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Immunoglobulin yolk targeting spike 1, receptor binding domain of spike glycoprotein and nucleocapsid of SARS-CoV-2 blocking RBD-ACE2 binding interaction

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ABSTRACT

Coronavirus disease (COVID)-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has become a global pandemic disease that has social and economic chaos. An alternative mitigation strategy may involve the use of specific immunoglobulin (Ig)-Y derived from chicken eggs. Our study aimed to evaluate the neutralizing potential of specific IgY targeting S1, receptor-binding-domain (RBD) of spike glycoprotein and nucleocapsid (N) of SARS-CoV-2 to inhibit RBD and angiotensin-converting-enzyme-2 (ACE2) binding interaction. Hy-Line Brown laying hens were immunized with recombinant S1, RBD spike glycoprotein, and nucleocapsid (N) of SARS-CoV-2. The presence of specific S1,RBD,N-IgY in serum and egg yolk was verified by indirect enzyme-linked immunosorbent assay (ELISA). Specific S1,RBD,N-IgY was purified and characterized from egg yolk using sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE), and was subsequently evaluated for inhibition of the RBD-ACE2 binding interaction in vitro. Specific IgY was present in serum at 1 week post–initial immunization (p.i.i), whereas its present in egg yolk was confirmed at 4 weeks p.i.i. Specific S1,RBD,N-IgY in serum was able to inhibit RBD-ACE2 binding interaction between 4 and 15 weeks p.i.i. The results of the SDS-PAGE revealed the presence of bands with molecular weights of 180 kDa, indicating the presence of whole IgY. Our results demonstrated that S1,RBD,N-IgY was able to inhibit RBD-ACE2 binding interaction in vitro, suggesting its potential use in blocking virus entry. Our study also demonstrated proof-of-concept that laying hens were able to produce this specific IgY, which could block the viral binding and large production of this specific IgY is feasible.

1. Introduction

The outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was acknowledged as a global pandemic by the World Health Organization (WHO) on March 11, 2020. This disease is not only a threat to human health but also causes critical social and economic burden worldwide. As of June 2022, over 533 million cases have been confirmed and over 6.3 million deaths have been reported globally (WHO, 2022). At the end of 2020, COVID-19 vaccination began in many countries, and several COVID-19 vaccines had been made commercially available by the first half of 2021. However, most of the vaccines developed have only been able to reduce the severity of the disease and do not prevent infection [1]. Thus, an effective nonvaccine prophylactic approach is necessary as an alternative mitigation strategy to prevent SARS-CoV-2 infection. The majority of drugs and symptomatic therapy only reduce symptoms and complications caused by the virus, while the immunity obtained through vaccination actually takes time to work effectively, so other therapeutic methods that act faster and more efficiently are needed. Immunotherapy with the principle of passive immunity can be an alternative solution for the treatment and prevention of COVID-19 [2].

Passive immunity using specific antibodies has the potential to

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prevent infectious diseases, and the development of such polyclonal antibodies may be more efficient compared to monoclonal ones. Specific antibodies can recognize and neutralize the foreign materials, such as viruses and bacteria [3]. Laying hens have been known as a biological factory of specific antibody production for various antigen types [4]. This immunization process, which is meant to provide significant protection as passive immunity to chicks during the hatching period, is based on specific antibodies being effectively transferred through blood to yolk as IgY. Immunoglobulin Y (IgY) is the main type of antibody produced by the avian immune system and is considered analogous to immunoglobulin G (IgG) which can be found in blood serum, placenta and mammalian colostrum functionally [5]. Its structural similarity to immunoglobulin G (IgG) makes IgY an alternative to passive immune therapy or immunotherapy which is rapidly developing today. IgY is more hydrophobic than IgG and has good resistance to heat and pH. In addition, the absence of a hinge region in the IgY structure causes IgY to be more rigid than IgG and affects its resistance to proteolytic degradation, thereby minimizing the risk of protein damage [4]. Immunotherapy using IgY is considered to be relatively easier to produce and more cost-effective than other treatment methods [6]. IgY can be harvested at high yield from immunized chicken eggs, and it has been found to be safe and effective for prophylaxis against infectious diseases in both animals and humans [6,7].

SARS-CoV-2 contains four structural proteins: spike (S) glycoprotein, envelope, membrane, and nucleocapsid (N) proteins. Spike protein plays a key role in viral infection and pathogenesis, and most of its neutralizing epitopes have been found at the S1 subunit. The S1 protein is divided into the receptor-binding domain (RBD) and the N-terminal domain, and both have been identified as critical neutralizing epitopes [8,9]. The RBD is important for viral binding to the ACE2 receptor [10], whereas the N protein is often used as a marker in diagnostic assays [11]. Therefore, we considered S1, RBD of spike glycoprotein and N protein as excellent antigens for inducing specific IgY against SARS-CoV-2. Thus, this study aimed to evaluate the neutralizing potential of the specific IgY, which targeting the S1, RBD of spike glycoprotein and nucleocapsid (N) of SARS-CoV-2, to inhibit the RBD and angiotensin-converting-enzyme-2 (ACE2) binding interaction.

