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Judul Artikel : "**Comparison of Two Set Pathotypic-Specific Primers to Detect Newcastle Disease Virus"**

No	Kegiatan	Tgl/bulan/tahun	Halaman
1.	Submit artikel	16 Juni 2023	1
2.	Artikel masuk ke editor	21 Juni 2023	2
3.	Artikel dikirim ke reviewer	23 Juni 2023	3
4.	Hasil peer review	4 Juli 2023	4 - 8
5.	Tanggapan dari hasil peer review		9 - 10
6.	Dokumen perbaikan dari hasil peer review reviewer 1		11 - 27
7.	Dokumen perbaikan dari hasil peer review reviewer 2		28 - 59
8.	Artikel dinyatakan diterima	23 September 2023	60
9.	Proof Reading	18 Oktober 2023	64 - 71
10.	Artikel dipublikasikan	29 November 2023	73

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Tue, 04 Jul 2023, 11:22 AM

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An interesting study but need some revision, the manuscript need to add study limitation and add discussion a bit about the primer and also comparison to other study. Please see my comment on your manuscript.

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=> Reviewer # 1

An interesting study but need some revision, the manuscript needed to add study limitation and add discussion a bit about the primer and also comparison to other study. Please see my comment on your manuscript.

1. Reviewer comment line 66 (Did you do duplo or not? If yes. Please explain clearly).
2. Reviewer comment in line 203 (add more disscusion Does the primer is only spesific for NDV in some region/country? or How?).
3. Reviewer comment line 214 (add your study limitation, and add disscusion or suggestion for designing primer to differentiate between virulent and avirulent ND).

=> Reviewer # 2

1. The manuscript still requires extensive English editing

2. The identity % within your manuscript is over 31% and should not be more than 20%.

List of change made in manuscript: ID MH20230616090630

" **Comparison of two set pathotypic-specific primer to detect Newcastle Disease Virus**"

Responses to editor and reviewer comments :
We would like to thank to the editor and reviewers for their comments and useful suggestions. We have made changes in the manuscript according to their remarks. In the following text you will find answers to their inquiries and the change we have made (written in red)

Responses to reviewer comments:

Reviewer 1

An interesting study but need some revision, the manuscript need to add study limitation and add discussion a bit about the primer and also comparison to other study. Please see my comment on your manuscript.
Thank you for your comments and suggestions. We agree with your comments that the manuscript need to add study limitation and add discussion a bit about the primer and also comparison to other study. We have made some revisions. We hope it is in line with reviewer suggestion.

1. Reviewer comment line 66 (Did you do duplo or not? If yes. Please explain clearly.
   Thank you for your comments and suggestions. In this research, Characterization of ND virus pathotypes by RT-PCR using pathotype-specific primers developed by Kant et al. (1997) and Pham et al. (2005) done three times (as repetition). We added some information about that line 65 – 66.
   "The next step was to amplify the ND isolates using 4 (four) 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 dan second-step results"
   We hope it is in line with reviewer suggestion.

2. Reviewer comment in line 203 (add more disscusion Does the primer is only spesific for NDV in some region/country? or How?).
   Thank you for your comments and suggestions. We added some information in disscusion line 198 – 200 and line 203 - 207. We hope it is in line with reviewer suggestion.
   "This can be caused by mutations in the primary site, especially in the F2-S target. ND virus mutations affected by co-circulation of genetically distinct virus lineages with the predominant virus genotype circulating in a particular time period"
   "Isolate was used as a sample in Pham et al study obtained from ND outbreak in Japan in the 1980-an until 2000. The isolate categorized as NDV genotype VII (d) (Umali et al., 2013). Genotype VII is the most predominant NDV genotype that is responsible for most outbreaks in East Asian countries including Japan, Taiwan, Korea and China since the 1980s, constituting the fourth pandemic (Lien et al., 2007; Mase et al., 2002)"
   We hope it is in line with reviewer suggestion.

3. Reviewer comment line 214 (add your study limitation, and add disscusion or suggestion for designing primer to differentiate between virulent and avirulent ND).

Thank you for your comments and suggestions. We added some information in disscusion line 216 – 227.

"Our present study has certain limitation. This study was used small number of samples are isolated at certain time limits from one region, so It can't be explained whether this primer can still detect other sample from other countries at different periods of time. The nucleotides sequence at the F gen cleavage site has been shown to be a major determinant of virulent or avirulent NDV (Putri et al., 2017). In general, nucleotide sequence of virulent strains at least has three basic amino-acids (multi basic cleavage site) (OIE., 2012). Alteration of one of nucleotide base can change the amino acid motif. The alteration of amino acid caused by mutation or substitution associated with the many diverse genotypes of the virus (Putri et al., 2018). It is very appropriate to design and use primers targeting the F gen cleavage site to differentiate virulent and avirulent ND viruses. In addition, it is necessary to do extensive evaluation of the genomic changes of the ND virus, to anticipate mismatch between primers and viruses to avoid false-negative PCR results" We hope it is in line with reviewer suggestion.

