Research Article

Comparison of Two Set Pathotypic-Specific Primers to Detect Newcastle Disease Virus

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Abstract | The purpose of this research is to analyze the compatibility of two sets of pathotypic-specific primers to detect ND viruses circulating in Indonesia. This study used 4 ND isolates characterized by RT-PCR and amino acid sequencing (Putri et al. 2018). The 4 ND isolates used as isolates represented the NDV currently circulating in Indonesia. The study used 4 pathotype-specific primers. The first step of the study was to analyze the compatibility of the primers and the sequencing results of ND isolates using BioEdit® version 7.2 and MEGA version 11 sequence alignment editing software. The next step was to amplify the ND isolates using two sets of pathotype-specific primers. This amplification stage was carried out three times (as repetition). According to this study, it is known that virulent ND isolates can be amplified with pathotype-specific primers designed by Kant et al. (1997). However, the pathotype-specific primer (nested PCR) developed by Pham et al. (2005) could not amplify these isolates.

Keywords | Mismatch, nested PCR, Newcastle Disease, pathotype-specific primers, sequencing.

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INTRODUCTION

Newcastle Disease (ND) is a severe and very contagious respiratory disease in chickens. (Waheed et al., 2013). The disease caused by microorganism called virus which classified as Avian Paramyxovirus type-1 (APMV-1) (Alexander and Jones, 2000). In general, Newcastle Disease Viruses (NDV) based on their pathotypes are grouped into 4: velogenic, mesogenic, lentogenic and avirulent (Aldous and Alexander, 2001). Velogenic and mesogenic NDV strains are very strong viruses that have been found to cause ND outbreaks in many countries around the world. Lentogenic and avirulent virus strains are often used as live vaccines in disease control programs.

Determining the pathotype of NDV is generally carried out by isolating the virus from embryonated chicken eggs (ECEs), then testing it on Species Pathogen Free (SPF) chickens to measure the virulence of the virus using the intracerebral pathogenicity index (ICPI), an intravenous pathotype index (IVPI), and mean time to death (MDT) (OIE, 2012; Cattoli et al., 2011). Molecular techniques such as Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) have been developed by (Farooq et al., 2014; Lai et al., 2012; Rabalski et al., 2014) to identify the NDV. Amino acid sequencing is a further step that must be carried out to determine the pathotype of the NDV molecularly (Xiao et al,. 2012; Viljoen et al., 2005). Sequencing is highly costly, so it can be a limiting factor for ND diagnosis in the field and can directly influence ND control strategies.

The disease control development requires accurate molecular analysis of NDV using pathotype-specific ND primers. Aldous and Alexander (2001) recommended several primers, either universal or specific, to detect NDVs circulating in the world. The research by Kant et al. (1997) succeeded in determining the pathotype of the NDV using pathotype-specific primers. Pham et al. (2005) developed pathotype-specific primers known as nested PCR to detect NDV rapidly. Both the pathotype-specific primers have different amplification sites in the cleavage site of the ND F gene (Alexander, 2009; Madadgar et al., 2013). The NDV continued to undergo mutations (evolutionary distance 3–9%) Putri et al. (2018). Research was needed to determine whether these primers can still characterize NDVs quickly and accurately. This study aims to analyze the compatibility of two sets of pathotypic-specific primers developed by Kant et al. (1997) and Pham et al. (2005) to detect NDVs circulating in Indonesia.

MATERIALS AND METHODS

This study used 4 ND isolates characterized by RT-PCR and amino acid sequencing (Putri et al., 2018). The 4 ND isolates represent the NDV currently circulating in Indonesia. Characterization of NDV pathotypes by RT-PCR was carried out using pathotype-specific primers developed by Kant et al. (1997) and Pham et al. (2005). In this study, 4 pathotype-specific primer sets were used. Two primers are primers developed by Kant et al. (1997), and the other two primer sets are primers developed by Pham et al. (2005).