2. Material & methods

2.1. Study area and animal ethical approval

This study was performed in the poultry house of the Animal Care Integrated Laboratory, School of Veterinary Medicine & Biomedical Science, IPB University between January 2021 and May 2022. This study complied with the article 80 on "Research in Animal Health" of the Indonesian Law on Livestock and Animal Health UU/18/2009 and received animal ethics approval from the veterinary ethics commission of the School of Veterinary Medicine & Biomedical Science, IPB University, with reference number 014/KEH/SKE/XI/2020 on 3 November 2020.

2.2. Immunization of laying hens

Recombinant SARS-CoV-2 S1-spike glycoprotein with His-tag (N439K, GenScript®, New Jersey, US), RBD spike glycoprotein with Avi & His-tag (E484Q, L452R, GenScript®, New Jersey, US), and nucleocapsid protein (GenScript®, New Jersey, US) were prepared as antigens. Each recombinant protein was reconstituted in phosphate-buffered saline (PBS) to obtained a concentration of 1 mg/mL.

Three laying hens were immunized using S1, RBD and N recombinant protein. Initial immunization was performed at 28 weeks, using mixed recombinant protein with a concentration of 3 μg for each antigen, through intravenous route (IV) at *v. Brachialis*, and performed three days in a row using the same amount of antigen and route for hyper-immune condition. The second immunization (first booster) was performed at 7 days p.i.i. using the mixed recombinant protein (3 μg/antigen) with complete Freund's adjuvant (50% antigen in PBS, 50% adjuvant, v/v). Finally, the third immunization (second booster) [12] was performed at 21 days p.i.i. using the combination of recombinant protein (5 μg/antigen) with the incomplete Freund's adjuvant (50% antigen in PBS, 50% adjuvant, v/v) for two laying hens, and the combination of recombinant protein (5 μg/antigen) with the poultry adjuvant Montanide ISA 70 VG (30% antigen in PBS, 70% adjuvant, v/v) for one laying hen. The second and third immunizations were performed through the subcutaneous route (SC).

Serum and egg collection. Blood collections were performed pre- and post-immunization once a week, starting from one up to fifteen weeks p.i.i.. Blood was collected from *v. Brachialis* at 1.5 mL and preserved horizontally in an incubator at 37 °C until serum was formed. Serum was then collected and preserved in a freezer at −20 °C for further use. Eggs were collected daily, and the egg yolk was then separated and preserved in the freezer at −20 °C for further use. For ELISA test, the egg yolk was pooled once a week from each laying hen, diluted with PBS to obtain 50% v/v, and centrifuged at 2,500 rpm for 10 min to collect the supernatant. This supernatant was then preserved until further use.

Serum and egg yolk antibody detection using ELISA. The ELISA test was performed with the ID Screen® SARS-CoV-2 Double Antigen Multi-Species ELISA kit (ID.Vet, Grabels, France), targeting the specific N protein antibodies, to detect antibodies in serum and egg yolk. The ELISA was performed according to the manufacturer's instructions. Samples presenting S/P% $\leq$ 50% were considered negative, those between 50% and 60% were considered doubtful, $\geq$ 60% were considered positive.

IgY purification from a chicken egg. IgY was purified using the Pierce® Chicken IgY purification kit (Thermo Scientific, US) according to the manufacturer's instructions [13]. Eggs from the same hen were pooled together for IgY purification. We only used egg yolk that showed positive N protein antibodies in ELISA, whereas only egg yolk from two hens that has positive antibody. Yolk was separated from the egg white with an egg separator. 1 mL of yolk was transferred into a glass beaker and added five times the volume of the cold delipidation reagent very slowly while stirring with a low rotary stirrer until evenly mixed. After that, the mixture was covered and left overnight at 4 °C. The mixture was then centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was collected into a beaker glass and added with an equal volume of IgY precipitation reagent with gentle mixing for two minutes. The glass beaker was then covered and incubated at 4 °C for 1 h to overnight. Consequently, the suspension was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was removed, and the pellet (IgY) was dissolved by adding PBS with an equal to the original volume of egg yolk.

Protein concentrations were quantified using a spectrophotometer, according to the manufacturer's (Pierce® Chicken IgY purification kit, Thermo Scientific, USA) instructions. The purified IgY was diluted 20 times with PBS and measured at an absorbance of 280 nm (A280) in a quartz strip with a 1-cm path length.

