properties. The motifs of the amino cleavage site are ^{112}RRQKRF^{117} and ^{112}RRRKRF^{117}. Determination of

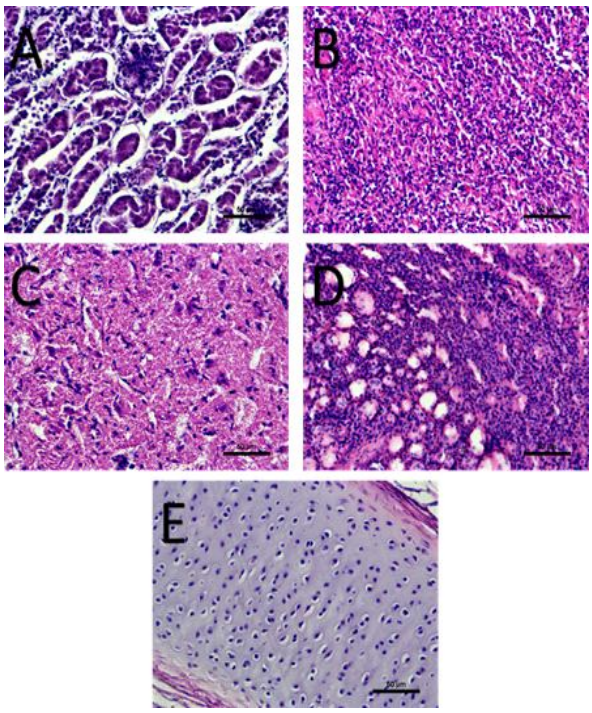

Fig 2: Histopathological changes in the internal organs of Gallus gallus infected by NDV. A: renal, B: liver, C: brain, D: lung and E: trachea. Bar: 50 µm.

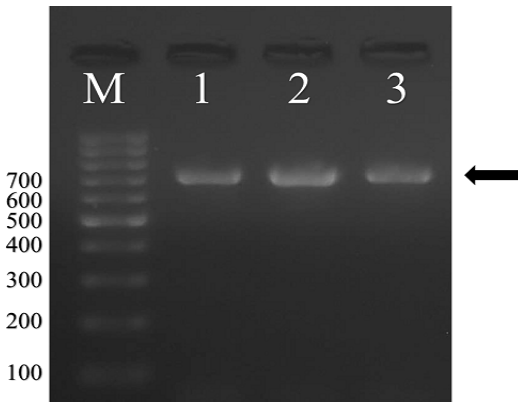

Fig 3: Fusion protein (F) gene amplification results. MSF1 (forward) and MSF2 (reverse) primers used for the RT-PCR amplification of NDV fusion protein (F) gene (product size of 700 bp). Lanes: M - marker, 1 - positive control, 2 - NDV1 and 3 - NDV2.

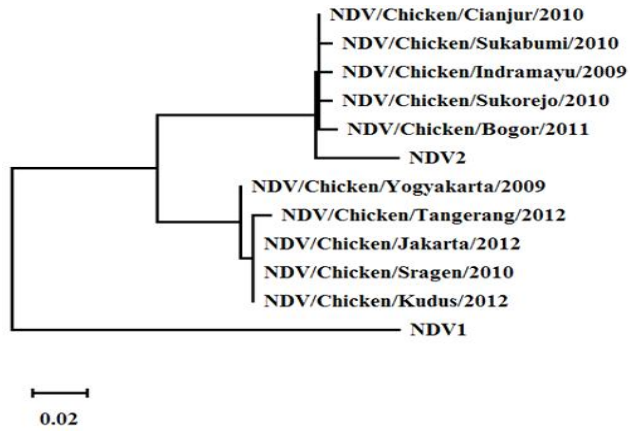

Fig 4: The molecular phylogenetic tree based on the fusion protein (F) gene of NDV from various viral isolates circulating in Indonesia at the GenBank®.