The first step of the study was to analyze the compatibility of the primers and the sequencing results of ND isolates using BioEdit® version 7.2 and MEGA version 11 sequence alignment editing software (Hall, 1999; Tamura et al., 2013). The next step was to amplify the ND isolates using 4 sets of pathotype-specific primers. This amplification step was carried out three times (as a repetition). The final step was to analyze the correlation between this research's first and second-step results. The data obtained were analyzed, displayed in images, and presented descriptively.

Isolation of ND Virus RNA

Viral RNA extraction was performed using QIAamp@ Viral RNA Mini Kit 52904 (Qiagen, Germany) from allantoic fluid according to the instructions provided by the manufacturer (Qiagen 2014). A total of 140 µl of sample was extracted and made more concentrated to a final volume of 60 µl. Then stored at -80 °C until needed.

ND Virus Amplification

In this study, amplification of the ND virus was carried out by Reverse Transcriptase-Polymerase Chain Reaction using the One-Step RT-PCR kit according to the manufacturer's instructions (Qiagen, Germany). The RT-PCR master mix had a total amount of 50 µl. It contained 2 µl of dNTPs, 2 µl of forward primer), 2 µl of reverse primer, 2 µl of RNA template, 10 µl of Onestep RT-PCR buffer Qiagen", 30 µl of water without any RNA-contaminating molecules, and 2 µl of an enzyme. The amplification for Matrix (M) gene was carried out at 45 °C cycle for 60 minutes, followed by initial denaturation at 95 °C for 5 minutes and 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 72 °C for 40 seconds and final extension at 72 °C for 10 minutes. The amplification of Fusion (F) gene was under the following cycle conditions: 45 °C for 60 minutes for c-DNA synthesis followed by initial denaturation at 94 °C for 5 minutes, and 35 cycles at 94 °C for 1 minute, 50 °C for 1 minute, 72 °C for 1 minute, and final extension for 5 minutes at 72 °C.

Primer

Four sets of primers were used in this study. Two sets of specific primers target the cleavage site of the F gene, namely: NDV-FA/FB, which is specific for virulent NDV, and NDV-FA/FC is specific for avirulent NDV strains (Kant et al., 1997), and the other two primer sets are Fout-S/Fout-AS and F2-S/F2-AS (Pham et al., 2000). The nucleotide sequences of all primers are presented in Table 1.

Electrophoresis

PCR products were seen by using electrophoresis on a gel made of 1. 5% agarose containing 0. 4 µg/ml ethidium bromide and looked at the products utilize a UV transilluminator. The flowchart of research activities for the identification of the NDV by RT-PCR using pathotype-specific primers can be seen in Figure 1.

Analysis of DNA Oligonucleotide Sequencing Results

Compatibility analysis of primers and sequencing results of ND isolates was performed using BioEdit® version 7.2 (Hall, 1999). Nucleotide sequence alignments were analyzed using MEGA version 11 sequence alignment editing software (Tamura et al., 2013).

RESULT AND DISCUSSION

Molecular techniques such as RT-PCR and amino acid sequencing can be used to determine the NDV pathotype (Aldous and Alexander, 2001; Miller et al., 2015; Wen et al., 2013). Recently, a pathotype-specific primer has been developed for detecting and identifying NDV efficiently (Kant et al., 1997; Ahmadi et al., 2014; Pham et al., 2005).

Table 1: Nucleotide sequences of primer used in the study

Gen	Code	Sequences	Position (bp)
Fusion	FA	5'-TTGATGGCAGGCCTCTTGC-3'	141–159
Fusion	FB	5'-AGCGT(C/T)TCTGTCTCCT-3'	395–380
Fusion	FC	5'-G(A/G)CG(A/T)CCCTGT(C/T)TCCC-3'	395–380
Fusion	Fout-S	5'-ATGGGCTCTACATCTTCTAC-3'	1–19
Fusion	Fout-AS	5'-CCATATTCCCACCAGCTAG-3'	720–738
Fusion	F2-S	5'-TTATCGGCAGTGTTAGCTCTT-3'	362–382
Fusion	F2-AS	5'-TCAGTAGGTACAAGTTGGAC-3'	627–648

Table 2: Compatibility analysis results between the FA/FB, FA/FC primer, and ND isolates.

