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Production, purification, and specificity serologically determination of immunoglobulin-Y (IgY) from chicken eggs against *Clostridium tetani* toxoid

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Abstract: Tetanus is a life-threatening bacterial disease in humans and many animal species caused by the neurotoxin tetanospasmin produced by *Clostridium tetani*. Antitoxins obtained from horses and humans are primarily used to treat this disease. However, there are several clinical side effects and disadvantages associated with the use of these antitoxins. Current techniques for diagnosing tetanus use monoclonal antibodies produced in mice. These antibodies have several advantages, such as their homogeneity and specificity. In contrast, a notable feature of polyclonal antibodies, especially egg yolk antibodies, i.e. immunoglobulin-Y (IgY), extracted from poultry, is that they can be generated in greater quantities than mammalian antibodies (IgG). In this study, 22-week-old chickens were immunized with *C. tetani* toxoid and adjuvant (Freund's complete and incomplete adjuvants) via injection into the chest muscle. The immunization process was completed by administering two booster injections at 4-week intervals. Total antibody titers were observed to reach their highest level in the serum of blood samples taken 14 days after the last immunization. IgY antibodies were isolated noninvasively from the eggs of immunized and nonimmunized chickens using the polyethylene glycol (6000) extraction protocol. Immunological analyses confirmed that the purified IgY antibodies were produced specifically for the *C. tetani* toxoid. The specific tetanus antibodies obtained in this study may be valuable therapeutic tools as alternatives to current treatments for tetanus in humans and domestic animals.

Key words: IgY antibody, tetanus, yolk antibodies, poultry

1. Introduction

Tetanus is an acute and sometimes fatal disease of the central nervous system caused by the toxin *Clostridium tetani* in humans and animals. Spores of these gram-positive microorganisms occur naturally in soil, polluted water, and animal feces, regardless of the geographic location [1]. *C. tetani* spores that enter organisms through open wounds pass into vegetative media under appropriate anaerobic conditions. These neurotoxins enter tissues, reach the lymphatic system, and are subsequently transferred to the central nervous system via the nervous system or bloodstream. The toxin blocks inhibitory neurotransmitters, causing the typical muscle stiffness and spasms associated with tetanus. The mortality rate reaches 100% in untreated patients [2]. Tetanospasmin (TeNT) neurotoxin is not generally found in the blood serum of patients diagnosed with tetanus. This is because very low TeNT levels are sufficient for developing disease symptoms [3]. When tetanus risk is suspected, prophylactic treatments are needed, including wound debridement and cleaning, antibiotic use, and TeNT immunoglobulin (Ig) injection. The Igs used prophylactically can neutralize only the circulating toxins since they cannot cross the blood-brain barrier. Based on clinical signs, rapid passive vaccination with equine antitetanus serum or hyperimmune human tetanus Ig is prescribed [4,5]. Antibodies used in passive immunization are protein molecules produced in response to pathogens. The ability of these peptides to bind easily to antigenic molecules has enabled them to be frequently used in many studies for diagnosis and treatment [6]. Laboratory animals such as rabbits, rats, mice, and guinea pigs or larger mammals such as horses, sheep, and goats are widely used for polyclonal antibody production. However, the production of these polyclonal antibodies includes practices that cause pain, such as prolonged bloodletting [7]. In recent years, isolating antibodies from egg yolk following hyperimmunization has emerged as a popular approach for producing significant amounts of antibodies. The method utilizes the principles of both natural and artificial

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passive immunity. Chickens are regularly immunized with specific antigens to ensure a continuous accumulation of antibodies in the egg yolk. The antibodies extracted from the egg yolk are subsequently used for human or animal immunotherapy and immunodiagnostic purposes. The antibacterial properties of chicken antibodies (IgY) have been one of the main areas of interest in IgY studies. Many reports have shown that IgYs exert immune function by preventing bacterial transmission or infection in vivo [8–14]. The phylogenetic distance between chickens and mammals makes producing yolk antibodies against generally conserved mammalian antigens more successful than in mammals [14]. In addition, these antibodies target more antigenic epitopes and recognize the same proteins in several species, making them more widely useful [15]. This study aimed to isolate anti-*C. tetani* IgY antibodies that could be used as an alternative to the current practice in diagnosing and treating tetanus toxin using IgY technology and confirm that they are specific for the toxin.