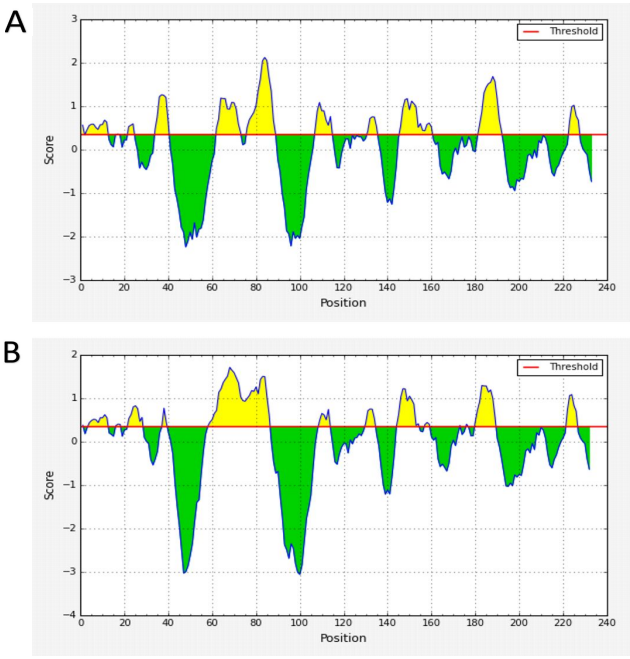

Fig 5: The predictive analysis results of B-cell epitopes from amino acid sequence of NDV1 isolate (A) and NDV2 isolate (B) fusion protein (F). The yellow area above the threshold (red line) is the area proposed as part of B-cell epitope. While the green area is not the area proposed as part of B-cell epitope. The default threshold value is 0.350.

Table 3: Prediction of B-cell epitopes and immunogenicity from amino acid sequence of NDV1 and NDV2 fusion protein (F).

Isolates	Peptides	Length	Score	Immunogenicity
NDV1	GHEVTSTKLE	10	5.64	Antigen
	AHSPRSS	7	6.81	Non-Antigen
	TRVEDSGSRLAP	12	10.74	Antigen
	CKMGSRPSTKNPAP	14	17.08	Antigen
	NSIDGRPL	8	6.27	Antigen
	LPKDKEACAKAPLDA	15	11.66	Non-Antigen
	SVTTSGGGRQG	11	12.81	Antigen
	HEVTSTKIE	9	4.71	Antigen
	PFRKAAS	7	4.61	Non-Antigen
NDV2	LEKTRVEESGSQSAHP RRNMGSKSSTGIP	29	33.06	Non-Antigen
	IPKDKEACAR	10	8.75	Non-Antigen
	GSVSTSGGRR	10	9.93	Antigen

molecular pathotypes in NDV is carried out in the F protein coding gene, because in this gene there are cleavage precursors that perform a fundamental contribution in the pathogenicity of the NDV. Generally, the nucleotide sequence at the cleavage site of the virulent NDV strain has a multibasic cleavage site of lysine (K) or arginine (R) at the positions of 112-116 and the amino acid phenylalanine (F) at the position of 117 (cleavage motif site ^{112}R/KRQR/K↓F^{117}) and the avirulent NDV strain has a monobasic cleavage site at the positions of 112-116 and amino acid leucine (L) at the position of 117 (cleavage site motif ^{112}G/EK/RQG/ER↓L^{117}) (Farooq *et al*., 2014; Chowdhary *et al*., 2020). On the other hand, the NDV pathogenicity can also be shaped based on many biological traits including the average of mean death time on ECEs aged 9-11 days, intravenous pathogenicity index in chickens aged six weeks, and intracerebral pathogenicity index in day old chicken (Farooq *et al*., 2014).

Prediction of B-cell epitope and protective antigens

The prediction results of B-cell epitopes and immunogenicity in NDV1 isolates exhibited the prediction of immunogenic epitopes which were “CKMGSRPSTKNPAP” peptide with BepiPred score of 17.08, “SVTTSGGGRQG” peptide with BepiPred score of 12.81, “TRVEDSGSRLAP” peptide with BepiPred score of 10.74, “NSIDGRPL” peptide with BepiPred score of 6.27 and “GHEVTSTKLE” peptide with BepiPred score of 5.64. This study revealed that only two obtained immunogenic epitope predictions in NDV2, they were “GSVSTSGGRR” peptide with BepiPred score of 9.93 and “HEVTSTKIE” peptide with BepiPred score of 4.71 (Table 3 and Fig 5). The bioinformatics approach may assist to the construction of epitope-based peptide vaccines. Several studies have predicted the candidates for epitope-based vaccines, such as the Zika virus (Adianingsih and Kharisma, 2019) and other viruses.

CONCLUSION

This investigation identified two ND viruses, NDV-1 and NDV-2, based on the fusion protein gene. Interestingly, the cleavage site motif of NDV1 is ^{112}GRQGRL^{117} (avirulent) and whereas for NDV2 is ^{112}RRRKRF^{117} (virulent). It also predicted that the “CKMGSRPSTKNPAP” peptide from NDV1 is able to be an immunogenic epitope candidate with a BepiPred linear epitope prediction score of 17.08. In summary, the results from this research suggested that NDV1 isolate can be used as a reference for vaccine design in Indonesia.