No	Isolate	Primers	Number of Mismatches	PCR Result
1.	NDV/Ck/Bogor/011	FA (Forward)	1	
		FB (Reverse)	2	+
		FC (Reverse)	7	-
2.	NDV/Ck/GnSindur/014	FA (Forward)	1	
		FB (Reverse)	3	+
		FC (Reverse)	6	-
3.	NDV/Ck/Cianjur/015	FA (Forward)	0	
		FB (Reverse)	5	-
		FC (Reverse)	1	+
4.	NDV/Ck/Bogor/015	FA (Forward)	0	
		FB (Reverse)	5	-
		FC (Forward)	1	+

Table 3: Compatibility analysis results between the Fout-S/Fout-AS and F2-S/F2-AS primers and ND isolates

No	Isolate	Primers	Number of Mismatches	PCR Result
1.	NDV/Ck/Bogor/011	Fout-S	3	+
		Fout-AS	2	
		F2-S	7	-
		F2-AS	6	
2.	NDV/Ck/GnSindur/014	Fout-S	3	+
		Fout-AS	2	
		F2-S	8	-
		F2-AS	6	
3.	NDV/Ck/Cianjur/015	Fout-S	3	+
		Fout-AS	1	
		F2-S	8	-
		F2-AS	5	
4.	NDV/Ck/Bogor/015	Fout-S	3	+
		Fout-AS	1	
		F2-S	8	-
		F2-AS	5	

A nested PCR method to identify the pathotype of the ND virus has also been developed (Green and Sanbrook, 2019).

The pathotype primers designed by Kant et al. (1997) have amplified targets on the cleavage site of the NDV F gene that have the same nucleotide sequence with forward primer (FA) sequence and a different sequence nucleotide

for reverse primer (FB and FC). The F gene cleavage site of NDV is the main determinant of virulence (Ahmadi, 2014; Yu et al., 2001; Madadgar et al., 2013). The cleavage site of F gene has nucleotide sequence that pairs with the forward primers (FA) and has a different nucleotide sequence for the reverse primers (FB and FC) (Putri et al., 2017). The reverse primer nucleotide sequence difference determines the NDV pathotype (Ahmadi et al., 2014). The alignment results of the primers nucleotide sequences FA/FB and FA/FC with the ND virus sequences are presented in Figure 1.

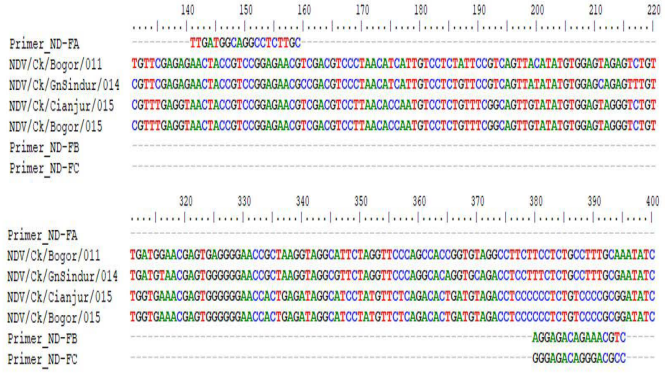

Figure 1: Complementary Nucleotide sequence between FA/FB, FA/FC primers and ND Isolates

Figure 1 shows that both primers in the 4 samples complement at nucleotide positions 141 – 159 for forward and 380 – 395 for reverse. These primers amplify all isolates by producing a 255 bp band. The amplification results of the 4 samples presented on Figure 2, and the analysis of the compatibility of the nucleotide sequences between the primers and the samples can be seen in Table 2.