2. Materials and methods

2.1. Experimental animals

This study used 25 chickens from the pure lines of broiler breeding chickens within the Eskişehir Transition Zone Agricultural Research Institute, Poultry Production and Feeding Department. Ethical approval to conduct this study was granted by the Eskişehir Osmangazi University Animal Experiments Local Ethics Committee dated 05/06/2021, numbered 843.

2.2. Toxoid

To immunize randomly selected chickens, the Cloteid 4 inj commercial vaccine, which is stated to contain 30 IU of tetanus toxoids per 1 mL in the package insert, was used as the tetanus toxoid. For this, 1 IU of tetanus toxoid was defined as 0.03384 mg [16].

2.3. Adjuvants

Freund's complete adjuvant (FCA, 5881, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and Freund's incomplete adjuvant (FIA, F5506, Sigma-Aldrich Chemie GmbH) were used to stimulate high antibody titers.

2.4. Immunization of the chickens

First, 1 mL of blood was taken from the wing vein for serological monitoring before and 14 days after each immunization from each experimental animal, aged 22 weeks. The serum was then separated from the blood, labeled with the experimental animal code, and stored at –18 °C. The chickens in the experimental group were injected with intramuscular antigen 3 times and once every 4 weeks. As shown in Table 1, to administer 150 µg/mL of toxin to the experimental groups, 167 mg of vaccine was combined with 333 mg of phosphate-buffered saline (PBS) and an equal volume of FCA, 500 mg. A total of 1 mL of antigen + adjuvant solution was mixed by vortexing and injected into 4 different regions of the chest muscles. After the first immunization, in the second and third injections, the same amount of toxoid and PBS mixture was combined

Table 1. Immunization procedure.

	Adjuvant used	Dose	Total volume	Application path	Age	Vaccination group
C. tetani toxoid + adjuvant	FCA	0.167 mL vaccine (150 µg/mL toxoid) + 0.333 mL PBS + 0.5 mL adjuvant	1 mL	Intramuscular	22 weekly	Experimental group
C. tetani toxoid + adjuvant	FIA	0.167 mL vaccine (150 µg/mL toxoid) + 0.333 mL PBS + 0.5 mL adjuvant	1 mL	Intramuscular	26 weekly	Experimental group
C. tetani toxoid + adjuvant	FIA	0.167mL vaccine (150 µg/mL toxoid) + 0.333 mL PBS+ 0.5 mL adjuvant	1 mL	Intramuscular	30 weekly	Experimental group
Adjuvant + PBS	FCA	0.5 mL FCA+ 0.5 mL PBS	1 mL	Intramuscular	22 weekly	Control group
Adjuvant + PBS	FIA	0.5 mL FIA+ 0.5 mL PBS	1 mL	Intramuscular	26 weekly	Control group
Adjuvant + PBS	FIA	0.5 mL FIA+ 0.5 mL PBS	1 mL	Intramuscular	30 weekly	Control group

with an equal volume of FIA, a total of 1 mL of antigen + adjuvant solution was injected into 4 different regions of the chest muscles, and immunization was completed. The immunization process was completed by injecting 500 mg of PBS and 500 mg of either FCA or FIA into the chest muscle of the experimental animals in the control group to a total volume of 1 mL. Eggs were collected 1 week after the completion of the immunization process, and the eggs collected for 2 months (eggs/day) were labeled daily and stored at 4 °C until the IgY isolation procedure.