IgY characterization. IgY characterization was determined with the SDS-PAGE method. We used a discontinuous system SDS-PAGE, consisting of 12% separating gel and 4% stacking gel. The electrophoresis procedure started by making a running gel with a 12% concentration consisting of 14 mL 30% acrylamide stock, 10 mL distilled water, 5 mL of 3 M Tris HCl pH 8.9 solution, 0.4 mL SDS 12% solution, 0.02 mL tetramethylethylenediamine (TEMED), 14.4 mL distilled water, and 0.4 mL ammonium persulfate solution (0.5 g in 4.5 mL distilled water). The stacking gel was made by mixing 1 mL of 30% acrylamide stock, 7.54 mL of distilled water, 1.25 mL of 0.5 M Tris HCl pH 7 solution, 10% SDS solution of 0.1 mL, 0.005 mL TEMED, and 0.1 mL ammonium persulfate solution (0.5 g in 4.5 mL of distilled water).

Samples were prepared by adding a protein sample and loading buffer with (4:1) ratio, which was then heated at 60 °C for 3 min. The loading buffer consisted of 10 mL of 10% SDS, 5 mL of glycerin, 5 mL of Tris HCl 0.5 M pH 8.8, 0.5 mL of bromphenol blue, 5 mL of 2-

mercaptoethanol solution, and 10 mL of distilled water. A protein sample (10 μL) and marker (ExcelBand™) were loaded into each well, and the separation process was conducted by electrophoresis (100 V, 180 min). Protein bands were stained by coomassie blue 0,1% staining solution for 3 h. The gel was subsequently submerged into a de-staining solution 40% for 24 h. The estimated molecular weight of the protein was based on a comparison with common molecular weight markers [14].

Binding inhibition of RBD-ACE2 interaction. Evaluation of the blocking or inhibition capacity was performed using the cPass SARS-CoV-2 neutralization antibody detection kit (GenScript®, New Jersey, US). This kit allows rapid detection of total neutralizing antibodies (NAbs) in a sample by mimicking the interaction between the virus and the host cell. For a virus to infect a host cell, a viral RBD protein first needs to interact with the host cell's membrane receptor protein (ACE2). The bottom of the microplate well of the kit was coated with the protein hACE2, which is the receptor of the SARS-CoV-2 virus. The conjugate used in this kit is a SARS-CoV-2 RBD protein conjugated with the enzyme horseradish peroxidase (HRP), and the substrate is tetramethylbenzidine (TMB).

The test phase was initiated by mixing the sample and the positive and negative controls with the RBD-HRP conjugate (1:1). Furthermore, 100 μL of the sample and positive and negative control, which had been mixed with conjugate, were added to each microplate well and then incubated for 15 min at 37 °C. After incubation, the washing step was continued four times using a 260 μL wash solution. The next step was to add 100 μL of substrate solution and then incubate again for 15 min at a temperature of 20 °C – 25 °C. After incubation, the reaction was stopped by adding 50 μL of stop solution, and the optical density was then read using a spectrophotometer or ELISA reader with a wavelength of 450 nm.

The positive and negative cutoff values for SARS-CoV-2 neutralizing antibody detection were used for the interpretation of the inhibition rate through the following formula:

$$Inhibition = \left(1 - \frac{ODsample}{ODnegativecontrol}\right) \times 100\%$$

Samples presenting a cutoff greater than or equal to 30% were considered positive (SARS-CoV-2 neutralizing antibody detected), whereas those with less than 30% were considered negative.

2.3. Data analysis

All data obtained from this study were analyzed descriptively.

3. Results

3.1. Specific IgY in serum and egg yolk

The specific antibodies were determined using the ID Screen® SARS-CoV-2 Double Antigen Multi-Species ELISA kit (ID.Vet), which is only targeting nucleocapsid (N) antibodies. The presence of specific N-IgY was detected in serum and egg yolk at 1 week p.i.i and 4 weeks p.i.i., respectively. All laying hens showed specific IgY in serum; however, only two hens showed specific IgY in egg yolk. IgY peaking at 4 weeks p.i.i in serum showed an S/P ratio of 538.9, whereas IgY at 5 weeks p.i.i in egg yolk showed an S/P ratio of 375.87. Specific N-IgY in serum last until 15 weeks p.i.i, whereas in egg yolk, they only last until 9 weeks p.i.i. (Figs. 1 & 2).

3.2. Characterization of IgY

SDS-PAGE analysis of purified IgY from two hens revealed the presence of three protein bands with molecular weights of 180.65 kDa, 69.75 kDa, and 27.52 kDa, respectively, indicating the presence of whole IgY (180.65 kDa), Fc heavy chain (69.75 kDa), and Fab light chain (27.52 kDa) (Fig. 3). We performed IgY purification from the egg yolks of two hens separately, and concentrations of 49.48 mg/mL and 18.92 mg/mL were obtained.