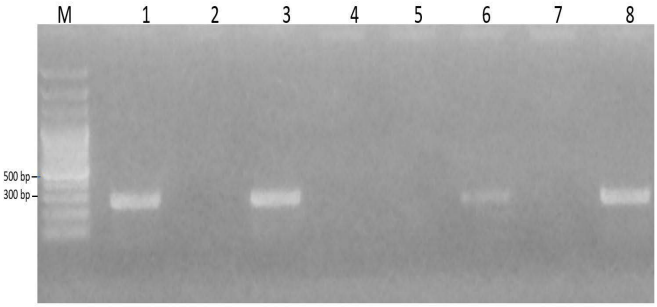

Figure 2: Complementary Nucleotide Sequence between Fout-S/Fout-AS primers and ND Isolates

Table 2 illustrates variations in nucleotide mismatches between the FA/FB primers and the nucleotide sequences of the ND isolates. FA/FB primers could amplify NDV/Ck/Bogor/011 and NDV/Ck/GnSindur/014 isolates, while NDV/Ck/Cianjur/015 and NDV/Ck/Bogor/015 isolates could be amplified using FA/FC primers. The isolates NDV/Ck/Bogor/011 and NDV/Ck/GnSindur/014 had 6 – 7 nucleotide mismatches with FA/FC primers, and two other isolates had 5 nucleotide mismatches with FA/FC primers. The result is in line with Putri's research, 2017 which showed that isolates NDV/Ck/Bogor/011 and NDV/Ck/GnSindur/014 were virulent ND isolates, while isolates NDV/Ck/Cianjur/015 and NDV/Ck/Bogor/015 were avirulent ND isolates. Template amplification will be less if the primer and template have more sequence differences (Kingsland and Maibaum, 2018). Ye et al. (2012) stated that at least 5 nucleotide mismatches between the primer and the template can prevent amplification interference.

Pham et al. (2005) developed nested PCR primers to differentiate virulent and avirulent ND viruses. Nested primers are designed to differentiate ND viruses based on their pathotype (Kho, 2000). Nested PCR performs two rounds of PCR. Each round has a different set of primers used to amplify the DNA. The results of the first amplification process are used as a template for the second PCR (Ghedira et al., 2009; Green and Sambrook, 2019). The Fout-S/Fout-AS primers work for an amplification target of 700 bp, and then the product was amplified using F2-S/F2-AS primers with a narrower target area (300 bp). The primer positions of Fout-S/Fout-AS and F2-S/F2-AS in the ND isolate sequences presented on Figure 3.

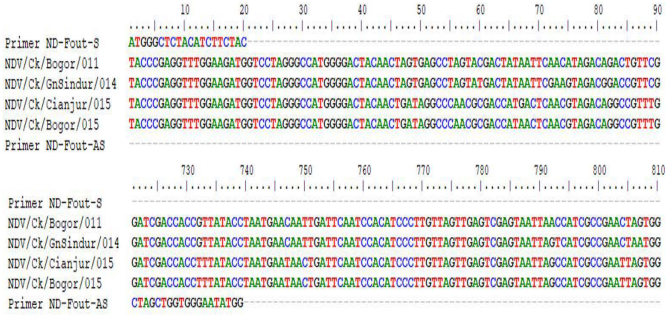

Figure 3: Fusion gene amplification results by FA/FB and FA/FC primers; M= Molecular size marker; 1, 2 = NDV/Ck/Bogor/011; 3, 4= NDV/Ck/GnSindur/014; 5, 6= NDV/Ck/Cianjur/015; 7, 8 = NDV/Ck/Bogor/015; 1, 3, 5, 7 = Amplification by FA/FB primer; 2, 4, 6, 8 = Amplification by FA/FB primer.

Figure 3 displays the primer positions of Fout-S/Fout-AS are at nucleotide positions 1 - 19 for forward and 720 - 748 for reverse. Based on the results of the primary amplification of Fout-S/Fout-AS, it was shown that all isolates used in this study could be amplified to produce a band of 700 bp (Figure 4). Furthermore, to determine the viral pathotype, the process was then proceeded by using the F2-S/F2-AS primer (Figure 5).

Figure 5 shows the primer positions of F2-S/F2-AS are at nucleotide positions 362 – 381 for forward and 627 – 648

for reverse. This primer amplified the ND isolate, resulting in a 300 bp band. Based on the results of the primer amplification of F2-S/F2-AS, all isolates used in this study could not be amplified. The analysis results of the primer compatibility of Fout-S/Fout-AS and F2-S/F2-AS for ND isolates can be seen in Table 3.

