Bukti Korespondensi Syarat Khusus (2)

Judul Artikel : "**Production of hyperimmune serum against genotype VII Newcastle disease virus in rabbits with several applications"**

No	Kegiatan	Tgl/bulan/tahun	Halaman
1.	Surat Pernyataan		1
2.	Submit Artikel	13 Oktober 2021	2
3.	Hasil Peer Review	23 Maret 2022	3
4.	Tanggapan dari hasil peer review		5
5.	Dokumen perbaikan dari hasil peer review		7
7.	Artikel dinyatakan diterima	4 April 2022	30
8.	Tanggapan atas komentar editor	9 Juni 2022	32
9.	Dokumen perbaikan atas komentar editor		33
10.	Proof Reading	16 Juni 2022	48
11.	Artikel dipublikasikan	7 Juli 2022	59

KEMENTERIAN PENDIDIKAN KEBUDAYAAN RISET DAN TEKNOLOGI
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SURAT PERNYATAAN

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Menyatakan bahwa artikel yang berjudul :

"Production of hyperimmune serum against genotype VII Newcastle disease virus in rabbits with several applications, yang dipublikasikan pada Jurnal: Journal of Advanced Veterinary and Animal Research, ISSN 2311-7710, Volume 9(2): 211-220"

Merupakan luaran dari riset DRPM skema Penelitian Dasar tahun 2020 pendanaan 2021 dengan judul :"**Potensi Antibodi *Newcastle Disease* Genotipe VII sebagai Reagen Imunodiagnostik dan Imunoterapi** ", yang dibiayai oleh Direktorat Riset dan Pengabdian Masyarakat, Kementerian Riset dan Teknologi, sesuai dengan Surat Perjanjian Pelaksanaan Hibah penelitian bagi Dosen Tahun Anggaran 2021, dengan nomor kontrak 378.4/PL15.8/2021 Tanggal 19 Juni 2021. Penelitian ini juga dijadikan sebagai syarat khusus tambahan point (1) "Pernah mendapat hibah penelitian kompetitif". Terdapatnya salah seorang pembimbing sebagai salah satu author dari penelitian tersebut karena keterlibatan beliau sebagai mitra dalam penelitian.

Demikian surat pernyataan ini dibuat, untuk dapat dipergunakan sebagaimana mestinya.

Bandar Lampung, 15 September 2024

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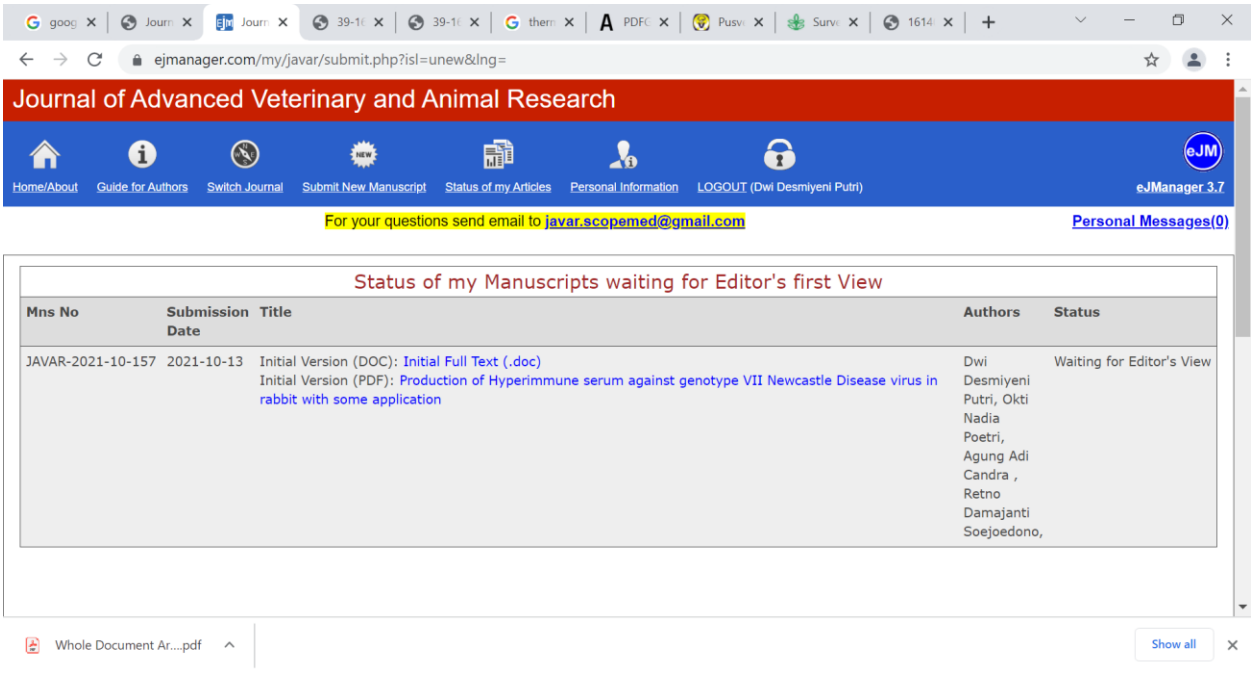
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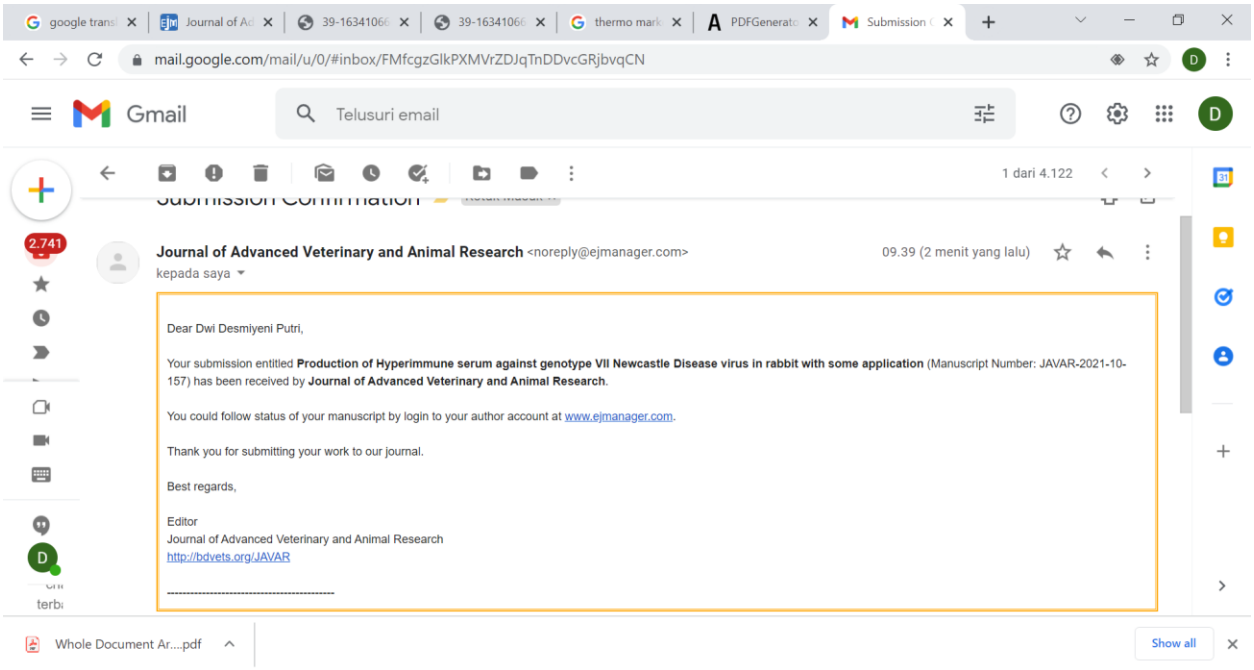
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Thank you for submitting your work to our journal.
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Article Revision Letter for Authors - (JAVAR-2021-10-157)

Noreply eJManager <noreply@ejmanager.com> 23 Maret 2022 pukul 23.10
Kepada: desmiyenidwi@gmail.com

Dear Dwi Desmiyeni Putri,

Your manuscript entitled \"Production of Hyperimmune serum against genotype VII Newcastle Disease virus in rabbit with some application\" (Ms.Nr. JAVAR-2021-10-157) was reviewed by expert reviewers of the Journal of Advanced Veterinary and Animal Research. As an initial decision, your manuscript was found interesting but some revisions have to be made before it can reach a publishable value.
Please answer all the comments below point-by-point in an accompanying response letter to your revised submission.

You should send your revised manuscript via the online system of ScopeMed on my.ejmanager.com.

Sincerely yours,

Nazmul H. Nazir, PhD
Editor-in-Chief
Journal of Advanced Veterinary and Animal Research

COMMENTS for Authors:

=> Reviewer # 1

The article described the production of hyperimmune serum against NDV genotype VII, with some applications. The authors described that they could produce immunity titer. However, the following points should be incorporated before acceptance.

1. Knowledge gap is weak in the introduction section. Please incorporate several recent references, on which novelty will stand for.

2. Results are ok.

3. Discussion section needs improvement considering recent references and novelty. Please revise this section considering latest references and novelty.

4. Reference section is not compatible with the journal style. Check and revise the references accordingly.

Good luck.

=> Reviewer # 2

The author found better titer on Day 38. The concentration of antibody is high. However, after injecting into the body the concentration will reduced. Moreover, biological half-life of immunoglobulin is there. Question is how long the protection will sustain? And, the protective antibody titer should be specified.

Serum preparation can be modified considering the presence of complement because complement may present even after centrifugation. How did you do that confirmation? Complement will hamper the reaction results, isn\'t it?

Reference style is not compatible with the journal. Please check the published articles and do the modification accordingly.

Letter

Article Title: Production of Hyperimmune serum against genotype VII Newcastle Disease virus in rabbit with some application

Letter Subject: Article Revision Letter for Authors - (JAVAR-2021-10-157)

Letter:
Dear Dwi Desmiyeni Putri,

Your manuscript entitled "Production of Hyperimmune serum against genotype VII Newcastle Disease virus in rabbit with some application" (Ms.Nr. JAVAR-2021-10-157) was reviewed by expert reviewers of the Journal of Advanced Veterinary and Animal Research. As an initial decision, your manuscript was found interesting but some revisions have to be made before it can reach a publishable value.
Please answer all the comments below point-by-point in an accompanying response letter to your revised submission.

You should send your revised manuscript via the online system of ScopeMed on my.ejmanager.com.

Sincerely yours,

Nazmul H. Nazir, PhD
Editor-in-Chief
Journal of Advanced Veterinary and Animal Research

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Good luck.

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The author found better titer on Day 38. The concentration of antibody is high. However, after injecting into the body the concentration will reduced. Moreover, biological half-life of immunoglobulin is there. Question is how long the protection will sustain? And, the protective antibody titer should be specified.

Serum preparation can be modified considering the presence of complement because complement may present even after centrifugation. How did you do that confirmation? Complement will hamper the reaction results, isn't it?

Reference style is not compatible with the journal. Please check the published articles and do the modification accordingly.

Letter Sent Date: Mar 23, 2022

List of change made in manuscript: Ms. Nr. JAVAR-2021-10-157
"Production of Hyperimmune serum against genotype VII Newcastle Disease virus in rabbit with several applications"

We would like to thank to the 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 blue italics)

=> Reviewer # 1

The article described the production of hyperimmune serum against NDV genotype VII, with some applications. The authors described that they could produce immunity titer. However, the following points should be incorporated before acceptance.

1. Knowledge gap is weak in the introduction section. Please incorporate several recent references, on which novelty will stand for.

Thank you for your comments and suggestions. We agree with your comments that knowledge gap is weak in the introduction section. We have added some recent references on line 78 until line 84 in introduction part. We hope it is in line with reviewer suggestion.

2. Discussion section needs improvement considering recent references and novelty. Please revise this section considering latest references and novelty.

We agree with your comment. We added some recent references in discussion line 354-362. We hope it is in line with reviewer suggestion.

4. Reference section is not compatible with the journal style. Check and revise the references accordingly.

We agree with your comment. We adjusted it accordingly.

Reviewer # 2

The author found better titer on Day 38. The concentration of antibody is high. However, after injecting into the body the concentration will reduced. Moreover, biological half-life of immunoglobulin is there. Question is how Question is how long the protection will sustain long the protection will sustain? And, the protective antibody titer should be specified.

Thank you for your comments. I will try to answer this question.In this research we found antibody titer of ND reached 1024 HI titer on day 38th. Naturally. if the antibody going to be injected to other animals as passive immunity, the antibody titer will decline. It is caused the antibody have the half time in the body. To answer the Question how long the protection will sustain. In this study we have not applied Hyperimmune Serum as immunotherapy. For next step, the hyperimmune serum that produced in this study will be used as immunotherapy agent.

Hyperimmune Serum to be applied to chickens suspected or confirmed to be infected with ND. The symptoms of ND successfully treated through passively immunization with the use of HIS (Pansota et al, 2013). Use of HIS as immunotherapy for ND should be as early as possible after the exposure of birds to the NDV (Syarif et al, 2017). Based on OIE standard, the protective titer of ND at least 4log$_2$. We hope it has answered the reviewer's question.

Serum preparation can be modified considering the presence of complement because complement may present even after centrifugation. How did you do that confirmation? Complement will hamper the reaction results, isn't it?

