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

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1 **ORIGINAL ARTICLE**

2

3 The production of hyperimmune serum against genotype VII 4 Newcastle Disease virus in rabbit with several applications

5

6 **Statement of novelty:** Finding a method for the production of hyperimmune serum against 7 genotype VII Newcastle Disease Virus (NDV). The serum can be produced by immunizing 8 rabbit intravenously within 38 days and until the antibody reached 2^{10} of HI titer. Genotype VII 9 Newcastle Disease hyperimmune serum produced in this research have spesificity for Newcastle 10 disease virus and was proven by Agar Gel Precipitation Test and Western Blot Assay.

11

12 **Ethical approval (if needed):** This research has been approved by the Animal Care and Use 13 Committee of Research and Community Services Institution, IPB University with approval 14 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.

44 **INTRODUCTION**

45 Newcastle Disease (ND) is the one of important diseases in bird that is caused by Avian
46 paramyxovirus serotype-1 (APMV-1) which belongs to the family Paramyxoviridae [1, 2]. The
47 Newcastle Disease Virus (NDV) has the ability to infect more than 250 spesies of birds, and
48 infection by virulent strains can cause huge morbidity and mortality with significant symptoms
49 [3]. The wide range of susceptible hosts causes the persistence of NDV which becomes
50 endemic in many countries in the world. Virulent strains infection has resulted four panzootics
51 [4]. The first ND outbreak by virulent strain occurred in Java, Indonesia in 1926, and at the
52 same time an outbreak happened in England precisely in Newcastle upon Tyne region [5].
53 Newcastle Disease virus is an enveloped, unsegmented, single-stranded RNA genome
54 of roughly 15.2 kb [5, 6]. The NDV genome encodes six polypeptides namely the nucleocapsid
55 (NP) protein, phosphoprotein (P) protein, matrix (M) protein, fusion (F) protein,
56 hemagglutinin-neuraminidase (HN) protein, and the RNA-dependent RNA polymerase (L)
57 protein. The virus nucleocapsid core consists of NP proteins bound to RNA [7].
58 Newcastle Disease Virus may vary widely in the severity of the disease in birds [8].
59 Multiple factors can contribute to the severity of disease including species of host, immune
60 status, age, environmental conditions, secondary infections, the number of virus transmitted,
61 the mode of transmission and most importantly the pathotype of the infecting virus [9]. In
62 comparison, susceptible species is chickens, whereas geese and ducks do not show symptom;
63 therefore, waterfowl are considered as the natural reservoir for NDV. The F protein cleavage
64 site is known to be a major determinant of viral virulence during replication in host cells. [10,
65 11]. Based on the pathogenicity of the disease, ND can be classified into five pathotypes: -
66 Neurotrophic velogenic strain exhibiting respiratory and neurological symptoms with a high
67 mortality rate; Viscerotropic velogenic strain causing hemorrhagic and highly pathogenic
68 intestinal lesions; Mesogenic strain caused by viruses with rare respiratory and neurological
69 symptoms, while mortality is related to the age of susceptible birds; Viral lentogenic strains

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

72 Interaction between virus and environment including host immune system resulted in
73 NDV evolution and continues to produce new genotypes virus. Lately, infection of genotype
74 VII NDV caused high mortality of birds in several poultry farms in Indonesia [13, 14]. In
75 recent years, producing hyperimmune serum in animals is an important activity of many
76 research projects. The hyperimmune serum as a biological reagen will continue to be developed
77 for research needs and possibly also for commercial applications in the future such as for
78 therapy and development of immunodignostic tools[15]. The specific antibodies in
79 hyperimmune serum can be used for the treatment and control of disease in case of an outbreak
80 [16]. Hyperimmune serum is already used for the successful treatment of some disease like foot
81 and mouth diseases, tetanus and canine viral diseases [17] . Currently, the imported
82 hyperimmune serum used for diagnostic in poultry is very expensive and has been imported
83 from different countries of the world. Moreover, the imported strains of viruses may differ
84 from indigenous isolates showing non-specificity in diagnosis [17]. The development of the
85 serum for NDV currently circulating must be followed by the development of
86 immunodiagnostic tests, to obtain accurate test results. Therefore, it is necessary to produce
87 genotype VII Newcastle Disease hyperimune serum which can be used as immunodiagnostic
88 reagents.