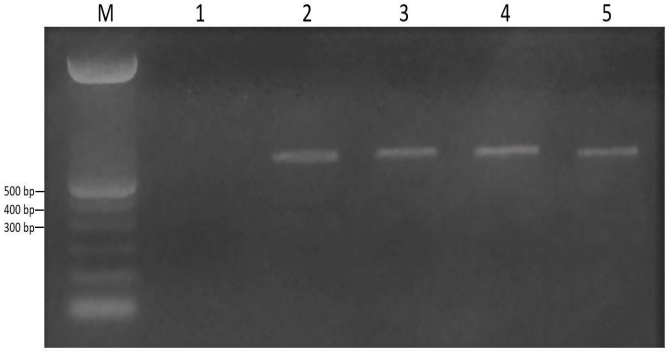

Figure 4: Fusion gene amplification results by Fout-S/Fout-AS primers; M= Molecular size marker; 1= Negative Control (H2O), 2 = NDV/Ck/Bogor/011; 3= NDV/Ck/GnSindur/014; 4 = NDV/Ck/Cianjur/015; 5 = NDV/Ck/Bogor/015

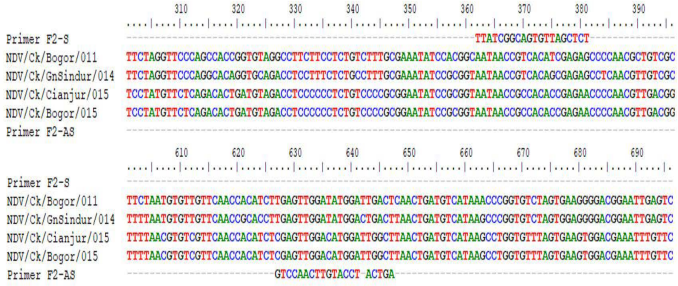

Figure 5: Complementary Nucleotide Sequence between F2-S/F2-AS primers and ND Isolates

Table 3 shows the analysis of the mismatch of the nucleotide sequences between the primers Fout and F2 and the nucleotide sequences of the target areas in the ND isolates. These results indicate that the Fout-S primer has 3 nucleotides mismatched with the nucleotide sequence of the target region in all isolates. In comparison, the Fout-AS primer had a mismatch of 1 – 2 nucleotide sequences with the nucleotide sequence of the target area in all isolates. PCR results using primers Fout-S and Fout-AS showed positive results for all isolates. This shows that the elongation and amplification process can still occur even though there are mismatches in several nucleotides but still causes the primer to stick to the template. A single mismatch in the r forward or reverse primer used may not affect the accuracy of target detection (Kamau et al., 2017). This mismatch does not always lead to false negative results because its effect depends on various factors such as number, position, and target (Chow et al., 2011; Ye et al., 2012). Several research have explored the effect of nucleotide mismatches with primer and demonstrated that targets can be amplified despite mismatches with primers. (Wiley, 2005; Sipos et al., 2007; Waterfall et al., 2002; Ghedira et al., 2009). Ye et al. (2012) stated that the primer must not fit all to the template. However, the 3' end of the primer must be completely aligned with the template DNA strand so that elongation can continue.

The F2-S primer had 7 – 8 nucleotide sequence mismatches with the target region nucleotide sequence in all isolates, while the F2-AS primer had 5 – 6 nucleotide sequence mismatches with the target region nucleotide sequence in all isolates. However, the difference of 5 – 7 nucleotides made the primers unable to amplify the target area, so the PCR results using the F2-S/F2-AS primers showed negative results for all isolates. The mismatch between the primer and the target DNA can influence pairing stability and can make it harder for the system to multiply copies of the template DNA (Yu et al., 2012). Single nucleotide mismatches in target annealing have lower negative effects than deletions or multi-nucleotide mismatches (Lefever et al., 2013). Selecting a suitable primer is one of the main things that influences PCR results (Kingsland and Maibaum, 2018). PCR results can be affected by several factors, as well as preparation of template DNA and PCR running reaction conditions, as well as good primer pair design, which is a critical factor in determining the success of amplification (Ye et al., 2012; Higgins et al., 2022).