Thank you for your comments and suggestions. We agree with your comment. In this study we have applied inactivation at 56°C for 30 min before used to evaluate the HI titer. We have added the information on line 131 in materials and method. We hope it is in line with reviewer suggestion.

Reference style is not compatible with the journal. Please check the published articles and do the modification accordingly.

We agree with your comment. We adjusted it accordingly

ORIGINAL ARTICLE

The production of hyperimmune serum against genotype VII Newcastle Disease virus in rabbit with several applications

Statement of novelty: Finding a method for the production of hyperimmune serum against genotype VII Newcastle Disease Virus (NDV). The serum can be produced by immunizing rabbit intravenously within 38 days and until the antibody reached 2^{10} of HI titer. Genotype VII Newcastle Disease hyperimmune serum produced in this research have spesificity for Newcastle disease virus and was proven by Agar Gel Precipitation Test and Western Blot Assay.

Ethical approval (if needed): This research has been approved by the Animal Care and Use Committee of Research and Community Services Institution, IPB University with approval number: 213-2021 IPB.

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Production of hyperimmune serum against genotype VII Newcastle Disease virus in rabbit with several applications

ABSTRACT

Objective : The aim of this research was to produce hyperimmune serum against genotype VII NDV with several applications.

Materials and Methods : Production of hyperimune serum against genotype VII NDV was performed on eight New Zealand White rabbits which were divided into four groups. Rabbits were immunized three times on 1st day, 14th day and 30th day. Blood sampling was carried out on the 8th day after third immunization.

Results : All groups showed the same pattern of HI titer results, HI titers would peak on 5th or 9th day after the second immunization, then decrease until 3rd day after the third immunization and increase again on 5th day after the third immunization. Rabbits immunized intravenously showed higher HI titers than the other groups. These results indicated that the intravenous route for hyperimmune serum production against genotype VII Newcastle Disease virus greatly affects the immune response result.

Conclusion : The production hyperimmune serum by intravenous immunization three times was able to produce the highest titer of 2^{10} at 38 days. The Hyperimmune serum had specificity for Newcastle Disease antigen based on the Agar Gel Precipitation Test and Western blot assay result.

Keywords: HI titer, Hyperimmune serum, Newcastle Disease.

INTRODUCTION

Newcastle Disease (ND) is the one of important diseases in bird that is caused by Avian paramyxovirus serotype-1 (APMV-1) which belongs to the family Paramyxoviridae [1, 2]. The Newcastle Disease Virus (NDV) has the ability to infect more than 250 spesies of birds, and infection by virulent strains can cause huge morbidity and mortality with significant symptoms [3]. The wide range of susceptible hosts causes the persistence of NDV which becomes endemic in many countries in the world. Virulent strains infection has resulted four panzootics [4]. The first ND outbreak by virulent strain occurred in Java, Indonesia in 1926, and at the same time an outbreak happened in England precisely in Newcastle upon Tyne region [5].

Newcastle Disease virus is an enveloped, unsegmented, single-stranded RNA genome of roughly 15.2 kb [5, 6]. The NDV genome encodes six polypeptides namely the nucleocapsid (NP) protein, phosphoprotein (P) protein, matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, and the RNA-dependent RNA polymerase (L) protein. The virus nucleocapsid core consists of NP proteins bound to RNA [7].

Newcastle Disease Virus may vary widely in the severity of the disease in birds [8]. Multiple factors can contribute to the severity of disease including species of host, immune status, age, environmental conditions, secondary infections, the number of virus transmitted, the mode of transmission and most importantly the pathotype of the infecting virus [9]. In comparison, susceptible species is chickens, whereas geese and ducks do not show symptom; therefore, waterfowl are considered as the natural reservoir for NDV. The F protein cleavage site is known to be a major determinant of viral virulence during replication in host cells. [10, 11]. Based on the pathogenicity of the disease, ND can be classified into five pathotypes: - Neurotrophic velogenic strain exhibiting respiratory and neurological symptoms with a high mortality rate; Viscerotropic velogenic strain causing hemorrhagic and highly pathogenic intestinal lesions; Mesogenic strain caused by viruses with rare respiratory and neurological symptoms, while mortality is related to the age of susceptible birds; Viral lentogenic strains

present with mild respiratory infection; and Asymptomatic enteric strain exhibiting no clinical sign or asymptomatic[12].

Interaction between virus and environment including host immune system resulted in NDV evolution and continues to produce new genotypes virus. Lately, infection of genotype VII NDV caused high mortality of birds in several poultry farms in Indonesia [13, 14]. In recent years, producing hyperimmune serum in animals is an important activity of many research projects. The hyperimmune serum as a biological reagen will continue to be developed for research needs and possibly also for commercial applications in the future such as for therapy and development of immunodignostic tools[15]. *The specific antibodies in hyperimmune serum can be used for the treatment and control of disease in case of an outbreak [16]. Hyperimmune serum is already used for the successful treatment of some disease like foot and mouth diseases, tetanus and canine viral diseases [17] . Currently, the imported hyperimmune serum used for diagnostic in poultry is very expensive and has been imported from different countries of the world. Moreover, the imported strains of viruses may differ from indigenous isolates showing non-specificity in diagnosis [17]*. The development of the serum for NDV currently circulating must be followed by the development of immunodiagnostic tests, to obtain accurate test results. Therefore, it is necessary to produce genotype VII Newcastle Disease hyperimune serum which can be used as immunodiagnostic reagents.

Hyperimune serum production can be carried out in various applications, with or without adjuvant and with its own advantages and disadvantages. Considering the numerous applications of hyperimune serum in research and clinical fields, the preparation method development of hyperimmune serum against pathogens is very important [15]. To be able to produce antibodies with high titers in a short time, it is necessary to conduct research on various immunization applications with or without adjuvant in inducing immunity. The aim of this research was to produce hyperimune serum againts genotype VII NDV with several applications efficient in time and cost.

MATERIALS AND METHODS

Ethical approval

This research has been approved by the Animal Care and Use Committee of Research and Community Services Institution, IPB University with approval number: 213-2021 IPB.

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Newcastle Disease Antigen

For the production of NDV hyperimune serum, characterized genotype VII NDV was used. Isolate was obtained from the repository of the Immunology Laboratory, Faculty of Veterinary Medicine, IPB University. The isolate was categorized as genotype VII NDV by PCR, sequencing dan filogenetic analysis [11, 13]. The antigen was prepared in fresh condition with and without adjuvant use.

Hyperimmune Serum Production

The production of hyperimune serum against genotype VII NDV was performed on eight New Zealand White rabbits aged 2,5 – 3,5 month with an average body weight of 2,5 kg that were formed into four groups. First group was rabbit immunized by emulsion of 1 ml isolate genotype VII NDV ($5\times10^{6.5}$ ELD_{50}/ml) and 1 ml Incomplate Freund's Adjuvant (IFA) subcutaneously; second group was rabbit immunized by emulsion of 0,5 ml isolate genotype VII NDV ($5\times10^{6.5}$ ELD_{50}/ml) and 0,5 ml IFA subcutaneously; third group was rabbit immunized by 1 ml isolate genotype VII NDV ($5\times10^{6.5}$ ELD_{50}/ml) subcutaneously and last group was rabbit immunized by 1 ml isolate genotype VII NDV ($5\times10^{6.5}$ ELD_{50}/ml) intravenously. The application and composition of the antigens used in this study are presented in Table 1.

Table 1. Composition and Aplication of Genotype VII NDV Isolate

Group	Volume		Aplication
	Antigen	IFA*)	
1	1 ml	1 ml	subcutaneously
2	0,5 ml	0,5 ml	subcutaneously

3	1 ml	-	subcutaneously
4	1 ml	-	intravenously

*) Incomplate Fruend's Adjuvant

Rabbits were immunized three times. First imunization was on 1st day and second immunization was on 14 th day and third immunization was on 30th day. Blood sampling was carried out on the 8th day after third immunization. Hyperimmune serum was collected by taking blood intra artery after the rabbits had been administered local anastethic agent into the ear. The procedure for making serum is as follows: The blood samples were kept at temperature ± 25°C for an hour and then kept overnight at 4°C t. The serum was separated manually and precipitated by centrifugation at 2500 rpm for 15 minutes. Futhermore, the serum was kept in collecting tube 1.5 ml and stored at -20°C until use. The rabbit blood samples were taken periodically to observe the Hemagglutinatin Inhibition (HI) antibody titer against genotype VII NDV. *Serum was inactivated at 56^{0}C for 30 min before used for the HI test.*

Serum Purification

Purification of ND hyperimmune serum was carried out by two stages. Precipitation by ammonium sulfate (4.1 M) was the first [18]. The first stage of serum precipitation was executed by stirring equal volumes of ammonium sulfate and serum solutions slowly, then incubating them overnight at 4°C. After that, the precipitate was centrifuged at 3000 xg for 20 minutes. The pellet was reconstituted by phosphate buffered saline pH 7.4 to obtain one-fourth of antibody volume. Hereafter was dialysis performed by puttingthe precipitate in a dialysis bag and stirred it in PBS pH 7.4 for 24 h at 4°C that was replaced every 8 h by PBS solution. The second step was hyperimmune serum purification using protein A purification kit (BioVision, USA) according to the manufacturer's instructions.

Serum Characterization by SDS-PAGE

The molecular weight of the purified ND hyperimun serum was measured using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique with the

concentration of separating gel 12% and 4% for the stacking gel [19]. The sample buffer (containing bromophenol blue, SDS, DTT and glycerol) 5 µl was mixed with serum sample (5 µl), and heated 65°C for five minutes to denature the protein. A total of 5 µl of marker protein (Thermo Scientific, USA) and 10 µl of hyperimun serum samples were used. Protein separation was carried out by electrophoresis at 100 V for 150 minutes. The electrophoresis gel was stained with Commasie Brilliat Blue for 30 minutes, followed by the addition of destaining solution for 24 hours.

Serum confirmation by Agar Gel Precipitation Test and Western Blot Assay

Determination the specificity of ND antibody can be done by several ways including Agar gel precipitation test (AGPT) and Western Blot Assay. ND antibody specificity was confirmed to two ND viruses [13] and other antigens such as Infectious Bursal Disease (IBD), and Avian Influenza (AI). The precipitation line in agarose gel indicated antigen and antibody interaction.

In order to detect Newcastle Disease viral protein, antigen genotipe VII NDV was run on SDS-PAGE gel. The SDS-PAGE result was transferred to Nitro Cellulose (NC) membranes. The membrane was blocked with Tris Buffer Saline (TBS) containing 0.05% Tween-20 (T-TBS) and 3% bovine serum albumin at 37^0C for 2 hours. After the T-TBS washing, the membrane was incubated with 1 : 2000 dilution of primary rabbit hyperimune serum (against NDV produced in this research) overnight and then washed by T-TBS. Afterwards the NC membrane was incubated in alkaline phosphatase conjugated secondary antibody at 37^0C for 2 hours. The membranes were washed and developed using Diaminobenzidine (DAB) substrate solution (Sigma) for 5-10 minutes. At the end of this procedure, the membrane was washed by distilled water to terminate enzyme reaction on the membrane.

RESULTS AND DISCUSSION

Antigen Preparation

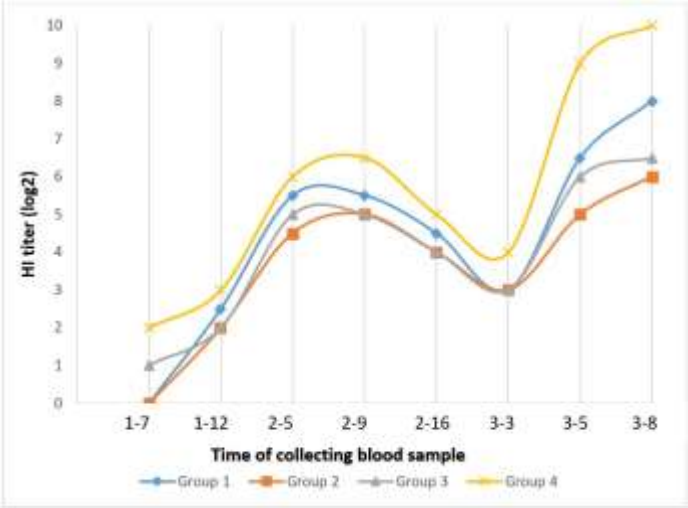
Antigens used in this research were genotype VII NDV characterized by PCR, sequencing, and phylogenetic analysis [11, 13]. The virus's ELD_{50} must be calculated to determine the virus's ability to kill 50% of Specific Pathogen Free embryos in eggs. The virus used in this study is genotype VII NDV with 5 x $10^{6.25}$/ml ELD_{50}. Before used, the virus must be filtered using a 0.45 millipore filter. Antigen preparation was different depending on group treatment. For first and second group, antigen have to be mixed with IFA before used. The antigen composition used was Antigen : IFA in 1:1 ratio. The making of Antigen-IFA emulsion was carried out by shaking the solution in a glass syringe with connector.