As a consequence of adding information, we also added 2 refference line 295 – 297 and 301 – 303.

Lien Y, Lee J, Su H, Tsai H, Tsai M, Hsieh C, Tsai S (2007) Phylogenetic characterization of Newcastle disease viruses isolated in Taiwan during 2003–2006. Vet Microbiol., 123:194–202.

Mase M, Imai K, Sanada Y, Sanada N, Yuasa N, Imada T, Tsukamoto K, Yamaguchi S (2002) Phylogenetic analysis of Newcastle disease virus genotypes isolated in Japan. J Clin Microbiol., 40:3826–3830.

Reviewer 2

1. The manuscript still requires extensive English editing

Thank you for your comments and suggestions. We agree with your comments that the manuscript requires extensive English. We have re-written and made technical corrections in revised text and we hope it is in line with suggestion of reviewer.

2. The identity % within your manuscript is over 31% and should not be more than 20%.

Thank you for your comments and suggestions. We agree with your comments that the manuscript have similirity over 31%. We have re-written and made technical corrections in revised text (written in red) and reduce the similarity less than 20%. We hope it is in line with suggestion of reviewer.

Comparison of two set pathotypic-specific primer to detect Newcastle Disease Virus

DWI DESMIYENI PUTRI*[1)], NURHAYATI[1)], INTAN KAMILIA HABSARI[1)], NI LUH PUTU IKA MAYASARI[2)]

[1)]Department of Animal Husbandry, Politeknik Negeri Lampung, Lampung, Indonesia

[2)] Division of Medical Microbiology, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia

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Abstract

This study was designed to analyze the compatibility of two sets of pathotypic-specific primers to detect ND viruses circulating in Indonesia. This study used 4 (four) ND isolates characterized by RT-PCR and amino acid sequencing (Putri et al. 2018). The four ND isolates were used as isolates representing the ND virus currently circulating in Indonesia. The study used 4 (four) pathotype-specific primers. The research's first step was to analyze the compatibility of the primers and ND isolate-sequencing results using the sequence alignment editor software BioEdit® Version 7.2 and MEGA version 11. The next step was to amplify the ND isolates using 2 (two) sets of pathotype-specific primers. This amplification stage was carried out three times (as repetition). Based on the results of this research, it is known that virulent ND isolates can be amplified with pathotype-specific primers developed 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.

INTRODUCTION

Newcastle Disease (ND) is a systemic respiratory disease in poultry that is acute and highly contagious (Waheed et al., 2013). Newcastle Disease is caused by Avian Paramyxovirus type-1 (APMV-1), which belongs to the genus Avulavirus, the family Paramyxoviridae (Alexander and Jones, 2000). Newcastle Disease viruses can be divided into four groups based on the pathotype: velogenic, mesogenic, lentogenic and avirulent (Aldous and Alexander, 2001). Velogenic and mesogenic ND virus strains are categorized as virulent ND viruses and have been

identified as causative agents of ND outbreaks in many countries worldwide. Lentogenic and avirulent virus strains are widely used in disease control programs as live vaccines.

Determination of NDV pathotype is generally carried out by isolating the virus from embryonated chicken eggs (TAB) and followed by in vivo tests such as the intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI) and mean death time (MDT) in chicken Species Pathogen Free (SPF) (OIE, 2012; Cattoli et al., 2011). Molecular methods such as Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) have been developed to identify ND viruses (Farooq et al., 2014; Lai et al., 2012; Rabalski et al., 2014). Amino acid sequencing is a further step that must be carried out to determine the pathotype of the ND virus 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 development requires accurate molecular analysis of ND viruses of pathotype-specific ND primers. Aldous and Alexander (2001) recommended several primers, either universal or specific, to detect ND viruses circulating in the world. The research by Kant et al. (1997) succeeded in determining the pathotype of the ND virus using pathotype-specific primers. Pham et al. (2005) developed pathotype-specific primers known as nested PCR to detect ND virus rapidly. Both the pathotype-specific primers have different amplification sites in the cleavage site of the ND virus F gene (Alexander, 2009; Madadgar et al., 2013). Newcastle Disease Virus continued to undergo mutations (evolutionary distance 3–9%) Putri et al., (2018). The research was needed to determine whether these primers can still characterize ND viruses quickly and accurately. This study was designed to analyze the compatibility of two sets of pathotypic-specific primers developed by Kant et al. (1997) and Pham et al. (2005) to detect ND viruses circulating in Indonesia.