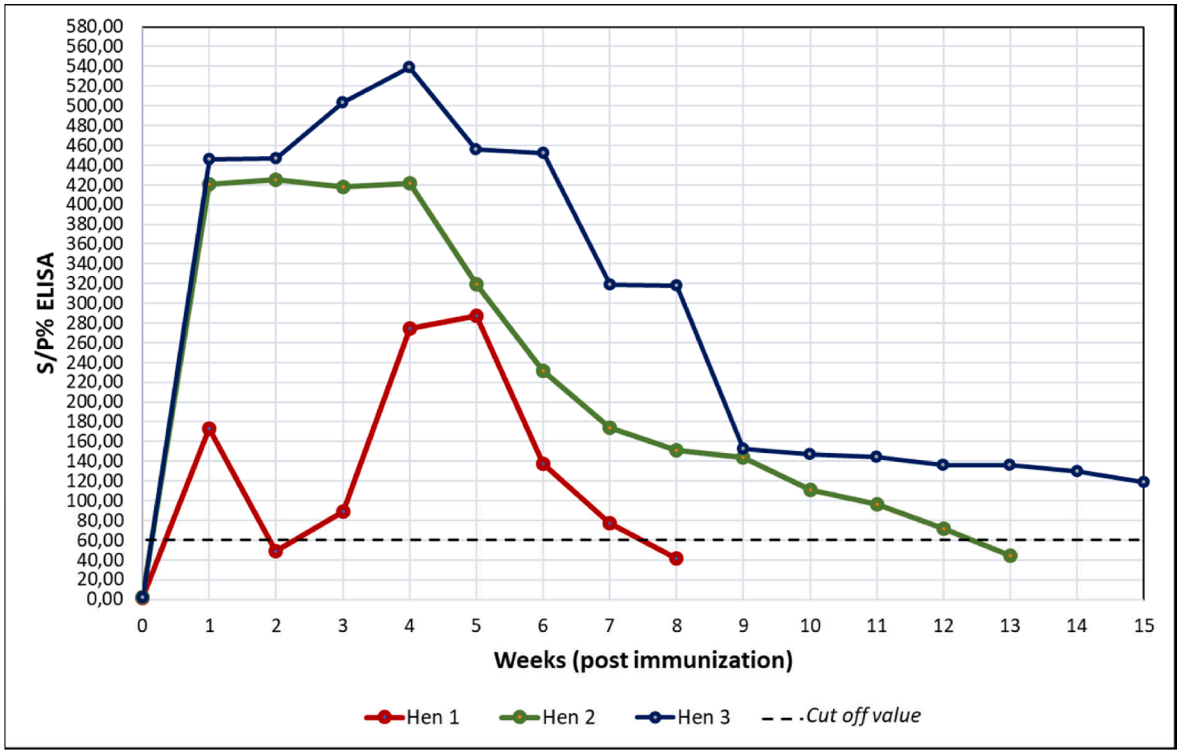

Fig. 1. ELISA S/P% of nucleocapsid antibodies in serum; initial immunization at week 0, first booster at week 1, and second booster at week 3.