Production of hyperimmune serum against genotype VII Newcastle Disease virus

The main purpose of hyperimmune serum production is to gain high titer with high antibody specificity against what continues to be a concern in animal welfare. Hyperimmune serum production needs a number of animals as a subject to a number of invasive treatment such as antigen injection and serum collection [20, 21]. This study used rabbits as a donor for antibody which received invasive treatment, namely immunization and serum collection. The rabbit is a popular animal to be used as a donor antibody under the reason of cost benefit ratio and easy to handle [22]. Moreover,rabbit is basically not related closely with chicken as a natural host of Newcastle Disease Virus [20]. This study used eight female rabbits aged 2,5 – 3 months as biological agents to produce hyperimmune serum against genotype VII NDV.
Hyperimmune serum production against genotype VII NDV was performed with and without adjuvants and applied subcutaneously and intravenously. Adjuvants work to increase the immune response through a "depot" effect mechanism that increases antigen presentation slowly. The adjuvant immunostimulatory properties can cause negative effect to animal because they induce inflammation and tissue destruction which potentially cause pain and distress [23]. The adjuvant used in this study was Incomplete Fruend's Adjuvant (IFA) because of it

196 minimizes pain and distress in rabbits while still retains the potency as immunostimulatory
197 agent.

198 The some factors can influence the immunization efficacy. They are divided into three
199 categories: (1) Antigen, including formulation, adjuvant, and dose; (2) recipients of vaccine; and
200 (3) route of immunization [24]. Hyperimmune serum against genotype VII NDV was produced
201 in several applications. In first and second groups, hyperimune serum production was carried
202 out by immunizing rabbits by antigen-IFA emulsion, while third and fourth group did not use
203 IFA in antigen preparation. Immunization in first, second, and third group was administered
204 subcutaneously, while in fourth group immunization was administered intravenously. In second
205 group, antigen volume was half of the first group. Newcastle Disease hyperimmune serum
206 produced in this research resulted from three times immunization to induce higher HI titer.
207 First immunization aims to introduce antigen to immune system especially the B cell, while
208 second and third injections are booster to modulate antibody production by B cells [25, 26].
209 The second immunization was carried out on the 14th day after the first immunization and the
210 third immunization was on the 16th day after the second immunization. Hyperimmune serum
211 titer against genotype VII NDV was measured with periodic HI test and hyperimmune serum
212 was collected on the 8th day after third immunization. The hyperimmune serum titer result is
213 shown in Figure 1.



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215 Figure 1. Hemaglutination Inhibition Titer after immunization

216

217 Based on Figure 1, the first group immunized with NDV – IFA emulsion showed HI
218 titer was already detected on the 12th day after first immunization and reached $2^{5,5}$ on the 5th day
219 and 9th day and then decreased on the 16th day after second immunization. The HI titer in this
220 group continued to decrease until the 3rd day after the 3rd immunization and then increased until
221 the 8th day after the 3rd immunization and reached 2^{8} of HI titer. The second group receiving
222 NDV - IFA emulsion (each volume 0.5 ml) showed hyperimmune serum against NDV
223 genotype VII detected on the 12th after first injection and the HI titer reached $2^{4.5}$ on the 5th day
224 after the second immunization and reached a peak on the 9th day after and then decreased on
225 the 16th day. The HI titer in this second group continued to decrease until the 5th day after the
226 third immunization and then increased reaching 2^{6} of HI titer on the 8th day after third
227 immunization. First and second group were different in the dose of antigen and adjuvant and
228 these conditions influenced the HI titer result. First and second group have difference in HI
229 titer of about 2 log. Antigen quantity may affect the immune response and automatically
230 influence the number of antibodies produced [20, 21].
231 The group of rabbits that received NDV subcutaneously showed that HI titer could be
232 detected on the 7th day after first immunization. This group showed the same HI titer with
233 second group until the 3rd day after third immunization except on the 5th day after second
234 immunization, where this group reached 2^{5} of HI titer that was higher than second group.
235 Furthermore, on the 5th day after the third immunization, this group showed an antibody titer
236 reaching 2^{6}, and at the end of the serum collection on 8th day after the third immunization the
237 HI titer reached $2^{6,5}$. The group of rabbits that received genotype VII NDV subcutaneously
238 showed the same HI titers pattern with the second groups but with higher HI titers. Second
239 and third group have difference on volume antigen and adjuvant. Immunization using half the
240 dose (volume) and mixture with adjuvants produced almost the same antibody titer with

241 immunization using full dose of antigen only (without adjuvant). The difference occured at the
242 beginning of antibody formation. In the group that received NDV-IFA emulsion, the antibody
243 formation process needed longer time. Furthermore, at the end of the hyperimmune serum
244 production, the group that received NDV-IFA emulsion showed a 1 log higher HI titer.
245 Incomplete Freund's Adjuvant was used as water-in-oil emulsion with antigen for secondary
246 and booster injections to raise polyclonal and monoclonal antibodies [23]. Awate et al. [27]
247 stated that compared to injection of antigen alone, injection of antigen plus an adjuvant
248 generally permits the use of a much smaller quantity of antigen while greatly enhances the
249 serum antibody response. The adjuvants promote increased immune response slowly [23, 27].
250 In general, adjuvants permit smaller antigen use but still retains the ability to modulate the
251 immune response against the antigen. Samiullah et al. [28] can produce antibody for APMV-1
252 using adjuvant within 91 days and reach 1024 (2^{10}) HI titer with 4 and 5 times injection. Putri et
253 al. [29], produced antibody of Newcastle disease in New Zealand Rabbit via subcutaneous route
254 application for first and second injection which resulted in the same pattern of antibody titer
255 until the 16th day after second injection. Moreover, after third injection intravenously, that study
256 revealed higher antibody titer on the 8th day, reaching 2^9 of HI titer.
257 The last group, rabbits immunized by antigen Newcastle Disease intravenously, showed
258 that HI titer started to be detected on the 7th day after first immunization and reached HI titer
259 2^6 on the 5th day after the second immunization. It continued to increase until the 9th day with
260 the HI titer reaching $2^{6.5}$ and decreased on the 16th day. The HI titer continued to decrease until
261 the 5th day after the third immunization and then increased on the 8th day after the third
262 immunization and achieved 2^{10} of HI titer. Rabbits receiving intravenous immunization showed
263 higher antibody titers than the other groups. These results indicated that the intravenous route
264 application for hyperimmune serum production against genotype VII NDV greatly affects the
265 immune response result. The intravenous route has the potential for broad distribution of
266 antigen. Intravenous route will distribute the antigen, firstly to the spleen and secondarily to

lymph nodes. Intravenous may be the most effective and to be the route of choice for small particulate antigen such as cells, virions, or bacteria[30].

Serum Purification

Serum is a blood component that contains albumin and globulin proteins [31]. The serum component that can bind directly to the antigen is called the antibody [32]. Before being characterized, serum must be purified from other components. Separation of serum could be done by some purification methods [33]. Purification of hyperimmune serum in this study was done by ammonium sulfate (4.1 M) and protein A purification kit (BioVision). Ammonium sulphate is the oldest, easiest, and most economical methods which is used most frequently to precipitate, and thus concentrate immunoglobulins from serum [34]. The principle of ammonium sulfate purification is the ability of ammonium sulfate to bind immunoglobulin G (IgG) [35]. The second stage of hyperimmune serum purification was using protein A purification kit. Protein A, located in the surface protein of *Staphylococcus aureus* [36], has five domains that have ability to bind Fc fragment of IgG [37]. After purification of protein, it is important to know the concentration of protein in our samples. The antibody concentration on serum was determined by UV-Vis spectrophotometer at 280 nm wavelength. Based on the UV-Vis spectrophotometer result, the genotype VII ND antibody concentration is 1.97 µg/µl.

Serum Characterization by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the protein profile and the molecular weight of hyperimmune serum against genotype VII NDV. The SDS-PAGE result showed that purified serum by ammonium sulfate contained 5 protein bands and serum that had passed 2 stages of purification only contained 2 protein bands, which is the same with commercial standard antibody (Figure-3).

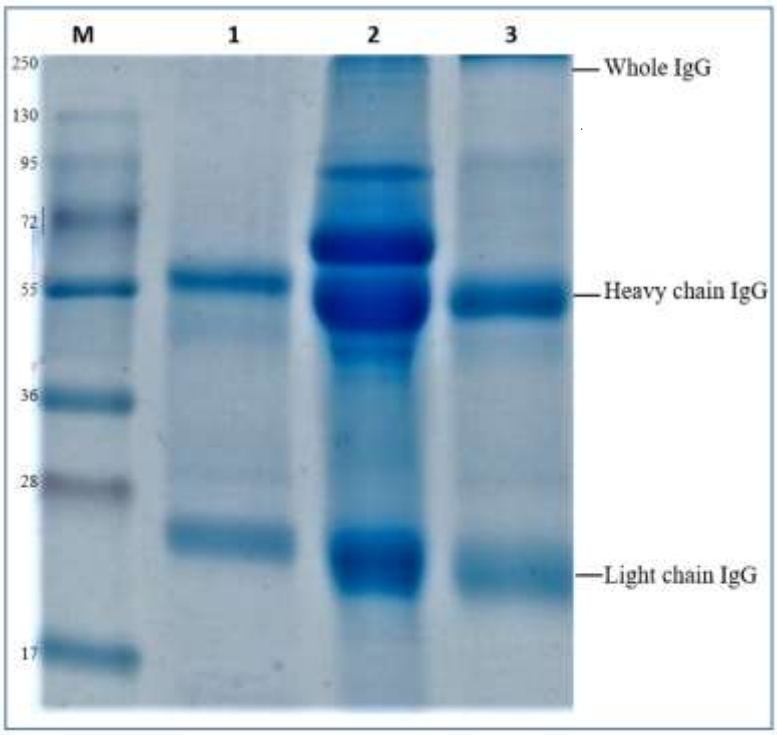


Figure-2: **SDS-PAGE result of Newcastle Disease (ND) hyperimmune serum**. (M) Protein marker; (1) Commercial standard antibody; (2) After purification by Ammonium sulfate; (3) After purification by protein purification kit A.

Determination of molecular weight of serum protein on SDS-PAGE was carried out by forming a linear curve based on the calculation of the relative mobility value (Rf) and the logarithm of the protein molecular weight. Based on the data in Table-2, linear regression curve with equation y = -0,1134x + 2,2379; R^2 = 0,9429 was obtained. The equations were used to determine the molecular weight of the standard antibody and purified serum samples which are presented in Table-3.

Table 2. The migration distance from the marker along with the Rf value.

Rf (cm)	MW (kDa)	Log MW
0,14	250	2,40
0,96	135	2,13
1,71	95	1,98
2,65	72	1,86
3,76	55	1,74

5,52	36	1,56
6,9	28	1,45
9,69	17	1,23
y = -0,1134x + 2,2379;		R^2 = 0,9429

Table 3. The migration distance and Molecular Weight of Hyperimmune serum against Newcastle Disease Virus

Rf (cm)	Log MW	MW (kDa)
Purification by Amonium Sulfate		
0,43	2,19	154,57
2,12	2,00	99,42
3,95	1,79	61,66
4,44	1,73	54,25
7,51	1,39	24,34
Purification by Protein A		
4,44	1,73	54,25
7,51	1,39	24,34

Based on the regression equation calculation, we found molecular weight of antibody standard was 154,57 kDa for whole IgG, heavy chain was IgG 54,25 kDa, and 24,34 kDa for light chain IgG. Molecular weight of immunoglobulin G was about 150 to 160 kDa [36]. Chemical treatments such as SDS will break the IgG molecule by the disulfide bond, causing the polypeptide to break into four separate chains. These chains are "heavy" chains with a molecular weight of 50 kDa and "light" chains with a molecular weight of about 25 kDa.The serum, which was purified by ammonium sulfate only, was detected to have 2 bans protein that were not the same as standard antibody in molecular weight 99,42 kDa and 61,66 kDa. Albumin is a protein found in serum with a molecular weight of 60 kDa [37]. In serum that has passed 2 purification stages, it only has 2 protein bands that are the same as standard antibodies.

Serum confirmation by Agar Gel Precipitation Test and Western Blot Assay

Serum confirmation is carried out to ensure that antibodies contained in Hyperimmune serum against NDV are only able to bind to NDV. Several methods can be used to ensure this, including AGPT and Western blot assay. The Agar Gel Precipitation Test has been applied to a

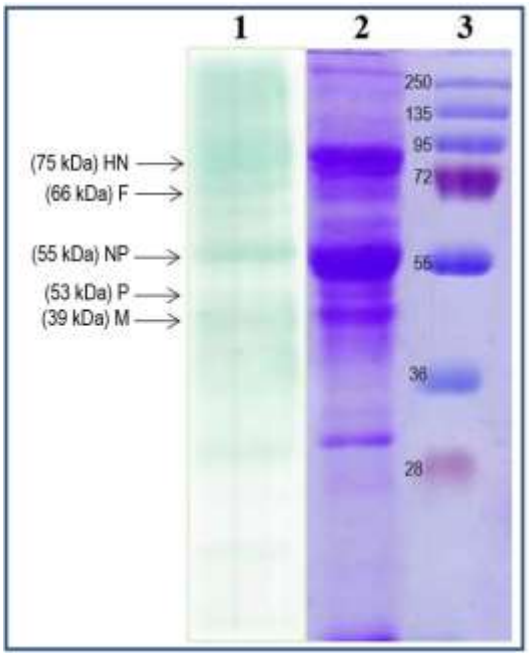
320 variety of avian diseases for the detection of precipitating antibodies. The confirmation results
321 of the ND antibodies specificity can be seen in Figure 4.