2.5. Isolation of IgY from the egg yolk

IgY was isolated using polyethylene glycol (PEG) 6000 (Sigma-Aldrich Chemie GmbH) precipitation following the protocol outlined in the Polson method [17]. In the first stage, eggs collected from immunized and nonimmunized chickens were broken, and the yolk and white were separated using a spoon. PBS, twice the volume of the egg yolk, was transferred to a Falcon tube. For the elimination of lipids and lipoproteins, 3.5% (w/v) PEG 6000 was added to the Falcon tube, which was vortexed and rolled by hand for 10 min. Then, the Falcon tubes were centrifuged at 7500 rpm at 4 °C for 30 min. The supernatants formed in the Falcon tubes were filtered through folded filter paper placed in the funnel and transferred to a new Falcon tube. Final protein precipitates were obtained after PEG 6000 precipitation/centrifugation at 8.5% (w/v) and 12%, respectively, to precipitate the total IgY from the supernatant of the first step in the second step. The last pellet was dissolved in 800 µL of PBS by careful vortexing using a glass pallet with the lid open and then transferred to the microdialysis capsule of Slide-A-Lyzer Dialysis Cassettes (No: 66012; Thermo Fisher Scientific Inc., Waltham, MA, USA). The mixture was dialyzed overnight on a magnetic stirrer in 0.1% sodium chloride (NaCl) solution in the capsule. The following day, the saline was replaced with PBS and dialyzed for another 3 h. When dialysis was completed, the final volume of IgY extract was transferred to an Eppendorf tube and stored at 4 °C. The protein concentration of the isolated IgY antibodies was measured via the Bradford protein assay method [18] using the Coomassie Plus Protein Assay Reagent. The 2 mg/mL of bovine serum albumin (BSA) concentration obtained from the protein measurement kit was used as a standard. The resulting blue color was measured using a Chromate 4300 microplate reader (Awareness Technology, Palm City, FL, USA) at 595 nm, and the amount of protein in the samples was calculated using a 4-parameter logistic regression analysis according to the standard curve created.

2.6. Immunological analysis

2.6.1. Total antibody titration

After the immunization process was completed, the components of the enzyme immunoassay (ELISA) Kit (Catalog MBS743332; MyBioSource, San Diego, CA, USA) (standards, conjugate, substrate A, substrate B, and stop solution) and the sera taken before and after each vaccination were brought to room temperature (20–25 °C). A total of 150 serum samples were diluted as specified in the manufacturer's instructions. Then, 100 µL of the standard was added to the first 6 wells (1A–1F) of the ELISA plates, and PBS was added to well 7 (blind control). Well 8 was left empty. Starting from well 2A, the serum samples were placed in the wells. In addition to PBS, 50 µL of conjugate was added to each well, mixed well, and incubated at 37 °C for 1 h. The plate was washed 5 times with the diluted wash solution in an automatic washer and dried. Then, 50 µL of substrate A and 50 µL of substrate B were added to each well, including the blank control well, and incubated at 37 °C for 15–20 min. Next, 50 µL of stop solution was added to each well, including the blank control well, and mixed well. The optical density of the samples was determined by measuring the absorbance at 450 nm spectrophotometrically in a microplate reader. The optical density values obtained were calculated according to a standard curve using the calculation method specified in the manufacturer's instructions.

2.6.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Bolt 4%–12% Bis-Tris Plus gel (Thermo Fisher Scientific Inc.) with different acrylamide concentrations was used for the SDS-PAGE. Samples prepared from the isolated IgYs were incubated in a thermal cycler (Longgene A300; Hangzhou Longgene Scientific Instruments Co., Ltd., Zhejiang, China) at 70 °C for 10 min and maintained at 4 °C for a while at the end of the incubation. The Invitrogen SeeBlue Plus 2 prestained standard ladder (Thermo Fisher Scientific Inc.) was used as a protein molecular weight determinant. The marker in well 1 and the IgY samples in the other wells were loaded carefully. Then, approximately 30–35 min of execution was carried out at 190 V. After washing, the gel was imaged using a BluPAD Dual LED blue/white light transilluminator (Topgen Biotechnology Co., Ltd., Zuoying District, Kaohsiung City, Taiwan).