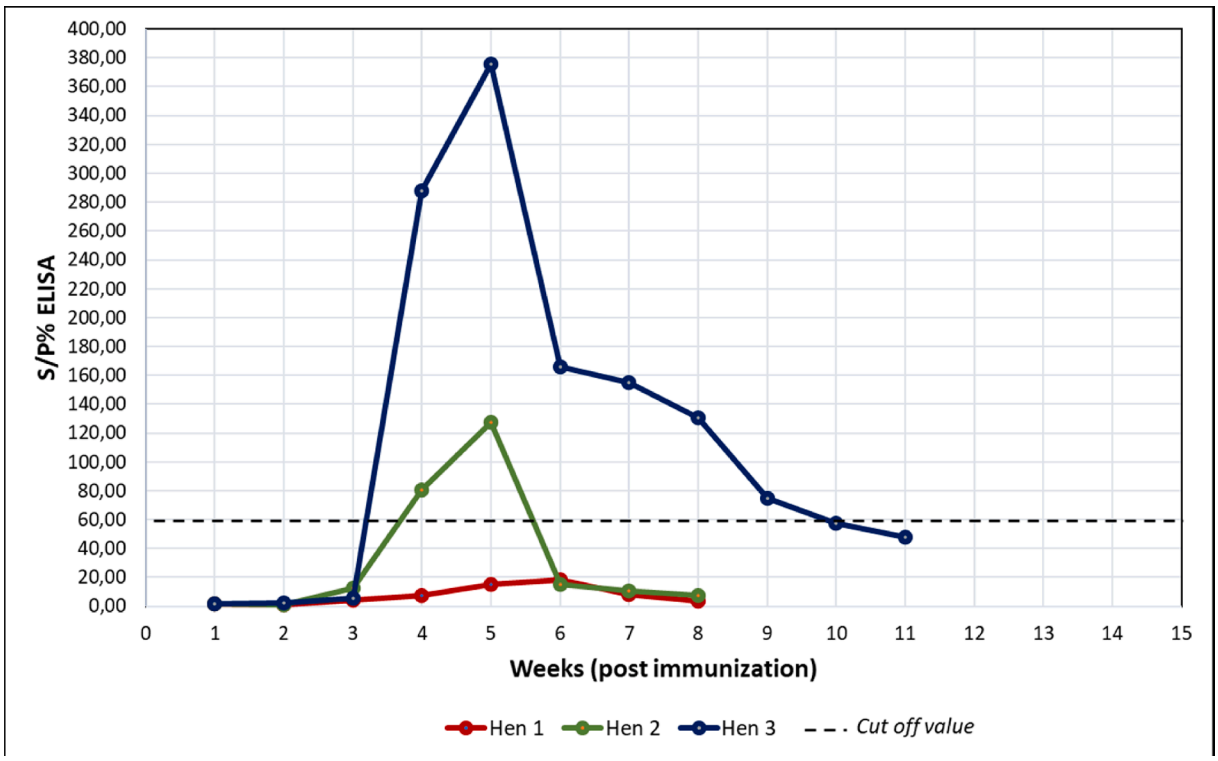

Fig. 2. ELISA S/P% of nucleocapsid antibodies in egg yolk; initial immunization at week 0, first booster at week 1, and second booster at week 3.

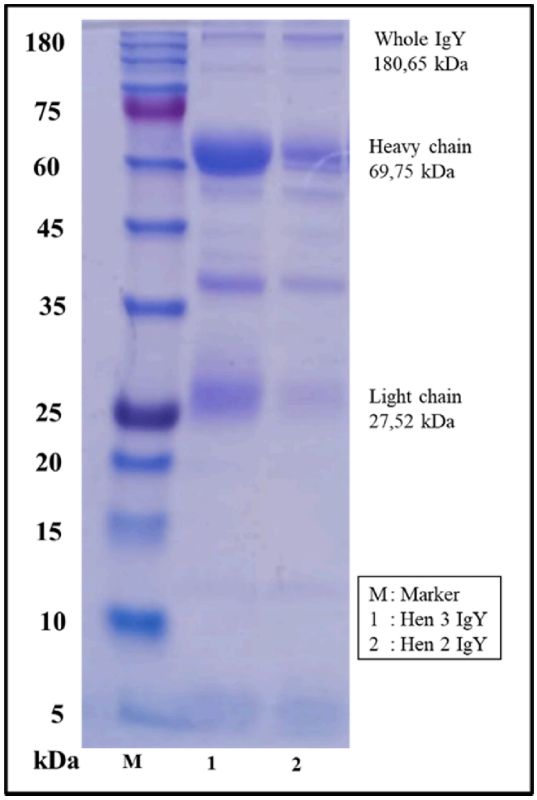

Fig. 3. Profile of the specific S1,RBD,N-IgY based on the SDS-PAGE method. M: Markers; 1: Hen 3 IgY; 2: Hen 2 IgY.

3.3. Binding inhibition capacity

Evaluation of the potential neutralization capacity showed that specific S1,RBD,N-IgY in serum could inhibit the RBD-ACE2 binding interaction, starting at 4 weeks p.i.i with concentrations of 990.42 U/mL, whereas neutralizing specific S1,RBD,N-IgY could be detected until 15 weeks p.i.i. (Table 1). Purified specific S1,RBD,N-IgY from egg yolk of two hens showed titer neutralization of 1009.45 U/mL and 1024.98 U/mL with a percent inhibition of 70.63% and 76.531%, respectively. A positive correlation was found between the neutralizing S1,RBD,N-IgY concentration and the inhibition value (%). Furthermore, the greater the inhibition value (%), the higher the concentration of the neutralizing S1,RBD,N-IgY (Fig. 5).

4. Discussion

SARS-CoV-2 is a positive-stranded virus (+ssRNA) belonging to the Coronaviridae family (order Nidovirales). SARS-CoV-2 is known to be the cause of the Coronavirus disease 2019 (COVID-19) pandemic, with 535,863,950 confirmed cases of COVID-19, including 6,314,972 deaths on 15 June 2022 [15]. Five variants of the SARS-CoV-2 virus have been identified since the beginning of the pandemic, namely the alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2), and omicron (B.1.1529) [16,17]. Continuous viral mutations will most likely affect the efficacy of the current vaccines. In addition, we know that most vaccines can only reduce the severity of the disease and do not prevent infection. Under these circumstances, it is necessary to devise and introduce novel prophylactic and therapeutic interventions for COVID-19, such as the IgY produced by laying hens. In our study, recombinant proteins were chosen as antigens due to low-risk consideration, recalling that SARS-CoV-2 is a virus that causes high morbidity and mortality rates.