322

323 **Figure-4:** Serum confirmation with Agar Gel Precipitation Test; (1) Avian Influenza Ag; (2)
324 Infectious Bursal Disease Ag; (3) Newcastle Disease virus Lasota (4) Newcastle Disease virus
325 Sato; (3) Newcastle Disease virus genotype VII (1); (4) Newcastle Disease virus genotype VII
326 (2); (7) Hyperimmune serum; Arrow (→): Precipitation line.

327 The antigen-antibody interaction on AGPT was characterized by precipitation line in
328 agarose gel. Agar Gel Precipitation Test result showed the line of precipitation formed on all
329 ND antigens, whereas in wells given Avian Influenza and Infectious Bursal Disease antigen, we
330 cannot found the precipitation line. This result indicated that the hyperimmune serum against
331 Newcastle Disease Virus produced in this research has specificity with NDV.

332 In addition to AGPT, the Western blot assay was also used to confirm whether the
333 antibody in the Newcastle Disease serum produced were able to bind to Newcastle Disease
334 virus proteins. By using Western blot method, researchers are able to identify specific proteins
335 from a complex mixture of proteins extracted from cells [40]. This stage begins with the
336 separation of viral proteins with SDS-PAGE followed by the transferring viral proteins to
337 nitrocellulose membranes. The Western blot assay result are presented in Figure-5.



338

339 **Figure-5. Western blot assay Antigen-antibody Newcastle Disease.** (1). Western blot assay
340 result; (2) SDS-PAGE result of Newcastle Disease virus; (3) Protein Marker.

341 Based on the SDS-PAGE results of NDV, there were 5 to 8 proteins recorded with a
342 molecular weight ranged from 28 kDa to 200 kDa. To know the molecular weight of each
343 protein band, the relative mobility must be determined first and then entered into the equation
344 $y = -0{,}1134x + 2{,}2379$; $R^2 = 0{,}9429$. Based on the regression equation, we obtained the
345 molecular weight of Newcastle Disease protein as presented in Table 6.

346 Table 6. Molecular weight of Newcastle Disease protein by SDS-PAGE

RF (cm)	Log MW	MW (kDa)
3,19	1,876154	75,19
3,67	1,821722	66,33
4,36	1,743476	55,40
4,52	1,725332	53,13
5,67	1,594922	39,35

347

348 Hemmatzadeh and Kazemimanesh [41] detected Newcastle Disease protein HN, F, NP,
349 P, and M with molecular weights approximately of 75; 66; 55; 53 and 39 kDa respectively, and
350 that those proteins can be detected by Western Blott Assay. This indicates that the antibodies
351 produced in this study were able to detect the Newcastle Disease virus protein.

The main goal in antibody production is to obtain high-titer, high-specificity antibody and still con-cerned in animal welfare. The study was successfully produced the Hyperimmune serum of Newcastle Disease in rabbit. *Hyperimmune serum can be used as an alternative and viable replacement of conventional antibodies and can be used in diagnostic assay of viruses [17].*

Hyperimmune serum can be used for large-scale screening of NDV carrier commercial and wild birds [17]. Hyperimmune serum against NDV can be used to decrease the morbidity and mortality rate in experimentally infected birds [16]. The passive immunization against Newcastle disease has also been attempted with promising results. The symptoms of ND in experimentally infected birds with NDV are successfully treated through passively immunization with the use of HIS [42]. The high doses of antibodies are also helpful in providing passive immunity by decreasing the mortality and morbidity in birds which are previously exposed the ND virus of velogenic strain. With increasing dose of HIS the mortality and morbidity is considerably reduced [42].

CONCLUSION

Hyperimmune serum against genotype VII Newcastle disease virus was successfully produced by various method of applications. The production of hyperimmune serum by three times intravenous immunization was able to produce the highest titer of 2^{10} at 38 days. The Hyperimmune serum has specificity for Newcastle Disease antigen based on the AGPT and Western blot assay result.

ABBREVIATIONS

NDV, Newcastle Disease Virus; HI, Hemaglutination Inhibition, IFA, Incomplete Freund's Adjuvant; ELD, Embryo lethal dose; AGPT, Agar Gel Precipitation Test; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTION

DDP executed the work (collection of data, analysis, and writing of manuscript); ONP participated in analysis and interpretation of data and writing of manuscript; AAC participated in designing the study and drafting of the manuscript; RDS participated in designing the study, analysis of data and drafting of the manuscript.

All authors read and approved the final manuscript.

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Original Article

Production of hyperimmune serum against the genotype VII Newcastle Disease virus in rabbit with several applications

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ABSTRACT

Objective : The purpose of this study was to produce hyperimmune serum against the genotype VII NDV with several applications.
Materials and Methods : Production of hyperimmune serum against the genotype VII NDV was performed on eight New Zealand White rabbits which divided into four groups. Rabbits were immunized three times on the 1st day, 14th day and 30th day. Blood sampling was carried out on the 8th day after the third immunization.
Results : All groups showed the same pattern of HI titer results, HI titers would peak on the 5th or the 9th day after the second immunization, then decrease until the 3rd day after the third immunization and increase again on the 5th day after the third immunization. Rabbits immunized intravenously showed higher HI titers than the other groups. These results indicated that the intravenous route for hyperimmune serum production against the genotype VII Newcastle Disease virus greatly affects the immune response result.
Conclusion : The production hyperimmune serum by intravenous immunization three times was able to produce the highest titer of 2^{10} at 38 days. The Hyperimmune serum had specificity for Newcastle Disease antigen based on the Agar Gel Precipitation Test and the Western blot assay result.

Keywords: HI titer, Hyperimmune serum, The Newcastle Disease

INTRODUCTION

Avian paramyxovirus serotype-1 (APMV-1) which belongs to the Paramyxoviridae family is the virus that cause Newcastle Disease (ND) [1, 2]. The Newcastle Disease Virus (NDV) can infect more than 250 spesies of birds, and infection by virulent strains can cause huge morbidity and mortality with significant symptoms [3]. The wide range of susceptible hosts causes the persistence of NDV which is endemic in many countries in the world. Virulent strains infection has resulted four panzootics [4]. The first outbreak of ND by virulent strain

happened in Java, Indonesia in 1926, and at the same time an outbreak occurred in England precisely in Newcastle upon Tyne region [5].

The Newcastle Disease virus is an enveloped, unsegmented, single-stranded RNA genome of roughly 15.2 kb [5, 6]. The NDV genome encodes six polypeptides, namely the nucleocapsid (NP) protein, phosphoprotein (P) protein, matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, and the RNA-dependent RNA polymerase (L) protein. The virus nucleocapsid core consists of NP proteins bound to RNA [7].

Newcastle Disease Virus may vary widely in the severity of the disease in birds [8]. Multiple factors can contribute to the severity of disease, including species of host, immune status, age, environmental conditions, secondary infections, the number of virus transmitted, the mechanism of transmission and most importantly the virulence of the infecting virus [9]. In comparison, susceptible species is chickens, whereas geese and ducks do not show symptoms; therefore, waterfowl act as the natural reservoir for NDV. The cleavage site of the F protein is a main determinant of viral virulence during replication in host cells. [10, 11]. Based on the pathogenicity of the disease, ND can be classified into five pathotypes: - Neurotrophic velogenic strain exhibiting respiratory and neurological symptoms with a high mortality rate; Viscerotropic velogenic strain causing hemorrhagic and highly pathogenic intestinal lesions; Mesogenic strain caused by viruses with rare respiratory and neurological symptoms, while the age of susceptible birds is related to mortality; Viral lentogenic strains present with mild respiratory infection; and Asymptomatic enteric strain exhibiting no clinical sign or asymptomatic[12].

Interaction between virus and the environment, including the host immune system resulted in NDV evolution and continues to produce new genotypes of virus. Lately, infection of genotype VII NDV caused high mortality of birds in several poultry farms in Indonesia [13, 14]. In recent years, producing hyperimmune serum in animals is an important activity of many research projects. The hyperimmune serum as a biological reagen will continue to be developed for research needs and possibly also for commercial applications in the future such as for therapy and development of immunodignostic tools [15]. The specific antibodies in hyperimmune serum can be used for the treatment and control of disease in case of an outbreak [16]. The hyperimmune serum is already used for the successful treatment of some disease like foot and mouth diseases, tetanus and canine viral diseases [17] . Currently, the hyperimmune serum used for diagnostic in poultry is imported from different countries of the world at very expensive. Moreover, the indigenous isolates may differ from the imported strains of viruses showing non-specificity in diagnosis [17]. The development of the serum for NDV currently circulating must be followed by the development of immunodiagnostic tests, to obtain accurate test results. Therefore, it is necessary to produce the genotype VII Newcastle Disease hyperimmune serum which can be used as immunodiagnostic reagents.

Hyperimmune serum production can be carried out in various applications, with or without adjuvant and with its own advantages and disadvantages. Considering the numerous applications of hyperimmune serum in research and clinical fields, the preparation method the development of hyperimmune serum against pathogens is very important [15]. To be able to produce antibodies with high titers in a short time, it is necessary to conduct research on various immunization applications with or without adjuvant in inducing immunity. The purpose of this study was to produce hyperimmune serum againts the genotype VII NDV with several applications efficient in time and cost.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee of Research and Community Services Institution, IPB University has approved this research with approval number: 213-2021 IPB.

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Newcastle Disease Antigen

For the production of NDV hyperimmune serum, characterized genotype VII NDV was used. Isolate was the repository sample from the Immunology Laboratory, Faculty of Veterinary Medicine, IPB University. We categorized isolate as genotype VII NDV based on PCR, sequencing and filogenetic analysis [11, 13]. The antigen was prepared in fresh condition with and without adjuvant use.

Hyperimmune Serum Production

This study used eight New Zealand White rabbits, aged 2,5 – 3,5 month with an average body weight of 2,5 kg for production of hyperimmune serum against the genotype VII NDV. We formed the rabbits into four groups. The first group was rabbit immunized subcutaneously by 1 ml of the genotype VII NDV isolate ($5\times10^{6.5}$ ELD_{50}/ml) and 1 ml Incomplate Freund's Adjuvant (IFA); the second group was rabbit immunized subcutaneously by 0,5 ml the genotype VII NDV isolate ($5\times10^{6.5}$ ELD_{50}/ml) and 0,5 ml IFA; the third group was rabbit immunized by 1 ml the genotype VII NDV isolate ($5\times10^{6.5}$ ELD_{50}/ml) subcutaneously and the last group was rabbit immunized by 1 ml of the genotype VII NDV isolate ($5\times10^{6.5}$ ELD_{50}/ml) intravenously. The application and composition of the antigens used in this study are presented in Table 1.

We immunized rabbits three times. First immunization on the 1st day and the second immunization on the 14 th day and the third immunization on the 30th day. Blood sampling was carried out on the 8th day after third immunization. Hyperimmune serum was collected by giving local anestethic agent into the ear and then taking blood intra arteries. The procedure for making serum is as follows: We kept blood samples at temperature ± 25°C for an hour and then kept overnight at 4°C. The serum was separated manually and precipitated by centrifugation at 2500 rpm for 15 minutes. Futhermore, we kept the serum in collecting tube 1.5 ml and stored at -20°C until use. The rabbit blood samples were taken periodically to observe the Hemagglutinatin Inhibition (HI) antibody titer against the genotype VII NDV. Serum was inactivated at 54^0C for 30 min before used for the HI test.

Serum Purification

Purification of hyperimmune ND serum was carried out by two stages. Precipitation by ammonium sulfate (4.1 M) was the first [18]. The first stage of serum precipitation was stirred equal volumes of serum solutions and ammonium sulfate slowly, then incubated them overnight at 4°C. After that, centrifugated precipitate at 3000 xg for 20 minutes. To obtain one-fourth of antibody volume, we reconstituted the pellet by phosphate buffered saline pH 7.4. Hereafter was dialysis performed by putting the precipitate in a dialysis bag and stirred it in PBS pH 7.4 for 24 h at 4°C that was replaced every 8 h by PBS solution. The second

step was hyperimmune serum purification using protein A purification kit (BioVision, USA) according to the manufacturer's instructions.

Serum Characterization by SDS-PAGE

We measured the molecular weight of the purified ND hyperimmune serum using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE using the concentration of separating gel 12% and 4% for the stacking gel [19]. The sample buffer (containing bromophenol blue, SDS, DTT and glycerol) 5 µl was mixed with serum sample (5 µl), and heated 65°C for five minutes to denature the protein. A total of 5 µl of marker protein (Thermo Scientific, USA) and 10 µl of hyperimmune serum samples were used. Protein separation using electrophoresis at 100 V for 150 minutes. The final process of serum characterization was staining the electrophoresis gel with Commasie Brilliat Blue for 30 minutes, and then addition of destaining solution for 24 hours.

Serum confirmation by Agar Gel Precipitation Test and Western Blot Assay

Determination, the specificity of ND hyperimmune serum done with several ways, including Agar Gel Precipitation Test (AGPT) and Western Blot Assay. ND antibody specificity was confirmed to two ND viruses [13] and other antigens such as Infectious Bursal Disease (IBD), and Avian Influenza (AI). The precipitation line in agarose gel indicated antigen and antibody interaction.