2.6.3. Western blot

An IBlot 2 dry blotting system (Thermo Fisher Scientific Inc.) was used to transfer the prepared SDS-PAGE gel to the nitrocellulose membrane. The gel was removed from the chamber and transferred to a nitrocellulose membrane within 7 min at 20 V by electroblotting under an electric field using the membrane transfer system (IBlot 2 Transfer Stacks) of the IBlot 2 device. After the transfer process, the gel on the membrane was cut off and kept in the prepared blocking solution for 5 min. Antibody administration was performed on an Invitrogen iBind Flex Western instrument (Thermo Fisher Scientific Inc.). As a confirmation antibody,

rabbit antichicken IgY (H+L) secondary antibody (HRP; Thermo Fisher Scientific Inc.) was used at a concentration of 1/10,000 as recommended by the manufacturer. The binding of IgY to secondary antibodies specific to the iBind Flex was assessed within 2.5 h. Then, the iBind Flex card was wetted with 10 mL of blocking solution, and 1 mL of the same solution was added to the area where the membrane was placed. The membrane was placed upside down on the card, fixed by going over it with a roller, and left in the device for 3 h. A working solution was prepared by adding 1 mL of peroxidase to 1 mL of luminol, after which the removed membrane was placed in the solution and incubated for 1 min. Then, western blot images of the membranes were recorded with the imagER Eyes program using a chemiluminescence (GenBox, ERBiotek, Ankara, Türkiye) imaging system.

2.6.4. Agar gel immunodiffusion assay (AGID)

The Cloteid 4 inj commercial vaccine was used as an antigen in the agar gel immunodiffusion test. Blood serum collected 14 days after the last vaccination was used as a positive control. IgYs isolated from the eggs of the experimental animals in the control group that were not given toxoids were used as a negative control. Because IgY antibodies do not have a hinge region, the flexibility of these molecules is limited. Therefore, they cause precipitation or agglutination only with high salt concentrations [19]. For this reason, the salt concentration that would provide the best precipitation of the agar to be used in the agar gel immunodiffusion test was determined. For this purpose, noble agar (A5431, Sigma-Aldrich Chemie GmbH) was prepared according to the method reported by the Office International des Epizooties (OIE) (2000) with NaCl concentrations of 8%, 12%, 16%, and 20%. The salt density of the agar used in the experiment was determined by determining the agar with the best precipitation among the agars prepared at different salt concentrations. After the agar was frozen, the porcelain beads were removed from it using forceps, and the appropriate wells were opened. The Cloteid 4 inj commercial vaccine was added to the middle of the prepared wells. Positive and negative controls and the IgY antibodies isolated from the experimental group were added to the surrounding wells. The petri dishes were incubated for 48 h at 37 °C in a suitable closed box in a humid environment. The petri dishes were examined with a single-focus light source on a dark background in a dark environment, after which the precipitates were checked.

2.6.5. Quick slide agglutination test

In the quick slide agglutination test, 10 µL of the Cloteid 4 inj commercial vaccine was dropped onto the slide for each test group as an antigen. The exact amount (10 µL) of *C. tetani*-specific IgY obtained from the experimental groups was added. IgYs and tetanus toxoids isolated from the control group were added in the same amount just below the experimental group on the same slide to be tested as a negative control. Observation of the typical aggregation within a few minutes was considered positive. A mixture with a homogeneous structure was evaluated as unfavorable.

3. Results

3.1. Clinical monitoring and immunization effects on chicken biology

All of the chickens were healthy throughout the experiment. No pain or discomfort was observed upon palpation, and no tissue damage was observed at the immunization site. No differences in growth kinetics were observed between the experimental and control groups.

3.2. Immunization findings in the blood serum

The total antibody titers increased after each vaccination in the experimental group immunized individually with *C. tetani* toxoid + adjuvant (FCA/FIA). The total antibody titers in the serum of blood samples taken 14 days after the last vaccination, as shown in Figure 1, reached the highest level. On the other hand, no significant change was observed in the total antibody titers of the control group given adjuvant (FCA/FIA) + PBS.

3.3. Protein concentration of the isolated anti-C. tetani IgY

The findings of the Bradford protein determination method [18] showed that the experimental groups had higher protein concentrations than the control group, as shown in Tables 2 and 3.

3.4. Immunological analyses

IgY samples loaded on the gel were examined via SDS-PAGE analysis. Bands with molecular weights between approximately 60 kDa (IgY heavy chain) and 25 kDa (IgY light chain), as shown in Figure 2, were visualized on the gel. To confirm the SDS-PAGE results, western blot analysis was performed, and the proteins were transferred to nitrocellulose membranes, which were visualized via a chemiluminescence imaging system using the imaging software program. Images obtained via western blot analysis, as shown in Figure 3, confirmed the presence of the isolated IgY antibodies.