Materials and Methods

This study used four ND isolates characterized by RT-PCR and amino acid sequencing (Putri et al., 2018). The four ND isolates were used as isolates representing the ND virus currently circulating in Indonesia. Characterization of ND virus 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, four pathotype-specific primer sets were used. The two primers are primers developed by Kant et al. (1997), and the two primer sets are pathotype-specific primers developed by Pham et al. (2005).

The study's first step was to analyze the primers' compatibility with the nucleotide sequences of the ND virus using BioEdit® sequence alignment editor software Version 7.2 (Hall, 1999) and MEGA version 11 (Tamura et al., 2013). The next step was to amplify the ND isolates using 4 (four) 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 dan second-step results. The data obtained were analyzed, displayed in images, and presented descriptively.

Isolation of ND Virus RNA

Viral RNA was extracted from the allantoic fluid according to the manufacturer's instructions (Qiagen 2014) using the QIAamp @ Viral RNA Mini Kit 52904 (Qiagen, Germany). A total of 140 µl of the sample was extracted and diluted to a final volume of 60 µl and stored at -80 °C until use.

ND Virus Amplification

Reverse Transcriptase-Polymerase Chain Reaction was performed using the One-step RT-PCR kit (Qiagen, Germany) according to the manufacturer's instructions. The RT-PCR master mix each consisted of 2 µl dNTPs (10 mM), 2 µl forward primer (10 pM), 2 µl reverse primer (10 pM), 2 µl RNA template, 10 µl 5× Onestep RT-PCR buffer Qiagen, 30 µl Rnase free water, and 2 µl Onestep RT-PCR enzyme were mixed until the final volume was 50 µl. Matrix (M) gene amplification 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. Fusion (F) gene amplification was carried out 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 ND virus, and NDV-FA/ The FC is specific for avirulent ND virus 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 visualized using electrophoresis on 1.5% agarose gel containing 0.4 µg/ml ethidium bromide and observed using a UV transilluminator. The flowchart of research

activities Characterization of Newcastle Disease Virus by RT-PCR using pathotype-specific primers can be seen in Figure 1.

Analysis of DNA Oligonucleotide Sequencing Results

The oligonucleotide sequencing results were analyzed using BioEdit® sequence alignment editor software Version 7.2 (Hall, 1999). Sequence alignment was performed using MEGA software version 11 (Tamura et al., 2013).

Result and Discussion

Molecular methods such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) and amino acid sequencing can be used to determine the pathotype of NDV (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) have also developed nested -PCR to identify the pathotype of the ND virus (Green and Sanbrook, 2019).

The Pathotype-primers developed 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 primar (FA) sequence and a different sequence nucleotide for reverse primar (FB and FC). The pathotype-specific primer developed by Kant et al. (1997) was designed based on the nucleotide sequence at the F gene cleavage site, which is the primary determinant of NDV virulence (Ahmadi, 2014; Yu et al., 2001; Madadgar et al., 2013). The F gene cleavage site has the same nucleotide sequence as 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 shows that the FA/FB and FA/FC primers in the four samples complement at nucleotide positions 141 – 159 for forward and 380 – 395 for reverse. These primers amplify all isolates by producing 255 bp band. The amplification results of the four samples can be seen in Figure 2, and the analysis of the compatibility of the nucleotide sequences between the primers and the samples can be seen in Table 2.

Table 2 illustrates variations in nucleotide mismatches between the FA/FB primers and the nucleotide sequences of the ND isolate. 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 a nucleotide mismatch 6 – 7 nucleotides with FA/FC primers, and isolates NDV/Ck/Cianjur/015 and NDV/Ck/Bogor/015 had a mismatch 5 nucleotides 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. The more nucleotide mismatch between primer and template, the less amplification will not occur (Kingsland and Maibaum, 2018; Ye et al., 2012) state 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 involves two sequential amplification reactions, each using a different

pair of primers. The product of the first amplification reaction is used as the 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 is 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 can be seen in Figure 3.

Figure 3 shows 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, then proceed with 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 a 300 bp band. Based on the results of the primer amplification of F2-S/F2-AS, showed that 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 4.