89 Hyperimune serum production can be carried out in various applications, with or
90 without adjuvant and with its own advantages and disadvantages. 24 Considering the numerous
91 applications of hyperimune serum in research and clinical fields, the preparation method
92 development of hyperimmune serum against pathogens is very important [15]. To be able to
93 produce antibodies with high titers in a short time, it is necessary to conduct research on
94 various immunization applications with or without adjuvant in inducing immunity. The aim of

95 this research was to produce hyperimune serum againts genotype VII NDV with several
96 applications efficient in time and cost.
97

98 **MATERIALS AND METHODS**

99 **Ethical approval**

100 This research has been approved by the Animal Care and Use Committee of Research
101 and Community Services Institution, IPB University with approval number: 213-2021 IPB.
102 .

103 **Newcastle Disease Antigen**

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

109 **Hyperimmune Serum Production**

110 The production of hyperimune serum against genotype VII NDV was performed on
111 eight New Zealand White rabbits aged 2,5 – 3,5 month with an average body weight of 2,5 kg
112 that were formed into four groups. First group was rabbit immunized by emulsion of 1 ml
113 isolate genotype VII NDV ($5\times10^{6.5}$ ELD_{50}/ml) and 1 ml Incomplate Freund's Adjuvant (IFA)
114 subcutaneously; second group was rabbit immunized by emulsion of 0,5 ml isolate genotype
115 VII NDV ($5\times10^{6.5}$ ELD_{50}/ml) and 0,5 ml IFA subcutaneously; third group was rabbit
116 immunized by 1 ml isolate genotype VII NDV ($5\times10^{6.5}$ ELD_{50}/ml) subcutaneously and last
117 group was rabbit immunized by 1 ml isolate genotype VII NDV ($5\times10^{6.5}$ ELD_{50}/ml)
118 intravenously. The application and composition of the antigens used in this study are presented
119 in Table 1.
120

121 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

122 *) Incomplate Fruend's Adjuvant

123

124 Rabbits were immunized three times. First imunization was on 1st day and second
125 immunization was on 14 th day and third immunization was on 30th day. Blood sampling was
126 carried out on the 8th day after third immunization. Hyperimmune serum was collected by
127 taking blood intra artery after the rabbits had been administered local anastethic agent into the
128 ear. The procedure for making serum is as follows: The blood samples were kept at temperature
129 ± 25°C for an hour and then kept overnight at 4°C t. The serum was separated manually and
130 precipitated by centrifugation at 2500 rpm for 15 minutes. Futhermore, the serum was kept in
131 collecting tube 1.5 ml and stored at -20°C until use. The rabbit blood samples were taken
132 periodically to observe the Hemagglutinatin Inhibition (HI) antibody titer against genotype VII
133 NDV. Serum was inactivated at 54°C for 30 min before used for the HI test.

134 **Serum Purification**

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

144 **Serum Characterization by SDS-PAGE**

145 The molecular weight of the purified ND hyperimun serum was measured using sodium
146 dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique with the
147 concentration of separating gel 12% and 4% for the stacking gel [19]. The sample buffer
148 (containing bromophenol blue, SDS, DTT and glycerol) 5 µl was mixed with serum sample (5
149 µl), and heated 65°C for five minutes to denature the protein. A total of 5 µl of marker protein
150 (Thermo Scientific, USA) and 10 µl of hyperimun serum samples were used. Protein separation
151 was carried out by electrophoresis at 100 V for 150 minutes. The electrophoresis gel was stained
152 with Commasie Brilliat Blue for 30 minutes, followed by the addition of destaining solution for
153 24 hours.