3.5. Serological confirmation of the specificity of the anti-*C. tetani* IgY

An AGID test was performed to confirm that the IgY antibodies were anti-C. tetani IgY. In the test, noble agar samples with different salt concentrations were prepared according to the method reported by the OIE (2000). The best precipitation was observed in agar prepared with 12% NaCl; thus, agar with this salt concentration was used for the test. As shown in Figure 4, as a result of the AGID test,

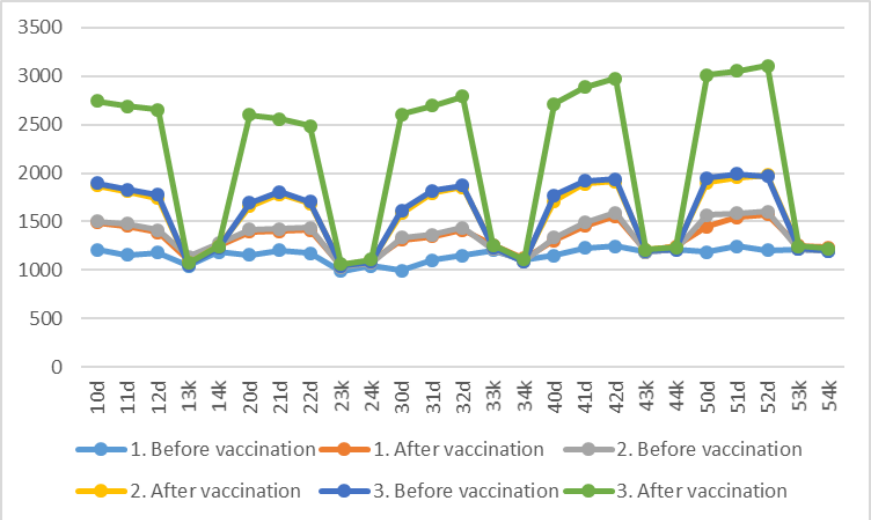

Figure 1. Changes in the total antibody titers after immunization. d: Experiment, k: control, A1: dam line 1 (10d–14d), A2: dam line 2 (20d–24k), A3: dam line 3 (30d–34k), B1: sir line 1 (40d–44k), and B2: sir line 2 (50d–54k).

Table 2. Bradford test results.

Sample	Absorbance	Result (µg/mL)	Sample	Absorbance	Result (µg/mL)
A Standard	1.239	2000.0	10d	1.382	4979.7
B Standard	1.215	1500.0	11d	1.519	7833.3
C Standard	1.100	1000.0	12d	1.522	7895.8
D Standard	1.055	750.0	13k	1.277	2458.4
E Standard	0.895	500.0	14k	1.078	903.6
F Standard	0.723	250.0	20d	1.379	4916.6
G Standard	0.623	125.0	21d	1.380	4937.5
H Standard	0.488	25.0	22d	1.491	7250.0
I Standard	0.464	0.0	23k	1.235	1654.7

(d): experiment, (k): control, (10,11,12,13,14): 1st dam line-A1,(20,21,22,23,24): 2nd dam line-A2, (30,31,32,33,34): 3rd dam line-A3, (40,41,42,43,44): 1st sir line-B1, (50,51,52,53,54): 2nd sir line-B2.

the images of the precipitates formed between the IgYs isolated from the experimental group and the tetanus toxoid group proved that the obtained egg yolk antibodies were produced specifically for *C. tetani* toxoid. Afterward, a rapid slide agglutination test was performed. As shown in Figure 5, within a few minutes, typical aggregations were observed to form in the experimental group and were considered positive. This finding again demonstrated that the isolated IgY antibodies were produced specifically for *C. tetani* toxoid. The mixture of the IgYs and antigens obtained from the control group in the test had a homogeneous structure and was evaluated as unfavorable.

4. Discussion

In this study, the total antibody titers increased after each immunization in the individually immunized experimental group. The results obtained from the ELISA support those of Schade et al., who reported that the antibody titer

Table 3. Bradford test results.