REFERENCES

Adianingsih, O.R. and Kharisma, V.D. (2019). Study of B cell epitope conserved region of the Zika virus envelope glycoprotein to develop multi-strain vaccine. Journal of Applied Pharmaceutical Science. 9: 98-103.

Aldous, E.W., Mynn, J.K., Irvine, R.M., Alexander, D.J. and Brown, I.H. (2010). A molecular epidemiological investigation of avian paramyxovirus type 1 viruses isolated from game birds of the order Galliformes. Avian Pathology Journal of the W.V.P.A. 39: 519-524.

Ansori, A.N.M., Kusala, M.K.J., Normalina, I., Indrasari, S., Alamudi, M.Y., Nidom, R.V., Santoso, K.P., Rachmawati, K. and Nidom, C.A. (2020). Immunoinformatic investigation of three structural protein genes in Indonesian SARS-CoV-2 isolates. Systematic Reviews in Pharmacy. 11: 422-434.

Chowdhary, M., Nashiruddullah, N., Roychoudhury, P., Bhat, A., Ahmed, J.A., Kour, K. and Sood, S. (2020). Molecular and virulence characterization of newcastle disease virus in fowls and pigeons from Jammu, India. Indian Journal of Animal Research. 54: 1279-1284.

Cornax, I., Miller, P.J. and Afonso, C.L. (2012). Characterization of live LaSota vaccine strain-induced protection in chickens upon early challenge with a virulent Newcastle disease virus of heterologous genotype. Avian Diseases. 56: 464-470.

Etriwati, Ratih, D., Handharyani, E. and Setiyaningsih, S. (2017). Pathology and immunohistochemistry study of Newcastle disease field case in chicken in Indonesia. Veterinary World. 10: 1066-1071.

Ewies, S.S., Ali, S., Tamam, S.M. and Madboulya, H.M. (2017). Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. Beni-Suef University Journal of Basic and Applied Sciences. 6: 232-237.

Farooq, M., Saliha, U., Munir, M. and Khan, Q.M. (2014). Biological and genotypic characterization of the Newcastle disease virus isolated from disease outbreaks in commercial poultry farms in Northern Punjab, Pakistan. Virology Reports. 3: 30-39.

Ganar, K., Das, M., Sinha, S. and Kumar, S. (2014). Newcastle disease virus: Current status and our understanding. Virus Research. 184: 71-81.

Kannaki, T.R., Priyanka, E. and Haunshi, S. (2019). Immunogenicity of virosome vaccine from Komarov mesogenic strain against Newcastle disease in chicken. Indian Journal of Animal Research. 53: 1651-1655.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution. 35: 1547-1549.

Lung, O., Pasick, J., Fisher, M., Buchanan, C., Erickson, A. and Ambagala, A. (2016). Insulated isothermal reverse transcriptase PCR (iiRT-PCR) for rapid and sensitive detection of classical swine fever virus. Transboundary and Emerging Diseases. 63: e395-e402.

Mohanambal, K., Selvaraju, G., Palanivel, K.M. and Rajeswar, J.J. (2018). Evaluation of immunopotentiating effect of medicinal plant products in commercial layer flock vaccinated against Newcastle disease. Indian Journal of Animal Research. 52: 1343-1346.

Nath, B. and Kumar, S. (2017). Emerging variant of genotype XIII Newcastle disease virus from Northeast India. Acta Tropica. 172: 64-69.

Nayan, V., Bhardwaj, A. and Singh, D. (2013). PAPP-A in Indian water buffalo (*Bubalus bubalis*) ovary: Phylogeny, expression, hormonal regulation, and sequence characterization. Agricultural Research. 2: 153-165.

Nayan, V., Onteru, S.K. and Singh, D. (2021). Epitope-based in silico peptide design yields peptide-directed antibodies that recognize the buffalo luteinizing hormone. International Journal of Biological Macromolecules. 176: 260-271.

Putri, N., Wulandari, I., Ernawati, R. and Rantam, F.A. (2021). Multi-epitope peptide vaccine prediction against Newcastle disease virus using immuno-informatics approaches. Bulgarian Journal of Veterinary Medicine. 24: 136-143.

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: 447-457.

Wahyono, N.D. and Utami M.M.D. (2017). A review of the poultry meat production industry for food safety in Indonesia. Journal of Physics: Conference Series. 953: 012125.