In order to detect Newcastle Disease viral protein, we run on the genotipe VII NDV antigen on SDS-PAGE gel. The SDS-PAGE result was transferred to Nitro Cellulose (NC) membranes. The membrane was blocked with Tris Buffer Saline (TBS) at 37^0C for 2 hours. After the T-TBS washing, incubate the membrane with 1 : 2000 dilution of primary rabbit hyperimmune serum (against NDV produced in this research) overnight and then washed by T-TBS. Afterwards, we incubated the NC membrane in alkaline phosphatase conjugated a secondary antibody at 37^0C for 2 hours. Then, washing and developing the membranes using Diaminobenzidine (DAB) substrate solution (Sigma) for 5-10 minutes. At the end of this procedure, we washed the membrane by distilled water to terminate enzyme reaction on the membrane.

RESULTS AND DISCUSSION

Antigen Preparation

Antigen used in this research were the genotype VII NDV characterized by PCR, sequencing, and phylogenetic analysis [11, 13]. The ELD_{50} of virus must be calculated to determine the virus ability to kill 50% of Specific Pathogen Free embryos in eggs. The virus used in this study is genotype VII NDV with 5 x $10^{6.25}$/ml ELD_{50}. Before used, the virus must be filtered using a 0.45 µm filter. Antigen preparation was different depending on group treatment. For the first and second group, antigen was mixed with IFA before used. The antigen composition used was Antigen : IFA in 1:1 ratio. Shake the solution in a glass syringe with connector to making Antigen-IFA emulsion.

Production of hyperimmune serum against the genotype VII Newcastle Disease virus

The main purpose of hyperimmune serum production is to gain high titer with high antibody specificity and continues to concern in animal welfare. Hyperimmune serum production needs a number of animals as a subject to a number of invasive treatment such as antigen injection and serum collection [20, 21]. This study used rabbits as a donor for hyperimmune serum which received invasive treatment, namely immunization and serum collection. The rabbit is a popular animal used as a donor antibody under the reason of cost benefit ratio and easy to handle [22]. Moreover, rabbit is basically not related closely with chicken as a natural host of Newcastle Disease Virus [20]. This study used eight female rabbits aged 2,5 – 3 months as biological agents to produce hyperimmune serum against the genotype VII NDV. Hyperimmune serum production against the genotype VII NDV was performed with and without adjuvants and applied subcutaneously and intravenously. Adjuvants work to increase the immune response through a "depot" effect mechanism that increases antigen presentation slowly. The adjuvant immunostimulant properties can cause negative effect to the animal because they induce inflammation and tissue destruction, which potentially cause pain and distress [23]. The adjuvant used in this study was Incomplete Fruend's Adjuvant (IFA) because of it minimizes pain and distress in rabbits while still retains the potency as immunostimulant agent.

Some factors can influence the immunization efficacy. They are divided into three categories: (1) Antigen, including formulation, adjuvant, and dose; (2) recipients of vaccine; and (3) the route of immunization [24]. Hyperimmune serum against genotype VII NDV was produced in several applications. For hyperimmune serum production, in the first and second groups, we immunized the rabbits with antigen-IFA emulsion, while the third and fourth group did not use IFA in antigen preparation serum. Immunization, in the first, second, and third group was administered subcutaneously, while in fourth group immunization was administered intravenously. In the second group, antigen volume was half of the first group. The Newcastle Disease hyperimmune serum produced in this research resulted from three times immunization to induce higher HI titer. First immunization aims to introduce antigen to immune system, especially the B cell, while the second and third injections are booster to modulate antibody production by B cells [25, 26]. The second immunization was carried out on the 14^{th} day after the first immunization and the third immunization was on the 16^{th} day after the second immunization. Hyperimmune serum titer against genotype VII NDV was measured with periodic HI test and hyperimmune serum was collected on the 8^{th} day after third immunization. The hyperimmune serum titer result is shown in Figure 1.

Based on Figure 1, the first group immunized with NDV – IFA emulsion showed HI titer was already detected on the 12^{th} day after the first immunization and reached $2^{5,5}$ on the 5^{th} day and the 9^{th} day and then decreased on the 16^{th} day after second immunization. The HI titer in this group continued to decrease until the 3^{rd} day after the 3^{rd} immunization and then increased until the 8^{th} day after the 3^{rd} immunization and reached 2^{8} of HI titer. The second group receiving NDV - IFA emulsion (each volume 0.5 ml) showed hyperimmune serum against NDV genotype VII detected on the 12^{th} after first the injection and the HI titer reached $2^{4.5}$ on the 5^{th} day after the second immunization and reached a peak on the 9^{th} day after and then decreased on the 16^{th} day. The HI titer in this second group continued to decrease until the 5^{th} day after the third immunization and then increased reaching 2^{6} of HI titer on the 8^{th} day after third immunization. The first and second group were different in the dose of antigen and adjuvant and these conditions influenced the HI titer result. First and second group have difference in HI titer of about 2 level of log. Antigen quantity may affect

the immune response and automatically influence the number of antibodies produced [20, 21].

The group of rabbits that received NDV subcutaneously showed that HI titer could be detected on the 7th day after first immunization. This group showed the same HI titer with the second group until the 3rd day after third immunization except on the 5th day after second immunization, where this group reached 2^5 of HI titer that was higher than the second group. Furthermore, on the 5th day after the third immunization, this group showed an antibody titer reaching 2^6, and at the end of the serum collection on the 8th day after the third immunization the HI titer reached $2^{6,5}$. The group of rabbits that received the genotype VII NDV subcutaneously showed the same of HI titers pattern with the second groups but with higher HI titers. The second and third group have difference on volume antigen and adjuvant. Immunization using a half dose (volume) and mixture with adjuvants produced almost the same antibody titer with immunization using full dose of antigen only (without adjuvant). The difference occure at the beginning of antibody formation. In the group that received NDV-IFA emulsion, the antibody formation process needed longer time. Furthermore, at the end of the hyperimmune serum production, the group that received NDV-IFA emulsion showed a 1 log higher HI titer. For the secondary and booster injections, we used Incomplete Freund's Adjuvant as water-in-oil emulsion with antigen to raise polyclonal and monoclonal antibodies [23]. Awate et al. [27] stated that compared to injection of antigen alone, injection of antigen plus an adjuvant generally permits the use of a much smaller quantity of antigen while greatly enhances the serum antibody response. The adjuvants promote increase immune response slowly [23, 27]. In general, adjuvants permit to use smaller antigen but still retains the ability to modulate the immune response against the antigen. Samiullah et al. [28] can produce antibody for APMV-1 using adjuvant within 91 days and reach 1024 (2^{10}) HI titer with 4 and 5 times injection. Putri et al. [29], produced antibody of Newcastle disease in New Zealand Rabbit with subcutaneous route application for first and second injection which resulted in the same pattern of antibody titer until the 16th day after second injection. Moreover, after third injection intravenously, that study revealed higher antibody titer on the 8th day, reaching 2^9 of HI titer.

The last group, rabbits immunized by antigen Newcastle Disease intravenously, showed that HI titer started to be detected on the 7th day after first immunization and reached HI titer 2^6 on the 5th day after the second immunization. It continued to increase until the 9th day with the HI titer reaching $2^{6.5}$ and decreased on the 16th day. The HI titer continued to decrease until the 5th day after the third immunization and then increased on the 8th day after the third immunization and achieved 2^{10} of HI titer. Rabbits receiving intravenous immunization showed higher antibody titers than the other groups. These results indicated that the intravenous route application for hyperimmune serum production against the genotype VII NDV greatly affects the immune response result. The intravenous route has the potential for broad distribution of antigen. Intravenous route will distribute the antigen, firstly to the spleen and secondarily to lymph nodes. Intravenous may be the most effective and to be the route of choice for small particulate antigen such as cells, virions, or bacteria[30].

Serum Purification

Serum is a blood component that contains albumin and globulin proteins [31]. The serum component that can bind directly to the antigen is called the antibody [32]. Before being characterized, serum must be purified from other components. Separation of serum could be done by some purification methods [33]. In this study, we purify the hyperimmune serum

by ammonium sulfate (4.1 M) and protein A purification kit (BioVision). Ammonium sulphate is the oldest, easiest, and most economical methods which used most frequently to precipitate, and thus concentrate immunoglobulins from the serum [34]. The principle of ammonium sulfate purification is the ability of ammonium sulfate to bind immunoglobulin G (IgG) [35]. The second stage of hyperimmune serum purification was using protein A purification kit. Protein A, located in the surface protein of *Staphylococcus aureus* [36], has five domains that have ability to bind Fc fragment of IgG [37]. After purification of protein, it is important to know the concentration of protein in our samples. In this study, we determine the antibody concentration on serum by UV-Vis spectrophotometer at 280 nm wavelength. Based on the UV-Vis spectrophotometer result, the genotype VII ND antibody concentration is 1.97 µg/µl.

Serum Characterization by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the protein profile and the molecular weight of hyperimmune serum against the genotype VII NDV. The SDS-PAGE result showed that purified serum by ammonium sulfate contained 5 protein bands and the serum that had passed 2 stages of purification only contained 2 protein bands, which is the same with a commercial standard antibody (Figure-2).

We determinate the molecular weight of serum protein on SDS-PAGE by forming a linear curve based on the calculation of the relative mobility value (Rf) and the logarithm of the protein molecular weight. Based on the data in Table-2, linear regression curve with equation y = -0,1134x + 2,2379; R^2 = 0,9429 was obtained. The equations were used to determine the molecular weight of the standard antibody and purified serum samples which are presented in Table-3.

Based on the regression equation calculation, we found the molecular weight of antibody standard was 154,57 kDa for whole IgG, heavy chain was IgG 54,25 kDa, and 24,34 kDa for light chain IgG. The molecular weight of immunoglobulin G was about 150 to 160 kDa [36]. Chemical treatments such as SDS will break the IgG molecule by the disulfide bond, causing the polypeptide to break into four separate chains. These chains are "heavy" chains with a molecular weight of 50 kDa and "light" chains with a molecular weight of about 25 kDa. In the serum, which purified by ammonium sulfate only, we detected 2 bans protein that not the same with standard antibody in molecular weight 99,42 kDa and 61,66 kDa. Albumin is a protein found in serum with a molecular weight of 60 kDa [37]. In serum that has passed 2 purification stages, it only has 2 protein bands that are the same as standard antibodies.

Serum confirmation by Agar Gel Precipitation Test and Western Blot Assay

Serum confirmation is carried out to ensure that antibodies contained in the hyperimmune serum against NDV are only able to bind to NDV. Several methods can be used to ensure this, including AGPT and Western blot assay. The Agar Gel Precipitation Test has been applied for detect precipitating antibodies in variety of avian diseases. The confirmation results of the ND antibodies specificity can be seen in Figure 3.

The antigen-antibody interaction on AGPT was characterized by precipitation line in agarose gel. The Agar Gel Precipitation Test result showed the line of precipitation formed

on all ND antigens, whereas in wells given the Avian Influenza and the Infectious Bursal Disease antigen, we cannot found the precipitation line. This result indicated that the hyperimmune serum against Newcastle Disease Virus produced in this research has specificity with NDV.

In addition for AGPT, the Western blot assay was also used to confirm whether the antibody in the Newcastle Disease serum produced able to bind to Newcastle Disease virus proteins. By using the Western blot method, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells [40]. This stage begins with the separation of viral proteins with SDS-PAGE followed by the transferring viral proteins to nitrocellulose membranes. The Western blot assay results are presented in Figure-4.

Based on the SDS-PAGE results of NDV, there were 5 to 8 proteins recorded with a molecular weight ranged from 28 kDa to 200 kDa. To know the molecular weight of each protein band, the relative mobility must be determined first and then entered into the equation $y = -0{,}1134x + 2{,}2379$; $R^2 = 0{,}9429$. Based on the regression equation, we obtained the molecular weight of the Newcastle Disease protein as presented in Table 4.

Hemmatzadeh and Kazemimanesh [41] detected Newcastle Disease protein HN, F, NP, P, and M with molecular weights approximately of 75; 66; 55; 53 and 39 kDa respectively, and that those proteins can be detected by Western Blott Assay. This indicates that the antibodies produced in this study were able to detect the Newcastle Disease virus protein. The main goal of antibody production is to obtain high-titer, high-specificity antibody and still concerned in animal welfare. The study was successfully produced the Hyperimmune serum of Newcastle Disease in rabbit. Hyperimmune serum can be used as an alternative and viable replacement of conventional antibodies and can be used in diagnostic assay of viruses [17].

Hyperimmune serum can be used for large-scale screening of NDV carrier commercial and wild birds [17]. The hyperimmune serum against NDV can be used to decrease the morbidity and mortality rate in experimentally infected birds [16]. The passive immunization against Newcastle Disease has also been attempted with promising results. The symptoms of ND in experimentally infected birds with NDV are successfully treated through passively immunization by use of HIS [42]. The high doses of antibodies are also helpful in providing passive immunity by decreasing the mortality and morbidity in birds which are previously exposed the ND virus of velogenic strain. With increasing dose of HIS the mortality and morbidity is considerably reduced [42].

CONCLUSION

Hyperimmune serum against the genotype VII Newcastle disease virus was successfully produced by various method of applications. The production of hyperimmune serum by three times intravenous immunization was able to produce the highest titer of 2^{10} at 38 days. The Hyperimmune serum has specificity for Newcastle Disease antigen based on the AGPT and Western blot assay result.