Sample	Absorbance	Result (µg/mL)	Sample	Absorbance	Result (µg/mL)
24k	1.180	1418.6	43k	1.053	813.6
30d	1.462	6645.8	44k	1.197	150.1
31d	1.401	6533.3	50d	1.482	7070.6
32d	1.502	7785.8	51d	1.390	5137.5
33k	1.089	903.6	52d	1.528	8318.4
34k	1.151	1251.7	53k	1.177	1403.5
40d	1.582	8109.7	54k	1.282	1878.4
41d	1.471	6909.7			
42d	1.515	7750.8			

(d): experiment, (k): control, (10,11,12,13,14): 1st dam line-A1,(20,21,22,23,24): 2nd dam line-A2, (30,31,32,33,34): 3rd dam line-A3, (40,41,42,43,44): 1st sir line-B1, (50,51,52,53,54): 2nd sir line-B2.

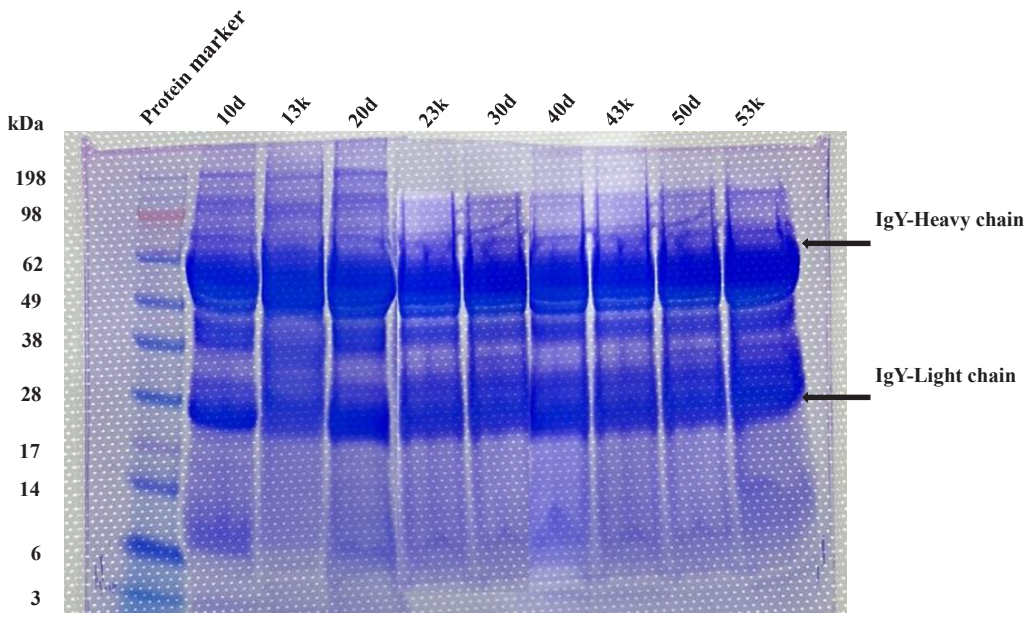

Figure 2. SDS-PAGE positive gel image. d: Experiment, k: control, protein marker: Invitrogen SeeBlue Plus 2 prestained standard ladder, A1: dam line 1 (10d), A2: dam line 2 (20d), A3: dam line 3 (30d), B1: sir line 1 (40d), and B2: sir line 2 (50d).

increased significantly after booster immunization [20]. Similarly, in a study by Fathi et al. on IgY against *E. coli* Shiga-like toxin, a significant difference in the titer of the antigen was reported between the experimental and control groups [21]. The PEG 6000 precipitation method was used herein to isolate IgY. The results showed that IgY antibodies isolated from egg yolk were specific and had stable titers. Similar results were obtained in many studies using the same method [21–23]. The protein concentrations of the isolated IgY antibodies were measured. The protein concentration was higher in the antibodies isolated from the experimental groups than in those from the control group. Similarly, in their IgY study against Rotaviruses, Bentes et al. reported that chickens