Table 4 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 showed that the nucleotide mismatch between the primers and the template can still cause the primer to stick to the template, and elongation and amplification processes can occur. A single mismatch in

backward or forward primers may not significantly impact target detection (Kamau et al., 2017). This mismatch is not necessary to produce a false negative result because the effect of the mismatch varies according to number, position, and target (probe, forward, or reverse primer) (Chow et al., 2011; Ye et al., 2012). Several studies have investigated the effect of a mismatch between target and primer and have shown that the target can be amplified even if it has some mismatch with the primer (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 affect duplex stability, which can then hinder the ability of the system to amplify template DNA (Yu et al., 2012). The negative effect of single-nucleotide mismatches on target annealing is lower than deletions or multi-nucleotide mismatches (Lefever et al., 2013). Selecting a suitable primer is one of the most important factors affecting PCR results (Kingsland and Maibaum, 2018). PCR results can be affected by many conditions, such as DNA template preparation and 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).

Based on 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

192 3-terminal region (defined as the last 5 nucleotides from the 3-terminal region) of the primers have
193 a much more significant effect (Lefever et al., 2013; Stadhouders et al., 2010; Brault et al., 2012),
194 as well as the mismatch of two bases at the 3' end generally preventing amplification (Ye et al.,
195 2012; Ghedira et al., 2009). Based on these results, the pathotype-specific primers (Fout-S/Fout-
196 AS and F2-S/F2-AS) developed by Pham et al. (2005) cannot be used to differentiate virulent and
197 avirulent ND viruses in NDV/Ck/Bogor/ 011; NDV/Ck/GnSindur/014; NDV/Ck/Cianjur/015 and
198 NDV/Ck/Bogor/015. This can be caused by mutations in the primary site, especially in the F2-S
199 target. ND virus mutations affected by co-circulation of genetically distinct virus lineages with the
200 predominant virus genotype circulating in a particular time period.
201 Putri et al., (2018) showed that NDV/Ck/Bogor/ 011 categorize as NDV genotype VII (h)
202 and NDV/Ck/GnSindur/014 categorize as NDV genotype VII (i), and NDV/Ck/Cianjur/015 and
203 NDV/Ck/Bogor/015 as NDV genotype II. Isolate was used as a sample in Pham et al study
204 obtained from ND outbreak in Japan in the 1980-an until 2000. The isolate categorized as NDV
205 genotype VII (d) (Umali et al., 2013). Genotype VII is the most predominant NDV genotype that
206 is responsible for most outbreaks in East Asian countries including Japan, Taiwan, Korea and
207 China since the 1980s, constituting the fourth pandemic (Lien et al., 2007; Mase et al., 2002).
208 Wild birds have played a role in the circulation of VII viruses across the Far East Asian countries
209 (Umali et al., 2013).
210 Changes in viral DNA or mutations are a form of virus dynamics to adapt to the
211 environment to survive (Sobhanie, 2021). Hypervariable region analysis of the F gene Newcastle
212 Disease isolate showed amino acid substitution in five mutation points in the F gene of all isolates
213 (Putri et al., 2018). Viruses accumulate mutations in their genomes when adapting to animal hosts.
214 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 should result in stronger template binding for better assay sensitivity (Brault et al., 2012). Our present study has certain limitation. This study was used small number of samples are isolated at certain time limits from one region, so It can't be explained whether this primer can still detect other sample from other countries at different periods of time. The nucleotides sequence at the F gen cleavage site has been shown to be a major determinant of virulent or avirulent NDV (Putri et al., 2017). In general, nucleotide sequence of virulent strains at least has three basic amino-acids (multi basic cleavage site) (OIE., 2012). Alteration of one of nucleotide base can change the amino acid motif. The alteration of amino acid caused by mutation or substitution associated with the many diverse genotypes of the virus (Putri et al., 2018). It is very appropriate to design and use primers targeting the F gen cleavage site to differentiate virulent and avirulent ND viruses. In addition, it is necessary to do extensive evaluation of the genomic changes of the ND virus, to anticipate mismatch 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 isolates NDV/Ck/Bogor/011, NDV/GnSindur/014 NDV/CK/Bogor/015 and NDV /Ck/Cianjur/015. It is crucial to monitor the target of diagnostic PCR tests for any possible future mutations as the virus continues to evolve in its host.

The Author's Contribution

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

Conflict of Interest

The author's country has no conflict of interest.

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Comparison of two set pathotypic-specific primer to detect Newcastle Disease Virus DWI DESMIYENI PUTRI*1), NURHAYATI1), INTAN KAMILIA HABSARI1), NI LUH PUTU IKA MAYASARI2)

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14**gmail.com** **Abstract This study was** designed **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 were used as isolates representing the ND virus currently circulating in Indonesia. The study used 4 pathotype-specific primers. The research's first step was to analyze the compatibility of the primers and ND isolate-sequencing results

11**using the sequence alignment editor software** BioEdit® **Version 7.2**

and MEGA version 11. 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).