List of abbreviations

NDV, Newcastle Disease Virus; HI, Hemaglutination Inhibition, IFA, Incomplete Freund's Adjuvant; ELD, Embryo lethal dose; AGPT, Agar Gel Precipitation Test; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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Conflict of interests

The authors declare that they have no competing interests.

Authors' contribution

DDP executed the work (collect data, analysis, and writing of manuscript); ONP participated in analysis and interpretation of data and writing of manuscript; AAC participated in designing the study and drafting of the manuscript; RDS participated in designing the study, analysis of data and drafting of the manuscript.
All authors read and approved the final manuscript.

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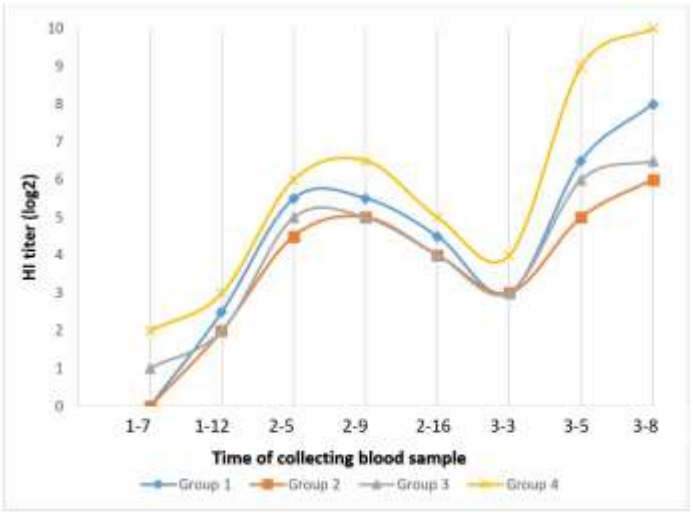

Figure 1. Hemaglutination Inhibition Titer after immunization

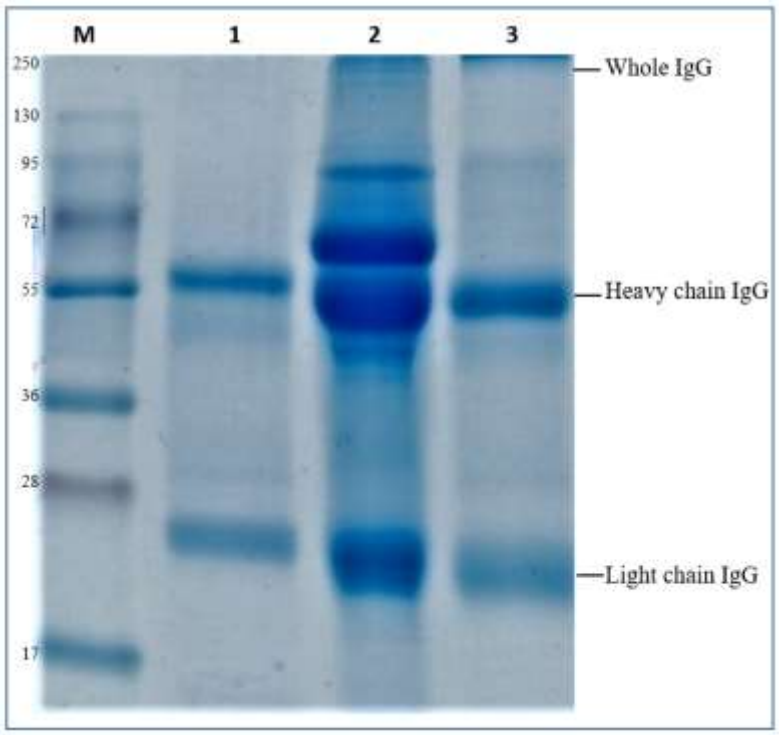

Figure-2: **SDS-PAGE result of Newcastle Disease (ND) hyperimmune serum**. (M) Protein marker; (1) Commercial standard antibody; (2) After purification by Ammonium sulfate; (3) After purification by protein purification kit A.

Figure-3: Serum confirmation with Agar Gel Precipitation Test; (1) Avian Influenza Ag; (2) Infectious Bursal Disease Ag; (3) Newcastle Disease virus Lasota (4) Newcastle Disease virus Sato; (3) Newcastle Disease virus genotype VII (1); (4) Newcastle Disease virus genotype VII (2); (7) Hyperimmune serum; Arrow ( ): Precipitation line.

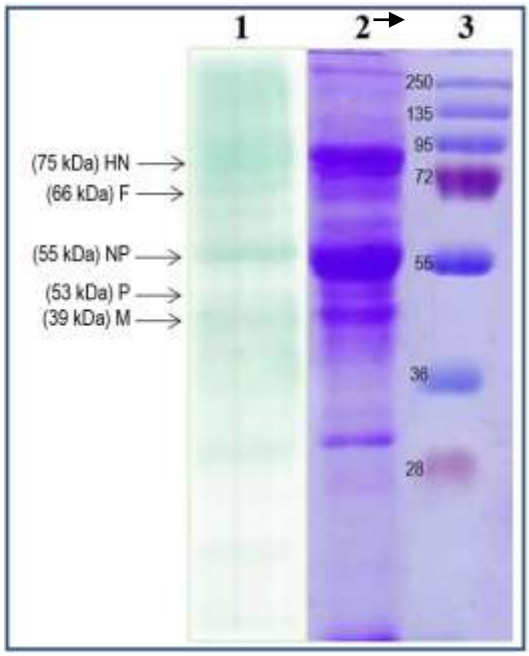

Figure-4. Western blot assay Antigen-antibody Newcastle Disease. (1). Western blot assay result; (2) SDS-PAGE result of Newcastle Disease virus; (3) Protein Marker.

Table 1. Composition and Aplication of The Genotype VII NDV Isolate

Group	Volume		Aplication
	Antigen	IFA*)	
1	1 ml	1 ml	subcutaneously
2	0,5 ml	0,5 ml	subcutaneously
3	1 ml	-	subcutaneously
4	1 ml	-	intravenously

*) Incomplate Fruend's Adjuvant

Table 2. The migration distance from the marker along with the Rf value.

Rf (cm)	MW (kDa)	Log MW
0,14	250	2,40
0,96	135	2,13
1,71	95	1,98
2,65	72	1,86
3,76	55	1,74
5,52	36	1,56
6,9	28	1,45
9,69	17	1,23
y = -0,1134x + 2,2379;		R^2 = 0,9429

Table 3. The migration distance and Molecular Weight of Hyperimmune serum against Newcastle Disease Virus

Rf (cm)	Log MW	MW (kDa)
Purification by Amonium Sulfate		
0,43	2,19	154,57
2,12	2,00	99,42
3,95	1,79	61,66
4,44	1,73	54,25
7,51	1,39	24,34
Purification by Protein A		
4,44	1,73	54,25
7,51	1,39	24,34

Table 4. The Molecular weight of Newcastle Disease protein by SDS-PAGE

RF (cm)	Log MW	MW (kDa)
3,19	1,876154	75,19
3,67	1,821722	66,33
4,36	1,743476	55,40
4,52	1,725332	53,13
5,67	1,594922	39,35

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ORIGINAL ARTICLE

Production of hyperimmune serum against genotype VII Newcastle disease virus in rabbits with several applications

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ABSTRACT

Objective: This study aimed to produce hyperimmune serum against genotype VII Newcastle disease virus (NDV) with several applications.
Materials and Methods: Production of hyperimmune serum against genotype VII NDV was performed on eight New Zealand white rabbits divided into four groups. Rabbits were immunized three times on the 1st day, the 14th day, and the 30th day. Blood sampling was carried out on the 8th day after the third immunization.
Results: All groups showed the same pattern of hemagglutination inhibition (HI) titer results. HI titers would peak on the 5th or the 9th day after the second immunization, then decrease until the 3rd day after the third immunization, and increase again on the 5th day after the third immunization. Rabbits immunized intravenously showed higher HI titers than the other groups. These results indicate that the intravenous route for hyperimmune serum production against genotype VII Newcastle disease virus greatly affects the immune response result.
Conclusions: The production of hyperimmune serum by intravenous immunization three times was able to produce the highest titer of 2^{10} at 38 days. The agar gel precipitation test and the Western blot assay showed that the hyperimmune serum was specific for the Newcastle disease antigen.

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HI titer; hyperimmune serum; Newcastle disease

Introduction

Avian paramyxovirus serotype-1 (APMV-1) which belongs to the paramyxoviridae family is the virus that causes Newcastle disease (ND) [1,2]. The Newcastle disease virus (NDV) can infect more than 250 species of birds, and infection by virulent strains can cause high morbidity and mortality with significant symptoms [3]. The wide range of susceptible hosts causes the persistence of NDV, which is endemic in many countries in the world. Virulent strains of infection have resulted in four panzootics [4]. The first outbreak of ND by a virulent strain happened in Java, Indonesia, in 1926, and at the same time, an outbreak occurred in England, precisely in the Newcastle upon Tyne region [5].

The Newcastle disease virus has a 15.2 kb enveloped, unsegmented, single-stranded RNA genome [5,6]. The NDV genome encodes six polypeptides, namely the nucleocapsid protein (NP), phosphoprotein (P) protein, matrix (M) protein, fusion (F) protein, hemagglutinin–neuraminidase (HN) protein, and the RNA-dependent RNA polymerase (L) protein. The virus nucleocapsid core consists of NP bound to RNA [7].

The Newcastle disease virus may vary widely in the severity of the disease in birds [8]. Multiple factors can contribute to the severity of disease, including species of host, immune status, age, environmental conditions, secondary infections, the number of viruses transmitted, the mechanism of transmission, and most importantly, the virulence of the infecting virus [9]. In comparison, susceptible species are chickens, whereas geese and ducks do not show symptoms; therefore, waterfowls act as the natural reservoir for NDV. The cleavage site of the F protein is a main

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determinant of viral virulence during replication in host cells. [10,11]. Based on the pathogenicity of the disease, ND can be classified into five pathotypes: neurotrophic velogenic strain, exhibiting respiratory and neurological symptoms with a high mortality rate; viscerotropic velogenic strain, causing hemorrhagic and highly pathogenic intestinal lesions; mesogenic strains caused by viruses with rare respiratory and neurological symptoms, while the age of susceptible birds is related to mortality; viral lentogenic strains present with mild respiratory infection; and asymptomatic enteric strain, exhibiting no clinical signs or asymptomatic [12].

Interaction between viruses and the environment, including the host immune system, resulted in NDV evolution and continues to produce new genotypes of viruses. Lately, infection of genotype VII NDV has caused high mortality of birds in several poultry farms in Indonesia [13,14]. Recently, producing hyperimmune serum in animals has become an essential activity of many research projects. The hyperimmune serum as a biological reagent will continue to be developed for research needs and possibly also for commercial applications in the future, such as for the therapy and the development of immunodiagnostic tools [15]. The specific antibodies in hyperimmune serum can be used to treat and control diseases in the event of an outbreak [16]. The hyperimmune serum is already used to successfully treat some diseases like foot and mouth diseases, tetanus, and canine viral diseases [17]. Currently, the hyperimmune serum used for diagnostics in poultry is imported from different countries of the world at very high prices. Moreover, the indigenous isolates may differ from the imported strains of viruses, showing nonspecificity in diagnosis [17]. The development of the serum for NDV currently circulating must be followed by the development of immunodiagnostic tests to obtain accurate test results. Therefore, it is necessary to produce genotype VII Newcastle disease hyperimmune serum that can be used as an immunodiagnostic reagent.

Hyperimmune serum production can be carried out in various applications, with or without adjuvants, with its own advantages and disadvantages. Considering the numerous applications of hyperimmune serum in research and clinical fields, the preparation method for developing hyperimmune serum against pathogens is essential [15]. To be able to produce antibodies with high titers in a short time, it is necessary to conduct research on various immunization applications with or without adjuvant-inducing immunity. This study aimed to produce hyperimmune serum against genotype VII NDV with several applications efficient in time and cost.

Materials and Methods

Ethical approval

This research has been approved by the Animal Care and Use Committee of IPB University's Research and Community Services Institution. The approval number for this research is 213-2021 IPB.

Newcastle disease *antigen*

For the production of NDV hyperimmune serum, characterized genotype VII NDV was used. The isolate was the repository sample from the Immunology Laboratory, Faculty of Veterinary Medicine, IPB University. We classified the isolate as genotype VII NDV based on PCR, sequencing, and phylogenetic analysis [11,13]. The antigen was prepared in fresh condition with and without adjuvant use.

Hyperimmune serum production

This study used eight New Zealand white rabbits aged 2.5–3.5 months with an average body weight of 2,5 kg to produce hyperimmune serum against genotype VII NDV. We divided the rabbits into four groups. The first group was immunized subcutaneously with 1 ml of genotype VII NDV isolate ($5 \times 10^{6.5}$ ELD_{50}/ml) and 1 ml of incomplete Freund's adjuvant (IFA); the second group was immunized subcutaneously with 0.5 ml of genotype VII NDV isolate ($5 \times 10^{6.5}$ ELD_{50}/ml) and 0.5 ml IFA; the third group was immunized with 1 ml of genotype VII NDV isolate ($5 \times 10^{6.5}$ ELD_{50}/ml) subcutaneously; and the last group was rabbits immunized with 1 ml of genotype VII NDV isolate ($5 \times 10^{6.5}$ ELD_{50}/ml) intravenously. Table 1 shows how the antigens used in this study.