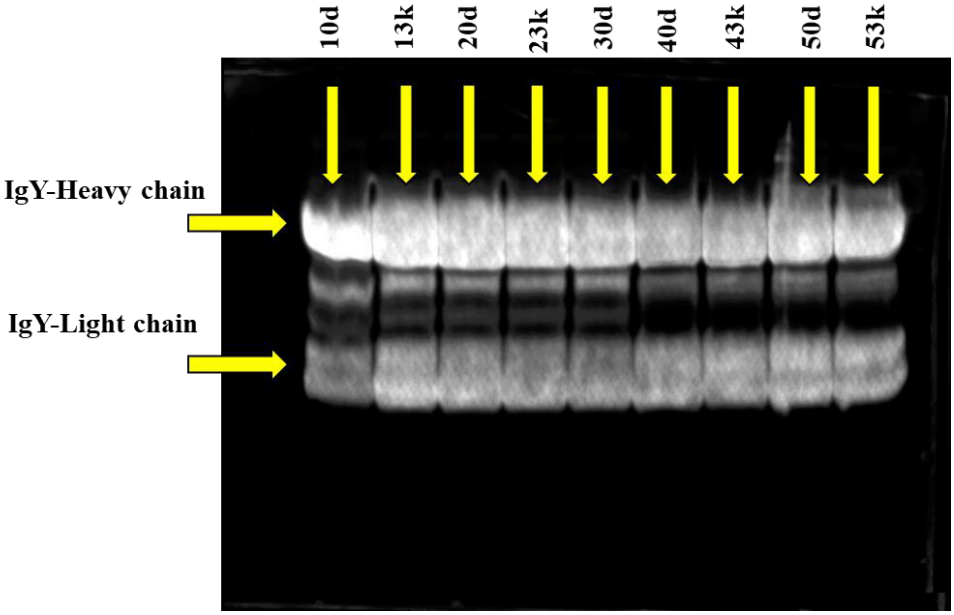

Figure 3. Western blot analysis chemiluminescence-positive image. d: Experiment, k: control, A1: dam line 1 (10d), A2: dam line 2 (20d), A3: dam line 3 (30d), B1: sir line 1 (40d), and B2: sir line 2 (50d).

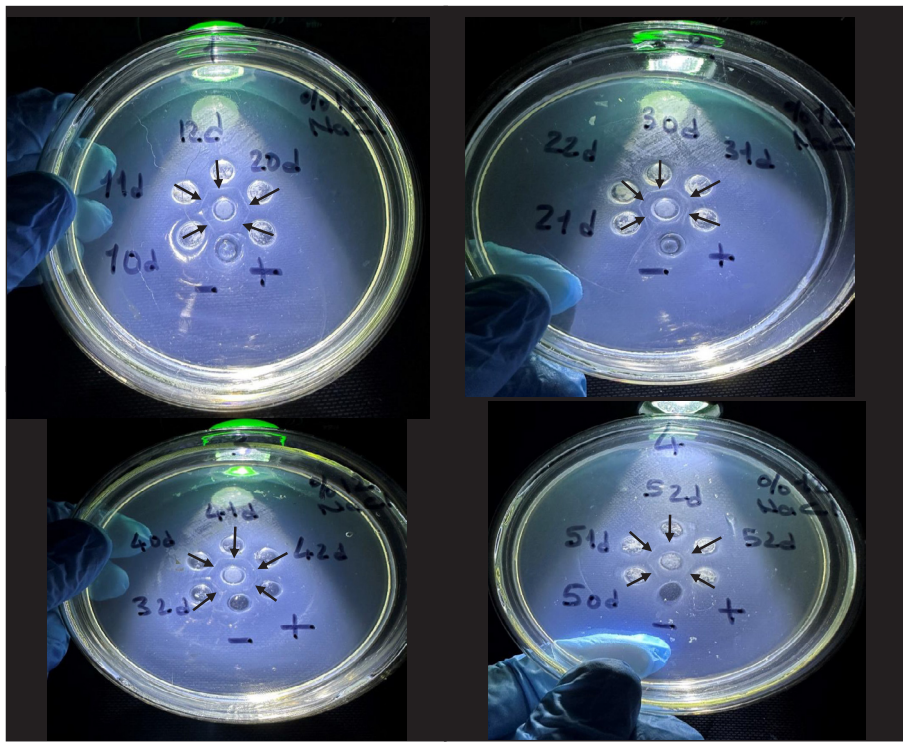

Figure 4. AGID test precipitation image. d: Experiment, k: control, A1: dam line 1 (10d), A2: dam line 2 (20d), A3: dam line 3 (30d), B1: sir line 1 (40d), and B2: sir line 2 (50d).