9**Based on the results** of **this research, it** is known **that**

virulent ND isolates can be amplified with

1**pathotype- specific primers developed 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.

28**INTRODUCTION Newcastle Disease (ND) is a systemic respiratory disease** in poultry **that is acute and**

highly contagious (Waheed et al., 2013). Newcastle

12**Disease is caused by Avian Paramyxovirus** type-**1 (APMV-1), which belongs to the genus Avulavirus, the family Paramyxoviridae**

(Alexander and Jones, 2000). Newcastle Disease viruses

40**can be divided into** 4 **groups based on** the pathotype: velogenic, **mesogenic**

, lentogenic and avirulent (Aldous and Alexander, 2001).

53**Velogenic and mesogenic** ND virus **strains are** categorized as **virulent**

ND viruses and

1**have been identified as causative** agents **of ND outbreaks in many countries** worldwide. Lentogenic and **avirulent** virus **strains** are widely **used**

in disease control programs as live vaccines. Determination of NDV pathotype is generally carried out by isolating the virus from embryonated chicken eggs (TAB) and

1**followed by in vivo tests such as** the **intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI) and mean death time (MDT) in** chicken Species **Pathogen Free (SPF**

) (OIE, 2012; Cattoli et al., 2011). Molecular methods such as

30**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR**) have **been developed to identify** ND **viruses**

(Farooq

18**et al., 2014**; Lai **et al**., 2012; **Rabalski et al., 2014**

). Amino acid sequencing is a further step that must be carried out to determine the pathotype of the ND virus 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 development requires accurate molecular analysis of ND viruses of pathotype- specific ND primers. Aldous and Alexander (2001) recommended several primers, either universal or specific, to detect ND viruses circulating in the world. The research by Kant et al. (1997) succeeded in determining the pathotype of the ND virus using pathotype-specific primers. Pham et al. (2005) developed pathotype-specific primers known as nested PCR to detect ND virus rapidly. Both the pathotype-specific primers have different amplification sites

18**in the cleavage site of the** ND virus **F gene**

(Alexander, 2009; Madadgar

50et al., 2013). Newcastle Disease Virus

continued to undergo mutations (evolutionary distance 3–9%) Putri et al., (2018). The research was needed to determine whether these primers can still characterize ND viruses quickly and accurately. This study was designed to analyze the compatibility of two sets of pathotypic-specific primers developed

47by Kant et al. (1997) and Pham et al

. (2005) to detect ND viruses 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 representing the ND virus currently circulating in Indonesia. Characterization of

20ND virus pathotypes by RT-PCR was carried out using pathotype- specific primers

37developed by Kant et al. (1997) and Pham et al. (2005). In this

study, 4 pathotype-specific primer sets were used. The two primers are primers developed by

49Kant et al. (1997), and the two primer

sets are

1pathotype-specific primers developed by Pham et al

. (2005). The first step was to analyze the primers' compatibility with the nucleotide sequences of the ND virus using

11BioEdit® sequence alignment editor software Version 7.2 (Hall, 1999

) and MEGA version 11 (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 dan second- step results. The data obtained were analyzed, displayed in images, and presented descriptively. Isolation of ND Virus RNA Viral

27RNA was extracted from the allantoic fluid according to the manufacturer's instructions (Qiagen 2014) using the QIAamp @ Viral RNA Mini Kit

52904 (Qiagen, Germany). A total of 140

52µl of the sample was extracted and diluted

26to a final volume of 60 µl and stored at -80 °C until use

. ND Virus Amplification Reverse Transcriptase-Polymerase Chain Reaction

2was performed using the One-step RT-PCR kit (Qiagen, Germany) according to the manufacturer's instructions. The RT-PCR master mix each consisted of 2 µl dNTPs (10 mM), 2 µl forward primer (10 pM), 2 µl reverse primer (10 pM), 2 µl RNA template, 10 µl 5× Onestep RT-PCR buffer Qiagen, 30 µl Rnase free water, and 2 µl Onestep RT-PCR enzyme were mixed until the final volume was 50 µl. Matrix (M) gene amplification 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. Fusion (F) gene

amplification

45was carried out under the following cycle conditions

:

245 °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

51cleavage site of the F gene

, namely:

1NDV-FA/FB, which is specific for virulent ND virus, and NDV-FA/ The FC is specific for avirulent ND virus 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