154 **Serum confirmation by Agar Gel Precipitation Test and Western Blot Assay**

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

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

169 procedure, the membrane was washed by distilled water to terminate enzyme reaction on the
170 membrane.
171

172 **RESULTS AND DISCUSSION**

173 **Antigen Preparation**

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

2

182 **Production of hyperimmune serum against genotype VII Newcastle Disease virus**

183 The main purpose of hyperimmune serum production is to gain high titer with high
184 antibody specificity against what continues to be a concern in animal welfare. Hyperimmune
14
185 serum production needs a number of animals as a subject to a number of invasive treatment
186 such as antigen injection and serum collection [20, 21]. This study used rabbits as a donor for
187 antibody which received invasive treatment, namely immunization and serum collection. The
188 rabbit is a popular animal to be used as a donor antibody under the reason of cost benefit ratio
189 and easy to handle [22]. Moreover,rabbit is basically not related closely with chicken as a natural
190 host of Newcastle Disease Virus [20]. This study used eight female rabbits aged 2,5 – 3 months
191 as biological agents to produce hyperimmune serum against genotype VII NDV.
192 Hyperimmune serum production against genotype VII NDV was performed with and without
193 adjuvants and applied subcutaneously and intravenously. Adjuvants work to increase the
194 immune response through a "depot" effect mechanism that increases antigen presentation

195 slowly. The adjuvant immunostimulatory properties can cause negative effect to animal because
196 they induce inflammation and tissue destruction which potentially cause pain and distress [23].
197 The adjuvant used in this study was Incomplete Fruend's Adjuvant (IFA) because of it
198 minimizes pain and distress in rabbits while still retains the potency as immunostimulatory
199 agent.

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

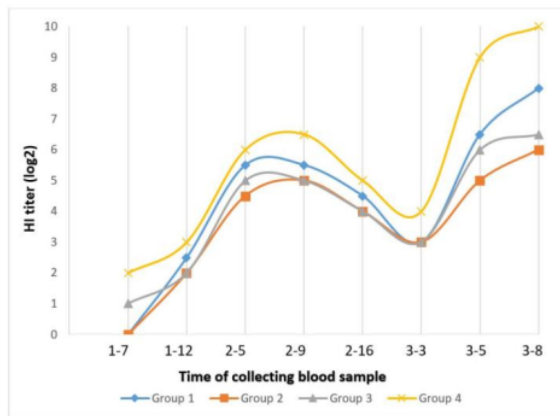


Figure 1. Hemaglutination Inhibition Titer after immunization

Based on Figure 1, the first group immunized with NDV – IFA emulsion showed HI titer was already detected on the 12th day after first immunization and reached $2^{5,5}$ on the 5th day and 9th day and then decreased on the 16th day after second immunization. The HI titer in this group continued to decrease until the 3rd day after the 3rd immunization and then increased until the 8th day after the 3rd 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 12th after 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 decrease until the 5th day after the third immunization and then increased reaching 2^{6} of HI titer on the 8th day after third immunization. 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 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

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 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 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 titers pattern with the second groups but with higher HI titers. Second and third group have difference on volume antigen and adjuvant. Immunization using half the dose (volume) and mixture with adjuvants produced almost the same antibody titer with immunization using full dose of antigen only (without adjuvant). The difference occured 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. Incomplete Freund's Adjuvant was used as water-in-oil emulsion with antigen for secondary and booster injections 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 increased immune response slowly [23, 27]. In general, adjuvants permit smaller antigen use 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 via 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

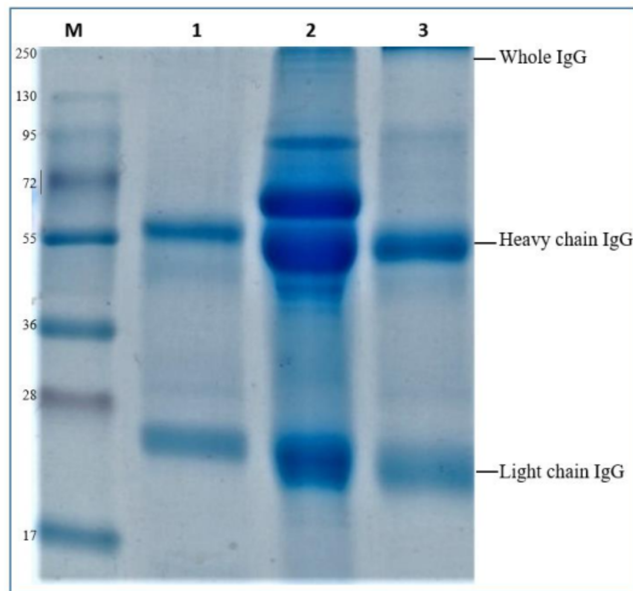
261 2^6 on the 5th day after the second immunization. It continued to increase until the 9th day with
262 the HI titer reaching $2^{6.5}$ and decreased on the 16th day. The HI titer continued to decrease until
263 the 5th day after the third immunization and then increased on the 8th day after the third
264 immunization and achieved 2^{10} of HI titer. Rabbits receiving intravenous immunization showed
265 higher antibody titers than the other groups. These results indicated that the intravenous route
266 application for hyperimmune serum production against genotype VII NDV greatly affects the
267 immune response result. The intravenous route has the potential for broad distribution of
268 antigen. Intravenous route will distribute the antigen, firstly to the spleen and secondarily to
269 lymph nodes. Intravenous may be the most effective and to be the route of choice for small
270 particulate antigen such as cells, virions, or bacteria[30].