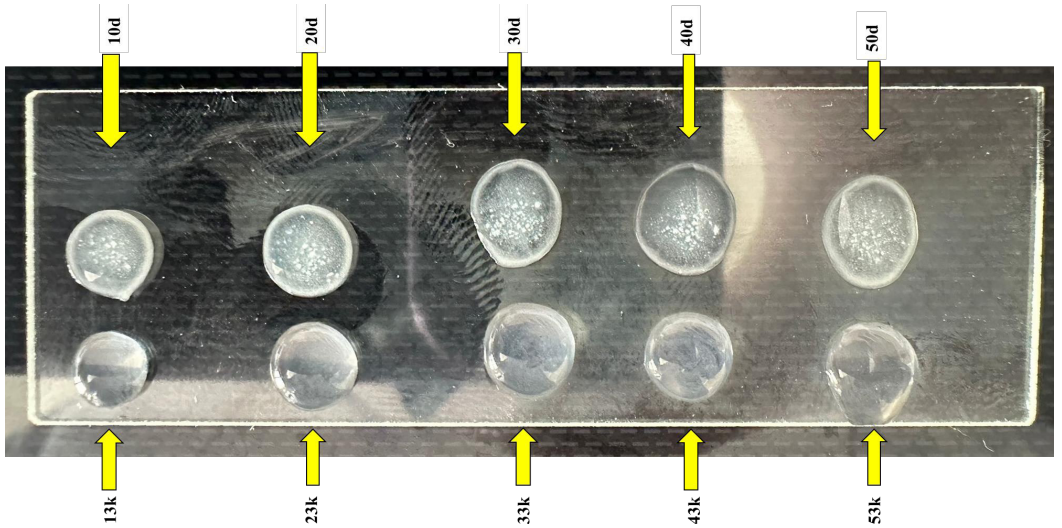

Figure 5. Quick slide agglutination image. d: experiment, k: control, A1: dam line 1 (10d), A2: dam line 2 (20d), A3: dam line 3 (30d), B1: sir line 1 (40d), and B2: sir line 2 (50d).

receiving 3 immunogenic doses had higher protein concentrations than those receiving 2 immunogenic and nonimmunogenic doses. The molecular weight of a standard egg yolk antibody is 180 kDa. This weight consists of 2 light chains of approximately 25 kDa each and 2 heavy chains of approximately 65 kDa each. In the current study, after SDS-PAGE, the IgY heavy chain was visualized in a band with a molecular weight of approximately 60 kDa. The 60 kDa band is further from the expected position on the gel. This may have been because the flow of some proteins may have been influenced by other components in the mixture, moving slightly further. However, another band weighing approximately 40 kDa was observed on the gel. It is thought that ovalbumin, a 42 kDa protein found abundantly in egg whites, contributes to the formation of this band. As a result of the AGID test, the precipitation between the IgYs isolated from the experimental group and the tetanus toxoid proved that the egg yolk antibodies obtained were produced specifically for *C. tetani* toxoid. After the AGID test, a quick slide agglutination test was performed, and it was observed that typical granules had formed in the experimental group within a few minutes. This result again showed that the isolated egg yolk antibodies were produced specifically for *C. tetani* toxoid.

5. Conclusion

Consequently, after a complete immunization period by antigen injection, IgY antibodies in the chicken blood were transferred to the egg to stimulate fetal immunity, and these antibodies could be purified from the egg yolk. These findings showed that chicken eggs are an ideal alternative source of mammal antibodies. The specific tetanus antibodies obtained from this study may be a valuable alternative therapeutic tool to current treatments for tetanus in humans and domestic animals. Moreover, a field for producing antidotes against various natural toxins could be created, and these antidotes could also be used as possible diagnostic tools.

Ethical approval

Ethical approval to conduct this study was granted by the Eskişehir Osmangazi University Animal Experiments Local Ethics Committee dated 05/06/2021, numbered 843.

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