We used S1 & RBD spike glycoprotein and N protein as the recombinant antigen. Among the structural proteins of *coronavirus*, the spike protein has an RBD, which that mediates virus attachment to the host cell, as a primary antigenic target of a vaccine neutralized by an

Table 1
Inhibition capacity of specific S1,RBD,N-IgY in serum.

Week th-[a]	Number of laying hens								
	1			2			3		
	Inhibition value (%)	Interp.	Conc. (U/mL)	Inhibition value (%)	Interp.	Conc. (U/mL)	Inhibition value (%)	Interp.	Conc. (U/mL)
1	−16,74	−	850,04	−21,85	−	839,68	−1,50	−	880,94
2	−14,19	−	855,21	−11,86	−	859,93	−16,22	−	851,10
3	11,26	−	906,83	65,54	+	1016,91	62,24	+	1010,21
4	52,48	+	990,42	82,13	+	1050,56	95,42	+	1077,51
5	76,65	+	1039,45	78,38	+	1042,95	95,72	+	1078,12
6	71,17	+	1028,33	69,14	+	1024,22	94,59	+	1075,84
7	65,69	+	1017,22	66,67	+	1019,20	90,17	+	1066,85
8	69,37	+	1024,68	61,64	+	1009,00	80,78	+	1047,82
9	64,19	+	1014,17	60,44	+	1006,56	80,93	+	1048,13
10	62,46	+	1010,67	56,38	+	998,34	63,29	+	1012,34
11	65,09	+	1016,00	46,47	+	978,24	53,98	+	993,46
12	60,44	+	1006,56	48,27	+	981,89	54,28	+	994,07
13	48,80	+	982,96	33,56	+	952,05	46,17	+	977,63
14	42,19	+	969,56	32,28	+	949,46	48,95	+	983,26
15	41,52	+	968,19	31,01	+	946,87	47,67	+	980,67

[a] Post-initial immunization (p.i.i), boosters were administered at weeks 1 and weeks 2.

antibody [18,19]. In this study, immunization was initiated with hyperimmune treatment by injecting antigen for three consecutive days intravenously, with two concurrent booster immunizations given subcutaneously (SC) to achieve a hyperimmune condition [20,21]. Initial immunization promotes primary immunity by introducing antigens to B and memory cells, and the intravenous (IV) route was selected to accelerate this process. Furthermore, administration of the booster dose was performed by mixing the antigen with adjuvants to stimulate high amounts of specific antibodies, which have the ability to persist for longer periods of time in the body, inducing a secondary immune response[22].

In this present study, we demonstrated that IgY targeting S1, RBD spike glycoprotein, and N protein of SARS-CoV-2 could be obtained from egg yolk of laying hens after three immunizations. This in turn yielded a concentration of pure IgY that were as high as 49.48 mg/mL and 18.92 mg/mL, respectively, which is in accordance with the findings of a previous study showing that IgY concentration can vary among individuals [23].

However, one of three laying hens used in this study demonstrated delayed production of specific IgY production in serum, and this hen was not able to transfer IgY to the egg yolk. This phenomenon might happened due to the different adjuvant used. The laying hens were boosted using an antigen mixed with SEPPIC adjuvant, that appeared to experience a slower increase in antibody S/P compared to the other two hens, which were boosted with the Freund's incomplete adjuvant. Freund's complete adjuvant is generally recommended as the first booster immunization due to its significant immune-stimulating effect [24]. In contrast, the Freund's incomplete adjuvants or adjuvants with a similar type of water-in-oil emulsion, one of which is SEPPIC MONTANIDE™ ISA 71 R VG., is commonly used as the next booster immunization step. SEPPIC adjuvants are characterized by a slower rate of inducing immunity compared to Freund's incomplete adjuvants, yet the immunity induced appears to be long-lasting [25]. An additional explanation for this might be the distinct difference in the concentration of the emulsifying active substance in the two adjuvants, which reached 10% in SEPPIC and approximately 3% in Freund's incomplete [26], thus potentially affecting the stability of the antigen depot with a slower antigen release mechanism. Consequently, it is hoped that the antigen can be introduced into the lymphatic system and immune effector cells more thoroughly [27]. Other explanation would be, the failure of chicken's immune system to transfer its antibody to the yolk, may be due to individual condition. However in this study we do not analyze the cause, since it is not our purpose of this study.