39nucleotide sequences of all primers are presented in Table 1. Electrophoresis

17PCR products were visualized using electrophoresis on 1.5% agarose gel containing 0.4 µg/ml ethidium bromide and observed using a UV transilluminator. The flowchart of

research activities

21Characterization of Newcastle Disease Virus by RT-PCR

1using pathotype-specific primers can be seen in Figure

1. Analysis of DNA Oligonucleotide Sequencing Results The oligonucleotide sequencing results

13were analyzed using BioEdit® sequence alignment editor software Version 7.2

(

29Hall, 1999). Sequence alignment was performed using MEGA software version 11 (Tamura et al., 2013

). Result and Discussion

22Molecular methods such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) and amino acid sequencing can be used

to determine

35the pathotype of NDV (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 (

36Kant et al., 1997; Ahmadi et al., 2014; Pham et al., 2005) have

also developed nested -PCR to identify the pathotype of the ND virus (Green and Sanbrook, 2019). The Pathotype-

1primers developed by Kant et al

. (1997) have amplified targets on

46the cleavage site of the NDV F gene that

have

1the same nucleotide sequence with forward primar (FA) sequence and a different sequence nucleotide for reverse primar (FB and FC

). The pathotype-specific primer developed

42by Kant et al. (1997) was designed based on the nucleotide

15sequence at the F gene cleavage site, which is the primary determinant of NDV virulence (Ahmadi, 2014; Yu et al., 2001; Madadgar et al., 2013). The

1F gene cleavage site has the same nucleotide sequence as 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

25FA/FB and FA/FC with the

ND virus sequences are presented in Figure 1. Figure 1 show that

25the FA/FB and FA/FC 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 255 bp band. The amplification results of the 4 samples

19can be seen in Figure 2, and the analysis of the

compatibility of the nucleotide sequences between the primers and the samples can be seen in Table 2. Table 2 illustrates variations in nucleotide mismatches between the FA/FB primers and the nucleotide sequences of the ND isolate. FA/FB primers could amplify

3NDV/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

3NDV/Ck/Bogor/011 and NDV/Ck/GnSindur/014

had a nucleotide mismatch 6 – 7 nucleotides with FA/FC primers, and isolates

3NDV/Ck/Cianjur/015 and NDV/Ck/Bogor/015

had a mismatch 5 nucleotides with FA/FC primers. The result is in line with Putri's research, 2017 which showed that isolates

3NDV/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. The more

41nucleotide mismatch between primer and template, the less amplification

will not occur (Kingsland and Maibaum, 2018; Ye et al., 2012) state that at least 5

43nucleotide 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).

8Nested PCR involves two sequential amplification reactions, each using a different pair of primers. The product of the first amplification reaction is used as the 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 is 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

19can be seen in Figure 3. Figure 3 shows 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, then proceed with 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 a 300 bp band. Based on the results of the primer amplification of F2-S/F2-AS, showed that 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 4. Table 4 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 showed that the nucleotide

33mismatch between the primers and the template can still cause the primer to stick to the

template, and elongation and amplification processes can occur. A single mismatch in backward

5or forward primers may not significantly impact target detection

(Kamau et al., 2017). This mismatch is

5not necessary to produce a false negative result because the effect of the mismatch varies according to number, position, and target (probe, forward, or reverse primer

) (Chow et al., 2011; Ye et al., 2012). Several

7studies have investigated the effect of a mismatch between target and primer and have shown that the target can be amplified even if it has

some mismatch with the primer (Wiley, 2005; Sipos

32et al., 2007; Waterfall et al., 2002; Ghedira et al., 2009; Ye et al., 2012

) stated that the primer must not fit all

48**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

23**DNA can affect duplex stability, which** can **then** hinder **the ability of** the **system to amplify template DNA**

(Yu et al., 2012).

5**The negative effect of single-nucleotide** mismatches **on target annealing is lower than deletions or** multi-**nucleotide mismatches**

(Lefever et al., 2013). Selecting a suitable primer

38**is one of the most important factors affecting** PCR results (Kingsland **and**

Maibaum, 2018). PCR results

6**can be** affected **by many conditions, such as** DNA **template preparation and reaction conditions**, as well as **good** primer **pair** design, which **is a critical factor**

in determining the success of amplification (Ye

9**et al., 2012**; Higgins **et al**., 2022). **Based on 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.