271 **Serum Purification**

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

286 **Serum Characterization by SDS-PAGE**

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



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

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

302

303 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

304 $y = -0,1134x + 2,2379$; $R^2 = 0,9429$

305 Table 3. The migration distance and Molecular Weight of Hyperimmune serum against

306 Newcastle Disease Virus

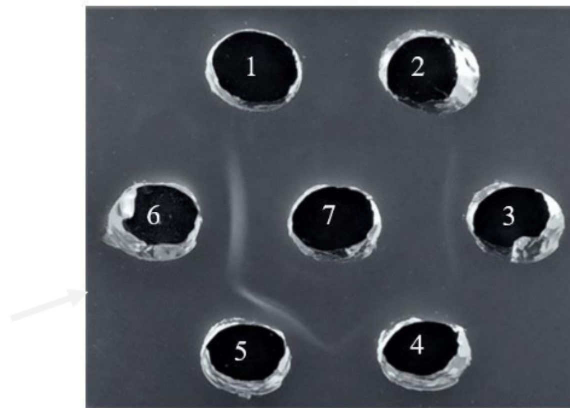
Rf (cm)	Log MW	MW (kDa)
Purification by Amonium Sulfate		
0,43	2,19	154,57
2,12	2,00	99,42
3,95	1,79	61,66
4,44	1,73	54,25
7,51	1,39	24,34
Purification by Protein A		
4,44	1,73	54,25
7,51	1,39	24,34

307

308 Based on the regression equation calculation, we found molecular weight of antibody
309 standard was 154,57 kDa for whole IgG, heavy chain was IgG 54,25 kDa, and 24,34 kDa for
310 light chain IgG. Molecular weight of immunoglobulin G was about 150 to 160 kDa [36].
311 Chemical treatments such as SDS will break the IgG molecule by the disulfide bond, causing
312 the polypeptide to break into four separate chains. These chains are "heavy" 21 chains with a
313 molecular weight of 50 kDa and "light" chains with a molecular weight of about 25 kDa.The
314 serum, which was purified by ammonium sulfate only, was detected to have 2 bans protein that
315 were not the same as standard antibody in molecular weight 99,42 kDa and 61,66 kDa. Albumin
316 is a protein found in serum with a molecular weight of 60 kDa [37]. In serum that has passed 2
317 purification stages, it only has 2 protein bands that are the same as standard antibodies.

318 **Serum confirmation by Agar Gel Precipitation Test and Western Blot Assay**

319 Serum confirmation is carried out to ensure that antibodies contained in Hyperimmune
320 serum against NDV are only able to bind to NDV. Several methods can be used to ensure this,
321 including AGPT and Western blot assay. The Agar Gel Precipitation Test has been applied to a
322 variety of avian diseases for the detection of precipitating antibodies. The confirmation results
323 of the ND antibodies specificity can be seen in Figure 4.