The results of the SDS-PAGE analysis revealed three protein bands with molecular weights of 180.65 kDa, 69.75 kDa, and 27.52 kDa, respectively, indicating the presence of whole IgY, Fc heavy chain, and Fab light chain. The whole chicken IgY has a molecular mass of 180 kDa, consisting of two heavy chains (67–70 kDa each) and two light chains (about 26 kDa each) [28], and its greater molecular mass is due to the increased number of heavy chain constant domains and carbohydrate chains [29].

Several studies have evaluated the potential of SARS-CoV-2 IgY [30,31,32], and confirmed the production of specific IgY that could target spike glycoprotein, S1 subunit, and S1 RBD. All studies demonstrated promising results with respect to IgY neutralizing activity against SARS-CoV-2, and our results are consistent with the findings of these studies. The present study confirmed that S1,RBD,N-IgY in serum and egg yolk has the potential to neutralize the SARS-CoV-2 virus (Table 1 and Figs. 4 and 5). Purified S1,RBD,N-IgY also demonstrated the ability to inhibit the binding interaction between RBD and ACE2 receptor. Furthermore, a positive correlation was found between the neutralizing S1,RBD,N-IgY concentration and the inhibition value (%). Specific S1, RBD,N- IgY binds to the S1 subunit, RBD, and neutralized spike glycoprotein, blocking their binding interaction to the ACE2 receptor and preventing infection. These findings are consistent with the results of a previous study published by Bao *et al.* [31], in which the authors demonstrated the potential of the specific IgY of the recombinant S1 protein in blocking the virus from entering the host. Based on our results, as well as the previous study, we suggest that the specific IgY can reliably provide protection against COVID-19.

Our present study has certain limitations. First, this study was designed as a preliminary study, hence a small number of laying hens was used, and we only performed three immunizations with a limited observation time. Consequently, we produced a specific IgY, which substantially limits the generalizability of our findings. Furthermore, we used comercially-laying hens, which although vaccinated, they were raised with minimum biosecurity standards, as opposed to using specified pathogen-free chicken raised floor-litter pens with maximum biosecurity. Therefore, the possibility of other pathogens affecting these laying hens cannot be excluded. Our study used recombinant proteins as an antigen, which might not be a cost-efficient approach for mass IgY production. However, this could be addressed by using a "relatively cheaper" antigen. Finally, our study did not evaluate the capacity of IgY in terms of inhibiting viral replication due the limited time available, and therefore future in vitro studies are warranted to address this issue.

5. Conclusion

This present study underlines the ability of a laying hen immune system to produce polyclonal specific IgY in the serum and egg yolk.

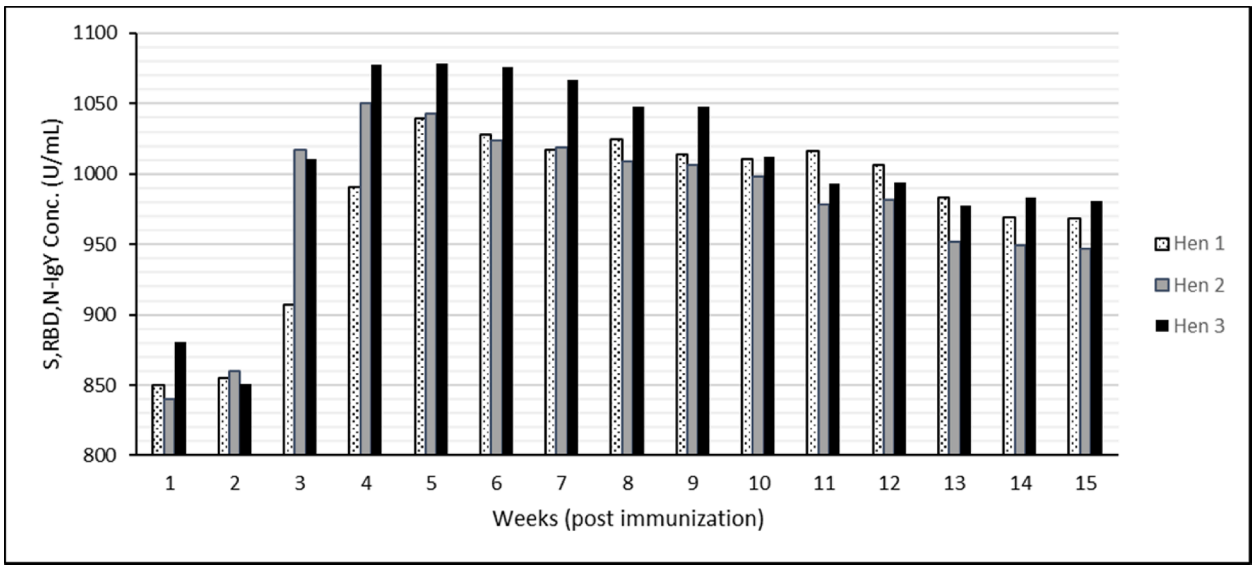

Fig. 4. Concentration of neutralizing specific S1,RBD,N-IgY (U/mL) 15 weeks after immunization in serum.