We immunized rabbits three times. The first immunization was on the 1st day, the second immunization on the 14th day, and the third immunization on the 30th day. Blood sampling was carried out on the 8th day after the third immunization. The hyperimmune serum was collected by giving a local anesthetic agent into the ear and then taking blood from the intra-arterial arteries. The procedure for making serum is as follows: we kept blood samples at a temperature ±25°C for an hour and then kept them overnight at 4°C. The serum was separated manually and precipitated by centrifugation at 2,500 rpm for 15

Table 1. Composition and aplication of genotype VII NDV isolate.

Group	Volume		Application
	Antigen	IFA[a]	
1	1 ml	1 ml	Subcutaneously
2	0.5 ml	0.5 ml	Subcutaneously
3	1 ml	-	Subcutaneously
4	1 ml	-	Intravenously

[a]Incomplete Fruend's adjuvant.

min. Furthermore, we kept the serum in a collecting tube of 1.5 ml and stored it at −20°C until use. The rabbit blood samples were taken periodically to observe the hemagglutination inhibition (HI) antibody titer against genotype VII NDV. Serum was inactivated at 54°C for 30 min before being used for the HI test.

Serum purification

Purification of the hyperimmune ND serum was carried out in two stages. The first was precipitation by ammonium sulfate (4.1 M) [18]. The first stage of serum precipitation was to stir equal volumes of serum solution and ammonium sulfate slowly, then incubate them overnight at 4°C. After that, we centrifuged the precipitates at 3,000× *g* for 20 min. To obtain one-fourth of the antibody volume, we reconstituted the pellet with phosphate-buffered saline pH 7.4. Hereafter, dialysis was performed by putting the precipitate in a dialysis bag and stirring it in PBS pH 7.4 for 24 h at 4°C, which was replaced every 8 h by PBS solution. The second step was hyperimmune serum purification using a protein A purification kit (BioVision, USA) according to the manufacturer's instructions.

Serum characterization by SDS-PAGE

We measured the molecular weight of the purified ND hyperimmune serum using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE, the concentration of separating gel was 12% and 4% for the stacking gel [19]. 5 µl of the sample buffer (containing bromophenol blue, SDS, DTT, and glycerol) was mixed with the serum sample (5 µl) and heated at 65°C for 5 min to denature the protein. 5 µl of marker protein (Thermo Scientific, USA) and 10 µl of hyperimmune serum samples were used. Protein separation was carried out using electrophoresis at 100 V for 150 min. The final process of serum characterization was staining the electrophoresis gel with Coomassie Brilliant Blue for 30 min and then adding a de-staining solution for 24 h.

Serum confirmation by agar gel precipitation test and Western blot assay

The specificity of ND hyperimmune serum can be determined in several ways, including agar gel precipitation test (AGPT) and Western blot assay. ND antibody specificity was confirmed for two ND viruses [13] and other antigens, such as infectious bursal disease (IBD) and avian influenza (AI). The precipitation line in the agarose gel indicated antigen and antibody interaction.

To detect Newcastle disease viral protein, we ran the genotype VII NDV antigen on an SDS-PAGE gel. The SDS-PAGE result was transferred to nitro cellulose (NC) membranes. The membrane was blocked with Tris-buffered saline (TBS) at 37°C for 2 h. After the T-TBS washing, we incubated the membrane with a 1:2,000 dilution of primary rabbit hyperimmune serum (against NDV produced in this research) overnight, and then washed it with T-TBS. Afterward, we incubated the NC membrane in alkaline phosphatase-conjugated with a secondary antibody at 37°C for 2 h. Then, we washed and developed the membranes using a diaminobenzidine substrate solution (Sigma) for 5–10 min. At the end of this procedure, we washed the membrane with distilled water to terminate the enzyme reaction on the membrane.

Results and Discussion

Antigen preparation

The antigen used in this research was genotype VII NDV, characterized by PCR sequencing and phylogenetic analysis [11,13]. The ELD_{50} of a virus must be calculated to determine the virus' ability to kill 50% of specific pathogen-free embryos in eggs. The virus used in this study is genotype VII NDV with $5 \times 10^{6.25}$/ml ELD_{50}. Before use, the virus must be filtered using a 0.45 µm filter. Antigen preparation was different depending on group treatment. The antigen was mixed with IFA before being used for the first and second groups. The antigen composition used was antigen: IFA in a 1:1 ratio. Shake the solution in a glass syringe with a connector to make an antigen–IFA emulsion.

Production of hyperimmune serum against genotype VII Newcastle disease virus

The main purpose of hyperimmune serum production is to gain a high titer with high antibody specificity, which continues to cause concern in animal welfare. Hyperimmune serum production needs several animals as subjects to invasive treatments such as antigen injection and serum collection [20,21]. This study used rabbits as a donor for hyperimmune serum, which received invasive treatment, immunization, and serum collection. Because of its low cost–benefit ratio and ease of handling, rabbits are widely used as donor antibodies [22]. Moreover, rabbits are not closely related to chickens as a natural host of the Newcastle disease virus [20]. This study used eight female rabbits aged 2.5–3.0 months as biological agents for producing hyperimmune serum against genotype VII NDV. Hyperimmune serum production against genotype VII NDV was performed with and without adjuvants and applied subcutaneously and intravenously. Adjuvants work to increase the immune response through a "depot" effect mechanism that increases antigen presentation slowly. The adjuvant immunostimulant properties can harm the animals because they induce inflammation and tissue destruction, which potentially causes pain and

distress [23]. The adjuvants used in this study was incomplete Fruend's adjuvant (IFA) because it minimizes pain and distress in rabbits while retaining the potency as an immunostimulant agent.

Some factors can influence the efficacy of immunization. They are divided into three categories: (1) antigen, including formulation, adjuvant, and dose; (2) recipients of the vaccine; and (3) the route of immunization [24]. Hyperimmune serum against genotype VII NDV was produced in several applications. For hyperimmune serum production, we immunized the rabbits with antigen–IFA emulsion in the first and second groups, while the third and fourth groups did not use IFA in antigen preparation. Immunization in the first, second, and third groups was administered subcutaneously, while in the fourth group, immunization was administered intravenously. In the second group, antigen volume was half of the first group. The Newcastle disease hyperimmune serum generated in this study resulted from three immunizations to induce a higher HI titer. The first immunization is intended to introduce antigen into the immune system, particularly the B cell, whereas the second and third immunizations are boosters designed to modulate antibody production by B cells [25,26]. The second immunization was carried out on the 14th day after the first immunization, and the third immunization was on the 16th day after the second immunization. The hyperimmune serum titer against genotype VII NDV was measured with a periodic HI test, and hyperimmune serum was collected on the 8th day after the third immunization. The hyperimmune serum titer result is shown in Figure 1.

Based on Figure 1, the first group immunized with NDV–IFA emulsion showed that the HI titer was already detected on the 12th day after the first immunization and reached $2^{5.5}$ on the 5th day and the 9th day and then decreased on the 16th day after the second immunization. The HI titer in this group continued to decline until the 3rd day after the 3rd immunization, then increased until the 8th day after the 3rd immunization, and reached a HI titer of 28. The second group receiving NDV–IFA emulsion (each volume 0.5 ml) showed hyperimmune serum against NDV genotype VII detected on the 12th day after the first injection, and the HI titer reached $2^{4.5}$ on the 5th day after the second immunization and reached a peak on the 9th day after and then decreased on the 16th day. The HI titer in this second group continued to decline until the 5th day after the third immunization and then increased, reaching 2^{6} HI titer on the 8th day after the third immunization. The first and second groups were different in the dose of antigen and adjuvant, and these conditions influenced the HI titer result. The first and second groups have a difference in HI titer of about 2 log levels. The amount of antigen can change the immune response and, in turn, the number of antibodies made [20,21].

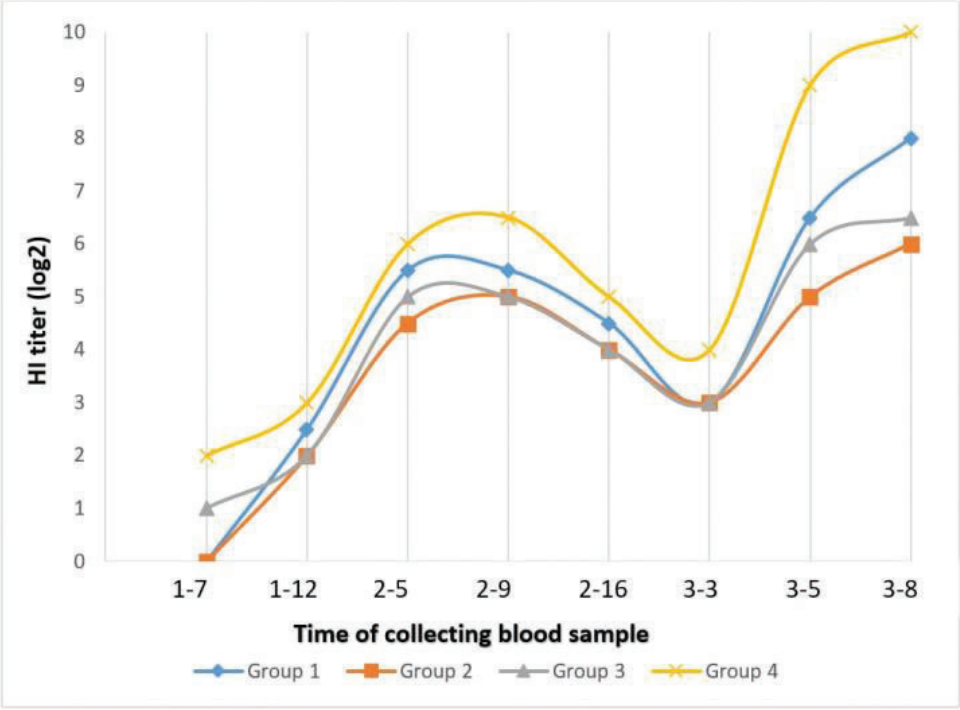

Figure 1. Hemagglutination inhibition titer after immunization.

The group of rabbits that received NDV subcutaneously showed that an HI titer could be detected on the 7th day after the first immunization. This group showed the same HI titer as the second group until the 3rd day after the third immunization, except on the 5th day after the second immunization, when this group reached a HI titer of 25, which was higher than the second group. Furthermore, on the 5th day after the third immunization, this group showed an antibody titer reaching 2^6, and at the end of the serum collection on the 8th day after the third immunization, the HI titer reached $2^{6.5}$. The group of rabbits that received genotype VII NDV subcutaneously showed the same HI titer pattern as the second group, but with higher HI titers. The second and third groups differ in antigen volume and adjuvant. Immunization using a half dose (volume) and mixture with adjuvants produced almost the same antibody titer as immunization using a full dose of antigen only (without adjuvant). The difference occurs at the beginning of antibody formation. The antibody formation process in the group that received NDV–IFA emulsion needed more time. Furthermore, at the end of the hyperimmune serum production, the group that received NDV–IFA emulsion showed a 1 log higher HI titer. For the secondary and booster injections, we used incomplete Freund's adjuvant as a water-in-oil emulsion with antigen to raise polyclonal and monoclonal antibodies [23]. Awate et al. [27] stated that compared to injection of antigen alone, injection of antigen plus an adjuvant generally permits the use of a much smaller quantity of antigen, while greatly enhancing the serum antibody response. The adjuvants promote an increased immune response slowly [23,27]. In general, adjuvants permit the use of smaller antigens but still retain the ability to modulate the immune response against the antigen. Samiullah et al. [28] produced an antibody for APMV-1 using adjuvant within 91 days and reach a 1024 (2^{10}) HI titer with 4 and 5 injections. Putri et al. [29] produced antibodies to Newcastle disease in New Zealand rabbits with subcutaneous route application for the first and second injection, which resulted in the same pattern of antibody titer until the 16th day after the second injection. Moreover, after the third injection intravenously, the study revealed a higher antibody titer on the 8th day, reaching HI titer of 2^9.

The last group of rabbits, immunized by antigen Newcastle disease intravenously, showed that HI titer started to be detected on the 7th day after the first immunization and reached HI titer 2^6 on the 5th day after the second immunization. It continued to increase until the 9th day, with the HI titer reaching $2^{6.5}$ and decreasing on the 16th day. The HI titer continued to decrease until the 5th day after the third immunization and then increased on the 8th day after the third immunization and achieved a 2^{10} HI titer. Rabbits receiving intravenous immunization showed higher antibody titers than the other groups. These results indicate that the intravenous route application for hyperimmune serum production against the genotype VII NDV dramatically affects the immune response result. The intravenous route has the potential for a broad distribution of antigens. Intravenous routes will distribute the antigen, firstly to the spleen and secondarily to the lymph nodes. Intravenous may be the most effective route for small particulate antigens, such as cells, virions, or bacteria [30].