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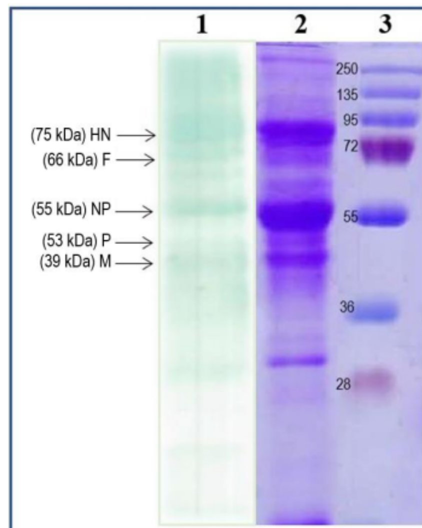
325 **Figure-4:** Serum confirmation with Agar Gel Precipitation Test; (1) Avian Influenza Ag; (2)
326 Infectious Bursal Disease Ag; (3) Newcastle Disease virus Lasota (4) Newcastle Disease virus
327 Sato; (3) Newcastle Disease virus genotype VII (1); (4) Newcastle Disease virus genotype VII
328 (2); (7) Hyperimmune serum; Arrow (→): Precipitation line.

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

334 In addition to AGPT, the Western blot assay was also used to confirm whether the
335 antibody in the Newcastle Disease serum produced were able to bind to Newcastle Disease
336 virus proteins. By using Western blot method, researchers are able to identify specific proteins

337 from a complex mixture of proteins extracted from cells [40]. This stage begins with the
338 separation of viral proteins with SDS-PAGE followed by the transferring viral proteins to
339 nitrocellulose membranes. The Western blot assay result are presented in Figure-5.



340

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

343 Based on the SDS-PAGE results of NDV, there were 5 to 8 proteins recorded with a
344 molecular weight ranged from 28 kDa to 200 kDa. To know the molecular weight of each
345 protein band, the relative mobility must be determined first and then entered into the equation
346 $y = -0{,}1134x + 2{,}2379$; $R^2 = 0{,}9429$. Based on the regression equation, we obtained the
347 molecular weight of Newcastle Disease protein as presented in Table 6.

348 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

349

350 Hemmatzadeh and Kazemimanesh [41] detected Newcastle Disease protein HN, F, NP,
351 P, and M with molecular weights approximately of 75; 66; 55; 53 and 39 kDa respectively, and
352 that those proteins can be detected by Western Blott Assay. This indicates that the antibodies
353 produced in this study were able to detect the Newcastle Disease virus protein.
354 The main goal in antibody production is to obtain high-titer, high-specificity antibody and still
355 con-cerned in animal welfare. The study was successfully produced the Hyperimmune serum of
356 Newcastle Disease in rabbit. Hyperimmune serum can be used as an alternative and viable
357 replacement of conventional antibodies and can be used in diagnostic assay of viruses [17].
358 Hyperimmune serum can be used for large-scale screening of NDV carrier commercial and wild
359 birds [17]. Hyperimmune serum against NDV can be used to decrease the morbidity and
360 mortality rate in experimentally infected birds [16]. The passive immunization against Newcastle
361 disease has also been attempted with promising results. The symptoms of ND in experimentally
362 infected birds with NDV are successfully treated through passively immunization with the use
363 of HIS [42]. The high doses of antibodies are also helpful in providing passive immunity by
364 decreasing the mortality and morbidity in birds which are previously exposed the ND virus of
365 velogenic strain. With increasing dose of HIS the mortality and morbidity is considerably
366 reduced [42].
367
368 **CONCLUSION**
369 Hyperimmune serum against genotype VII Newcastle disease virus was successfully
370 produced by various method of applications. The production of hyperimmune serum by three
371 times intravenous immunization was able to produce the highest titer of 2^{10} at 38 days. The
372 Hyperimmune serum has specificity for Newcastle Disease antigen based on the AGPT and
373 Western blot assay result.
374
375 **ABBREVIATIONS**

376 NDV, Newcastle Disease Virus; HI, Hemaglutination Inhibition, IFA, Incomplete Freund's
377 Adjuvant; ELD, Embryo lethal dose; AGPT, Agar Gel Precipitation Test; SDS-PAGE, Sodium
378 dodecyl sulphate polyacrylamide gel electrophoresis.
379

380 **ACKNOWLEDGMENT**

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382 Republic Indonesia in Basic Research Grant No. 378.4/PL15.8/PT/2021

383

384 **CONFLICT OF INTERESTS**

385 The authors declare that they have no competing interests.

386

387 **AUTHORS' CONTRIBUTION**

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

393

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