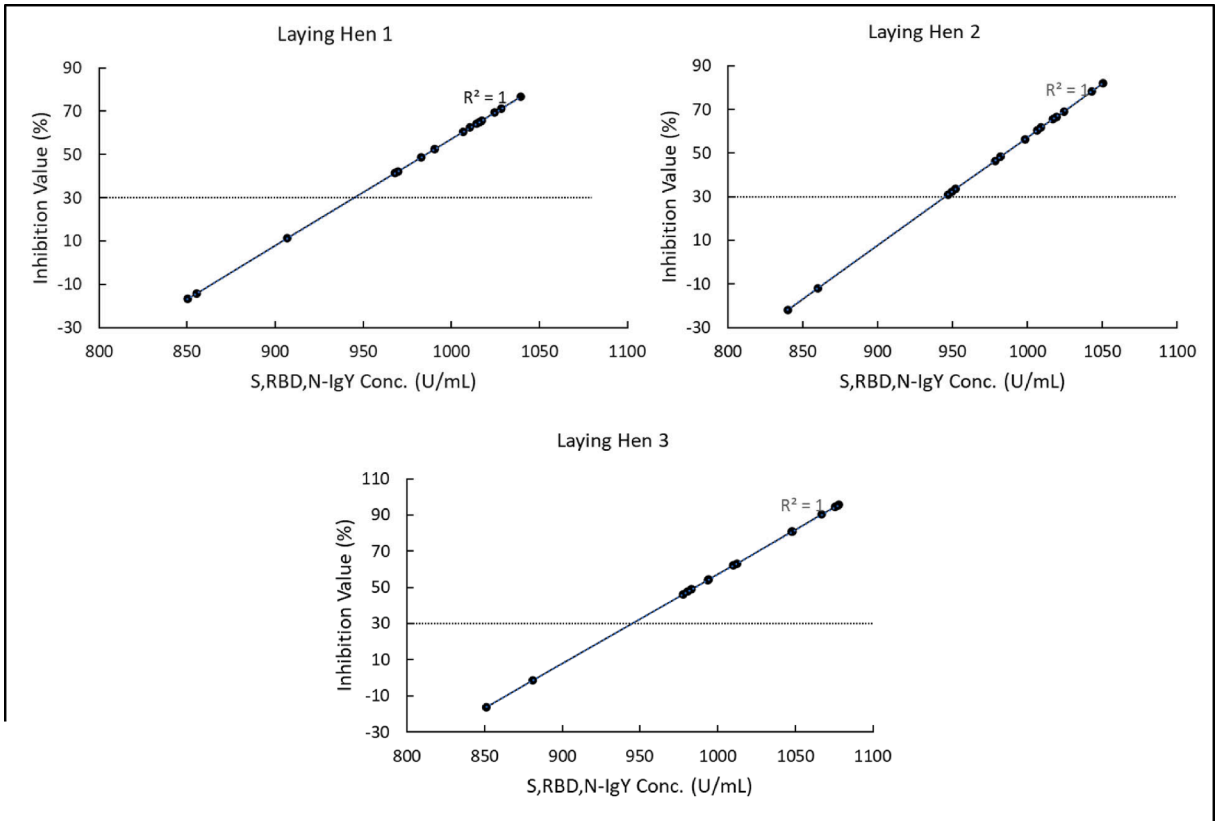

Fig. 5. Correlation of neutralizing S1,RBD,N-IgY concentration and inhibition value (%) in serum.

Recombinant S1 and RBD spike glycoprotein and N protein were able to induce specific S1,RBD,N-IgY with neutralizing capacity to inhibit the binding interaction between RBD and ACE2 receptor, suggesting the feasibility of specific anti-SARS-CoV-2 IgY production for controling COVID-19.

Authorship contributions

Category 1

Conception and design of study: M.E. Saputri, R.D. Soejoedono, E. Handharyani, O.N. Poetri; acquisition of data: M.E. Saputri, O.N. Poetri, S.A.R. Effendi, R. Nadila, S.A. Fajar; analysis and/or interpretation of data: M.E. Saputri, O.N. Poetri, S.A.R. Effendi, R. Nadila, S.A. Fajar.

Category 2

Drafting the manuscript: M.E. Saputri, R.D. Soejoedono, E. Handharyani, O.N. Poetri; revising the manuscript critically for important intellectual content: M.E. Saputri, R.D. Soejoedono, E. Handharyani, O. N. Poetri.

Category 3

Approval of the version of the manuscript to be published (the names

of all authors must be listed): M.E. Saputri, S.A.R. Effendi, R. Nadila, S.A. Fajar, R.D. Soejoedono, E. Handharyani, O.N. Poetri.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

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