Serum purification

The serum is a blood component that contains albumin and globulin proteins [31]. The serum component that can bind directly to the antigen is called an antibody [32]. Before being characterized, the serum must be purified from other components. Some purification methods could be used to separate serum [33]. In this study, we purified the hyperimmune serum with ammonium sulfate (4.1 M) and a protein A purification kit (BioVision). Ammonium sulfate is the oldest, easiest, and most economical method used most frequently to precipitate and thus concentrate immunoglobulins from the serum [34]. The principle of ammonium sulfate purification is the ability of ammonium sulfate to bind immunoglobulin G (IgG) [35]. The second stage of hyperimmune serum purification was using a protein A purification kit. Protein A, located in the surface protein of *Staphylococcus aureus* [36], has five domains that have the ability to bind the Fc fragment of IgG [37]. After the protein purification, it is important to know the protein concentration in our samples. In this study, we determined the antibody concentration in serum by a UV-Vis spectrophotometer at a 280 nm wavelength. Based on the UV-Vis spectrophotometer result, the genotype VII ND antibody concentration is 1.97 µg/µl.

Serum characterization by SDS-PAGE

SDS-PAGE was used to determine the protein profile and the molecular weight of hyperimmune serum against the genotype VII NDV. The SDS-PAGE result showed that purified serum by ammonium sulfate contained five protein bands, and the serum that had passed through two stages of purification only contained two protein bands, the same as with a standard commercial antibody (Fig. 2).

We determine the molecular weight of serum protein on SDS-PAGE by forming a linear curve based on calculating the relative mobility value (Rf) and the logarithm of the molecular protein weight. Based on the data in Table 2, a linear regression curve with equation $y = -0.1134x + 2.2379$ and $R^2 = 0.9429$ was obtained. The equations were used to determine the molecular weight of the standard antibody and purified serum samples, which are presented in Table 3.

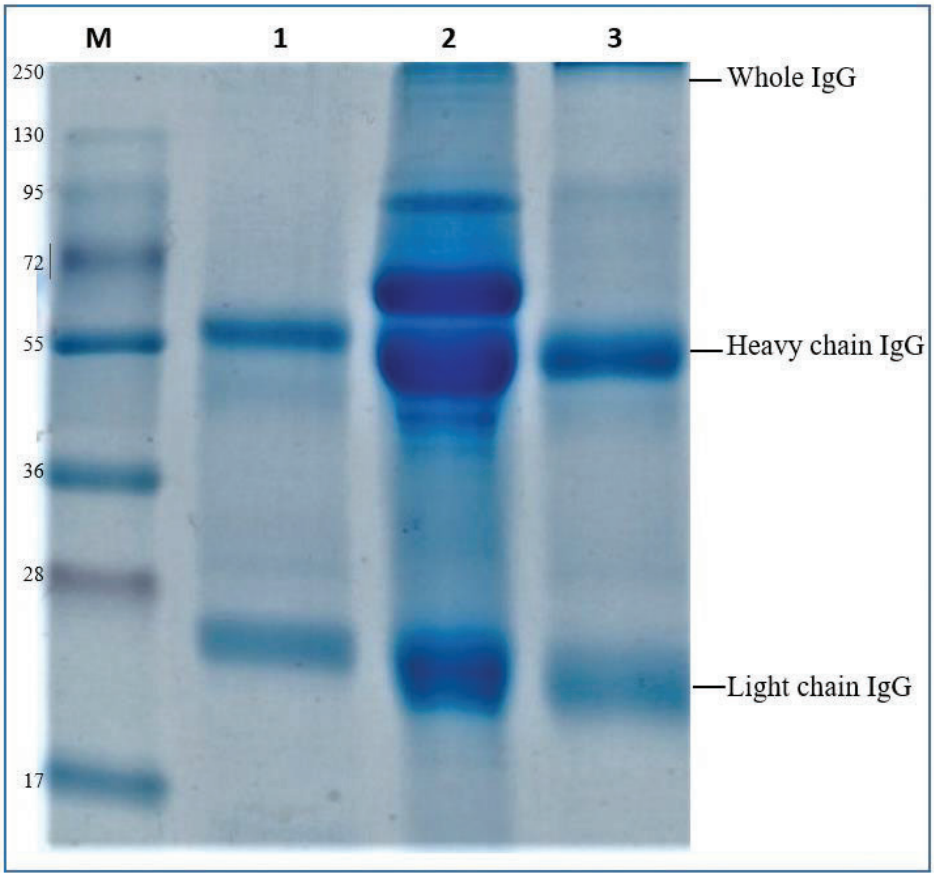

Figure 2. SDS-PAGE result of ND hyperimmune serum. (M) Protein marker; (1) commercial standard antibody; (2) after purification by ammonium sulfate; and (3) after purification by the protein purification kit A.

Table 2. The migration distance from the marker along with the Rf value.

Rf (cm)	MW (kDa)	Log MW
0.14	250	2.40
0.96	135	2.13
1.71	95	1.98
2.65	72	1.86
3.76	55	1.74
5.52	36	1.56
6.9	28	1.45
9.69	17	1.23

$y = -0.1134x + 2.2379$; $R^2 = 0.9429$.

Table 3. The migration distance and molecular weight of the hyperimmune serum against Newcastle disease virus.

Rf (cm)	Log MW	MW (kDa)
Purification by ammonium sulfate		
0.43	2.19	154.57
2.12	2.00	99.42
3.95	1.79	61.66
4.44	1.73	54.25
7.51	1.39	24.34
Purification by Protein A		
4.44	1.73	54.25
7.51	1.39	24.34

Based on the regression equation calculation, we found that the molecular weight of the antibody standard was 154.57 kDa for whole IgG; the heavy chain was IgG 54.25 kDa; and the light chain IgG was 24.34 kDa. Immunoglobulin G had a molecular weight of 150–160 kDa [36].Chemical treatments such as SDS will break the IgG molecule by the disulfide bond, causing the polypeptide to break into four separate chains. These chains are "heavy" chains with a molecular weight of 50 kDa and "light" chains with a molecular weight of about 25 kDa. In

the serum purified by ammonium sulfate only, we detected two banned proteins that were not the same as the standard antibody in molecular weight: 99.42 kDa and 61.66 kDa. Albumin is a protein found in serum with a molecular weight of 60 kDa [37]. In serum that has passed through two purification stages, it only has two protein bands that are the same as standard antibodies.

Serum confirmation by agar gel precipitation test and Western blot assay

Serum confirmation is carried out to ensure that the antibodies contained in the hyperimmune serum against NDV are only able to bind to NDV. Several methods can confirm this, including AGPT and Western blot assay. The agar gel precipitation test has been applied to detect precipitating antibodies in various avian diseases. The confirmation results of the ND antibody specificity can be seen in Figure 3.

The antigen–antibody interaction on AGPT was characterized by a precipitation line in the agarose gel. The agar gel precipitation test result showed the line of precipitation formed on all ND antigens, whereas in wells given the avian influenza and the IBD antigen, we could not find the precipitation line. This result indicated that this research's hyperimmune serum against Newcastle disease virus has specificity.

In addition to AGPT, the Western blot assay was also used to confirm whether the antibody in the Newcastle disease serum produced could bind to Newcastle disease virus proteins. By using the Western blot method, researchers can identify specific proteins from a complex mixture of proteins extracted from cells [40]. This stage begins with the separation of viral proteins with SDS-PAGE, followed by the transfer of viral proteins to nitrocellulose membranes. The Western blot assay results are shown in Figure 4.

Based on the SDS-PAGE results of NDV, 5–8 proteins were recorded with a molecular weight ranging from 28 to 200 kDa. To know the molecular weight of each protein band, the relative mobility must be determined first and then entered into the following equation: $y = -0.1134x + 2.2379$; $R^2 = 0.9429$. Table 4 shows the molecular weight of the Newcastle disease protein, which we got from the regression equation.

Hemmatzadeh and Kazemimanesh [41] detected Newcastle disease proteins HN, F, NP, P, and M with molecular weights of approximately 75, 66, 55, 53, and 39 kDa, respectively, and that Western blot assay can detect those proteins. This shows that the antibodies made in this study

Figure 3. Serum confirmation with the agar gel precipitation test. (1) Avian influenza (Ag); (2) IBD (Ag); (3) Newcastle disease virus (Lasota); (4) Newcastle disease virus (Sato); (5) Newcastle disease virus genotype VII (1); (6) Newcastle disease virus genotype VII (2); and (7) hyperimmune serum. Arrow: precipitation line.

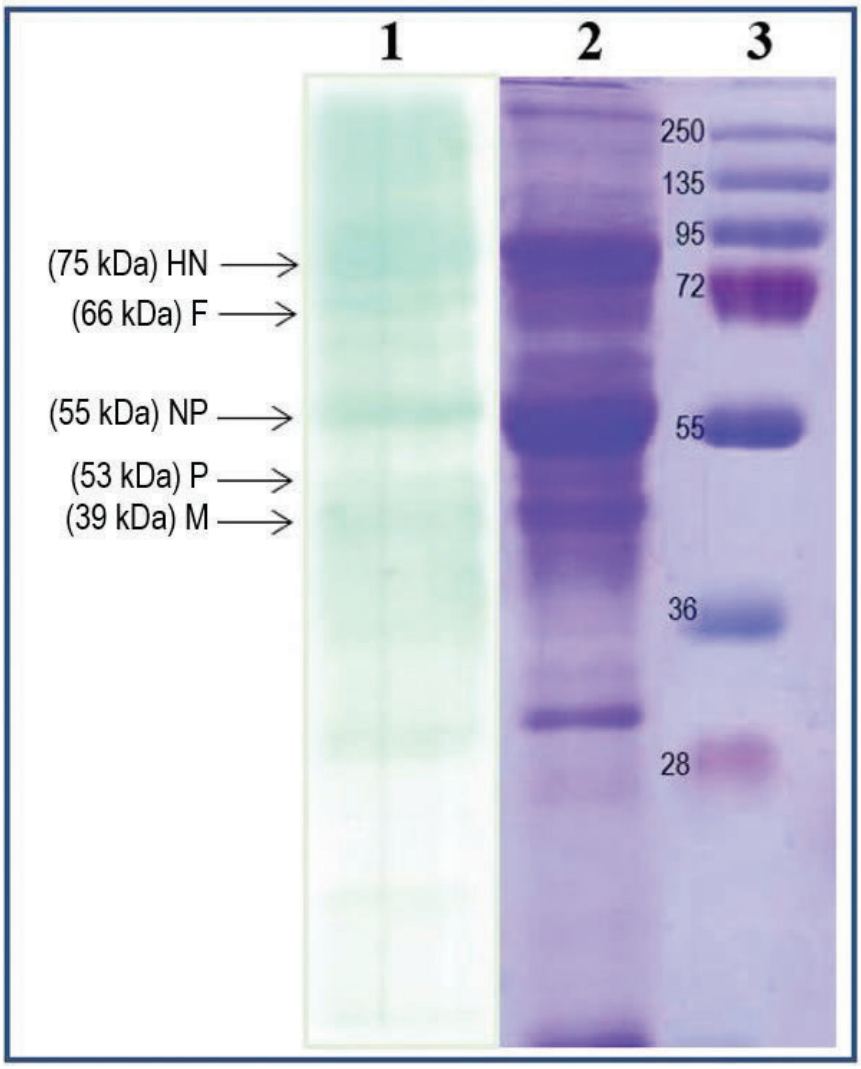

Figure 4. Western blot assay antigen–antibody Newcastle disease. (1) Western blot assay result; (2) SDS-PAGE result of Newcastle disease virus; and (3) protein marker.

Table 4. The molecular weight of Newcastle disease protein by SDS-PAGE.

RF (cm)	Log MW	MW (kDa)
3.19	1.876154	75.19
3.67	1.821722	66.33
4.36	1.743476	55.40
4.52	1.725332	53.13
5.67	1.594922	39.35

could find the protein caused by the Newcastle disease virus.

The main goal of antibody production is to obtain high-titer, high-specificity antibodies while still being concerned about the animal welfare. The study successfully produced the hyperimmune serum for Newcastle disease in rabbits. Normal antibodies can be replaced with hyperimmune serum, which can also be used to test for viruses [17].

Hyperimmune serum can be used for large-scale screening of NDV-carrying commercial and wild birds [17]. The hyperimmune serum against NDV can be used to decrease the morbidity and mortality rate in experimentally infected birds [16]. Passive immunization against Newcastle disease has also been attempted with promising results. The symptoms of ND in experimentally infected birds with NDV are successfully treated through passive immunization using HIS [42]. The high doses of antibodies are also helpful in providing passive immunity by decreasing the mortality and morbidity in birds previously exposed to the ND virus of velogenic strain. With an

increasing dose of HIS, mortality and morbidity are considerably reduced [42].

Conclusion

The various application methods successfully produced hyperimmune serum against the genotype VII Newcastle disease virus. The production of hyperimmune serum by three intravenous immunizations produced the highest titer of 2^{10} at 38 days. The hyperimmune serum has specificity for Newcastle disease antigen based on the AGPT and Western blot assay results.

List of abbreviations

NDV, Newcastle disease virus; HI, Hemagglutination inhibition; IFA, Incomplete Freund's adjuvant; ELD, Embryo lethal dose; AGPT, Agar gel precipitation test; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Conflicts of interest

The authors declare that they have no competing interests.

Authors' contribution

DDP executed the work (collect data, analysis, and writing of manuscript); ONP participated in the analysis and interpretation of data and writing of the manuscript; AAC participated in designing the study and drafting of the manuscript; and RDS participated in designing the study, analysis of data and drafting of the manuscript. All authors read and approved the final manuscript.

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