16**Mismatches located in the 3**-terminal **region (defined as the last 5 nucleotides** from **the 3**-terminal **region) of** the primers have **a**

much more significant effect (Lefever

34**et al., 2013**; Stadhouders **et al**., 2010; Brault **et al**., 2012), **as well as**

the mismatch of two bases at the 3' end generally preventing amplification (Ye

9**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 ND viruses in NDV/Ck/Bogor/ 011;

3**NDV/Ck**/GnSindur/**014; NDV/Ck**/Cianjur/**015 and NDV/Ck**/Bogor/**015**

. This can be caused by mutations in the primary site, especially in the F2-S target. ND virus mutations affected

4**by co-circulation of genetically distinct virus lineages with the predominant virus genotype circulating in a particular time period**

. Putri et al., (2018) showed that NDV/Ck/Bogor/ 011 categorize as NDV genotype VII (h) and NDV/Ck/GnSindur/014 categorize as NDV genotype VII (i),

3and NDV/Ck/Cianjur/015 and NDV/Ck/Bogor/015 as NDV genotype II

. Isolate was used as a sample in Pham et al study obtained from ND outbreak in Japan in the 1980-an until 2000. The isolate categorized as NDV genotype VII (d) (Umali et al., 2013).

4Genotype VII is the most predominant NDV genotype that is responsible for most outbreaks in East Asian countries including Japan, Taiwan, Korea and China since the 1980s, constituting the fourth pandemic

(Lien et al., 2007; Mase et al., 2002).

4Wild birds have played a role in the circulation of VII viruses across the Far East Asian countries

(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).

10Hypervariable region analysis of the F gene Newcastle Disease isolate showed amino acid substitution in five mutation 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

5should result in stronger template binding for better assay sensitivity

(Brault et al., 2012). Our present study has certain limitation. This study was used small number of samples are isolated at certain time limits from one region, so It can't be explained whether this primer can still detect other sample from other countries at different periods of time. The

1nucleotides sequence at the F gen cleavage site has been shown to be a major determinant of virulent or avirulent NDV

(Putri et al., 2017). In general, nucleotide sequence of

1virulent strains at least has three basic amino-acids (multi basic cleavage site

) (OIE., 2012). Alteration of one of nucleotide base can change the

1amino acid motif. The alteration of amino acid caused by mutation or substitution associated with the many diverse genotypes of the virus

(Putri et al., 2018). It is very appropriate to design and use primers targeting the F gen cleavage site to differentiate virulent and avirulent ND viruses. In addition, it is necessary to do extensive evaluation of the genomic changes of the ND virus, to anticipate mismatch 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 isolates NDV/Ck/Bogor/011, NDV/GnSindur/

3014 NDV/CK/Bogor/015 and NDV /Ck/Cianjur/015

.

5It is crucial to monitor the target of diagnostic PCR tests for any possible future mutations as the virus continues to evolve in

its host. The Author's Contribution DDP and N design the research; DDP and NPIM conducted experiment in laboratory; DDP, N, and IKH analyze the data; DDP and NPIM drafted the manuscript; DDP and IKH revised the manuscript.

44**Conflict of Interest The author's** country has **no** conflict **of**

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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 ND virus 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.

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 ND virus. 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 ND viruses circulating in the world. The research by Kant et al. (1997) succeeded in determining the pathotype of the ND virus using pathotype-specific primers. Pham et al. (2005) developed pathotype-specific primers known as nested PCR to detect ND virus rapidly. Both the pathotype-specific primers have different amplification sites in the cleavage site of the ND virus F gene (Alexander, 2009; Madadgar et al., 2013). The ND Virus continued to undergo mutations (evolutionary distance 3–9%) Putri et al. (2018). Research was needed to determine whether these primers can still characterize ND viruses 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 ND viruses 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 ND virus currently circulating in Indonesia. Characterization of ND virus 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 microliters of sample was extracted and made more concentrated to a final volume of 60 microliters. 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). 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 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.

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 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 4.

Table 4 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 ND viruses in all NDV isolates. This can be caused by mutations in the primary site, especially in the F2-S target. ND 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 ND viruses. In addition, it is necessary to do extensive evaluations of the genomic changes of the ND virus, 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. 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.

The 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.

Conflict of Interest

The author's country has no conflict of interest.

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Comparison Two Set Patotype

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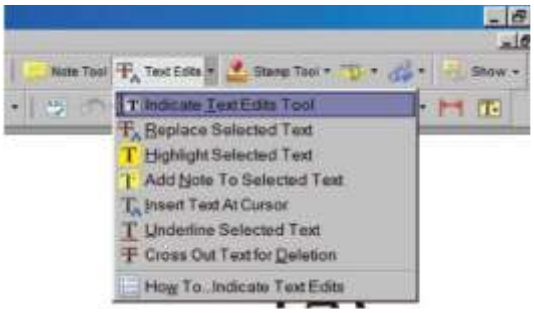

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Research Article

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 ND virus 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 ND virus. 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 ND viruses circulating in the world. The research by Kant et al. (1997) succeeded in determining the pathotype of the ND virus using pathotype-specific primers. Pham et al. (2005) developed pathotype-specific primers known as nested PCR to detect ND virus rapidly. Both the pathotype-specific primers have different amplification sites in the cleavage site of the ND virus F gene (Alexander, 2009; Madadgar et al., 2013). The ND Virus continued to undergo mutations (evolutionary distance 3–9%) Putri et al. (2018). Research was needed to determine whether these primers can still characterize ND viruses 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 ND viruses 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 ND virus currently circulating in Indonesia. Characterization of ND virus 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 microliters of sample was extracted and made more concentrated to a final volume of 60 microliters. 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.