Referring to the evaluation results in Figure 5, it can also be seen that the mismatch between the F2-S primer and the template occurs at the nucleotide at the 3' end. Mismatches located in the last 5 nucleotides from the 3-terminal region of the primers have a much more significant effect (Lefever et al., 2013; Stadhouders et al., 2010; Brault et al., 2012), as well as a mismatch of two nucleotides at the 3' end of the primer, can also inhibit amplification (Ye et al., 2012; Ghedira et al., 2009). Based on these results, the pathotype-specific primers (Fout-S/Fout-AS and F2-S/F2-AS) developed by Pham et al. (2005) cannot be used to differentiate virulent and avirulent NDV in all NDV isolates. This can be caused by mutations in the primary site, especially in the F2-S target. Newcastle Disease virus mutations are influenced by the presence of various types of viruses circulating at the same time.

Putri et al. (2018) showed that NDV/Ck/Bogor/ 011 is categorized as NDV genotype VII (h) and NDV/Ck/GnSindur/014 is categorized as NDV genotype VII (i), and NDV/Ck/Cianjur/015 and NDV/Ck/Bogor/015 as NDV genotype II. The isolate used as a sample in Pham et al study was obtained from ND outbreaks in Japan from the 1980s until 2000. The isolate was categorized as NDV

genotype VII (d) (Umali et al., 2013). Genotype VII is the most common type of NDV that causes the majority of outbreaks in East Asian countries like Japan, Taiwan, Korea, and China since the 1980s. This makes it the fourth widespread occurrence of the virus (Lien et al., 2007; Mase et al., 2002). Wild birds have helped spread the NDV genotype VII virus to countries in Far East Asia (Umali et al., 2013).

Changes in viral DNA or mutations are a form of virus dynamics to adapt to the environment to survive (Sobhanie, 2021). Analysis of the hypervariable region of the F gene in NDV showed that there were amino acid changes at five specific points in the F gene of all isolates (Putri et al., 2018). Viruses accumulate mutations in their genomes when adapting to animal hosts. Mutations in the viral gene at the primary target site will result in a false negative test result (Alkhatib et al., 2022). Modifying the nucleotide sequence in the primer will result in better template binding thereby increasing the sensitivity of the assay (Brault et al., 2012). The research we conducted has limitations. This research used a small number of samples isolated within certain time limits from one region, so it is uncertain if this primer can still detect other samples from other countries at different periods of time. The nucleotide sequence in the F gene cleavage region is the main determinant of virulent or avirulent NDV (Putri et al., 2017). In general, virulent NDV strains have a specific sequence of nucleotides that contains at least three basic amino acids. This sequence is known as a multi-basic cleavage site (OIE., 2012). Alteration of one of the nucleotide bases can change the motif of amino acid. Amino acid changes are caused by mutations or substitutions associated with various viral genotypes (Putri et al., 2018). It is very appropriate to design and use primers targeting the F gene cleavage site to differentiate virulent and avirulent NDVs. In addition, it is necessary to do extensive evaluations of the genomic changes of the NDV to anticipate mismatches between primers and viruses to avoid false-negative PCR results.

CONCLUSION

The pathotype-specific primer developed by Khan et al. 1997 can distinguish virulent and avirulent Indonesian ND isolates. Pathotype-specific primers (nested PCR) developed by Pham et al. 2000 could not differentiate virulent and avirulent Indonesian isolates because they had 5 – 8 nucleotide differences in Primer F2-S/F2-AS with all of isolates isolates. It is crucial to pay attention to the targets of the PCR diagnostic test to determine potential changes in the virus in the future along with changes in its host.

CONFLICT OF INTEREST

The author's country has no conflict of interest.

NOVELTY STATEMENT

This research obtained results that pathotype specific primers (nested PCR) developed by Pham et al. 2000 could not differentiate between virulent and avirulent isolates of the Indonesian NDV.

AUTHOR'S CONTRIBUTION

DDP and N designed the research; DDP and NPIM conducted the experiment in the laboratory; DDP, N, and IKH analyzed the data; DDP and NPIM drafted the manuscript; DDP and IKH revised the manuscript.

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