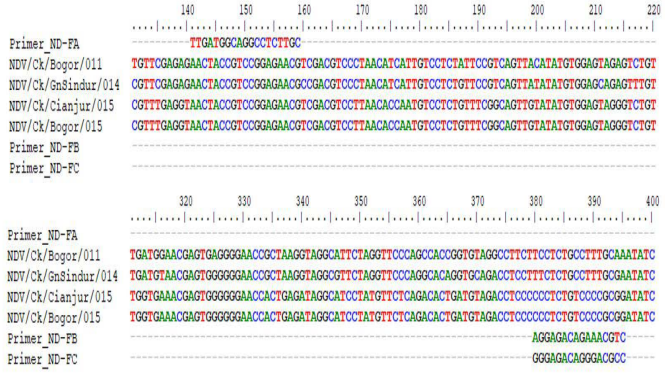

Figure 1:

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.

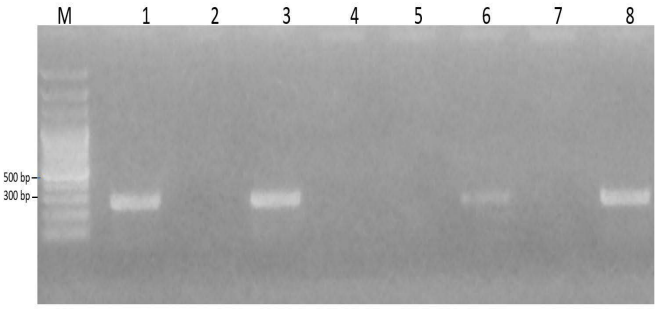

Figure 2:

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.

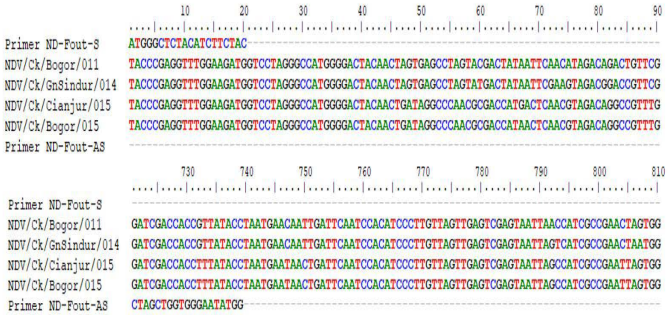

Figure 3:

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.

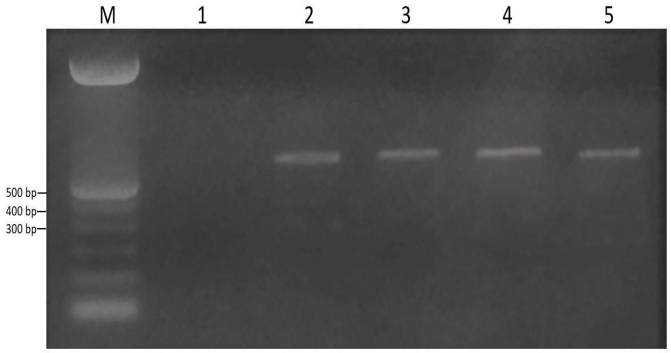

Figure 4.

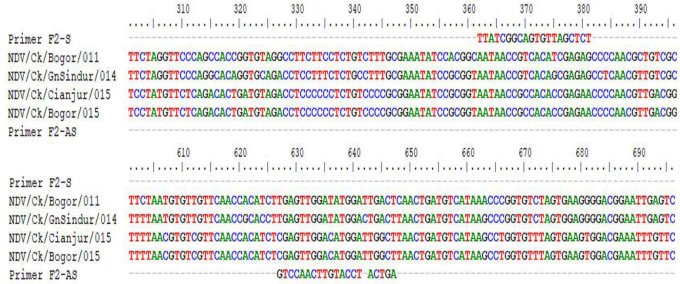

Figure 5.

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 ND viruses in all NDV isolates. This can be caused by mutations in the primary site, especially in the F2-S target. ND 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 (Suranie, 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 ND viruses. In addition, it is necessary to do extensive evaluations of the genomic changes of the ND virus 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

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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