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Original Article

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Abstract

The dog erythrocyte antigen (DEA) as a blood group antigen in dogs, especially DEA 1.1, has the highest immunogenicity. IgY is an excellent antibody for use in immunological assays involving mammalian sera. The aim of this study was to produce and purify IgY against DEA1.1 from chicken egg yolks for canine blood group detection. Hens were immunized by intramuscular vaccination. IgY was achieved after three booster immunizations by DEA 1.1-positive canine ghost cells. The extraction of total IgY from the egg yolk was carried out using precipitation by polyethylene glycol 6000 and dialysis. The purified protein and yield of IgY were 102.52 ± 0.13 mg/ml and 25.13 ± 2.45%. Electrophoretic separation of egg yolk IgY showed other bands that appeared between the heavy and light chains of IgY corresponding to molecular weights between 65 and 27 kDa. Canine red blood cells with DEA1.1 positive which agglutinated with monoclonal antibody against DEA1.1 in a Rapid Vet H detection kit reacted with polyclonal antibody against DEA1.1. Hemagglutination of three sample-polyclonal antibodies against DEA1.1 and two samples of canine red blood cell with DEA1.1 positive was shown at titer 4. The purified IgY can be standardized and relatively simple to perform, compared with standardization for blood typing. We successfully purified chicken IgY against DEA1.1 from egg yolks of immunized hens, which can be used for diagnostic reagent for canine blood group DEA1.1 detection. It is expected that IgY will play an increasing role in research and diagnosis in the future.

Keywords: dog, erythrocyte, DEA1.1, IgY, polyethylene glycol

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Introduction

Blood types are classified based on the types of antigen on the RBCs' surface that can induce the body's immune system to react. Blood types are usually present for the function of the RBC which can be in slightly different forms in different individuals (Hohenhaus, 2004). Dogs have at least six well characterized blood types, also known as dog erythrocyte antigens (DEA). The antigens are DEA1.1, 1.2, 3, 4, 5 and 7 (Kessler *et al*., 2010). The blood type considered to have most immunogenicity in dogs is DEA1.1. Acute hemolytic transfusion reactions only occur in DEA1.1 negative dogs. As these dogs do not have naturally occurring antibodies, a reaction will only be seen after the sensitization of the dog through exposure to DEA1.1 positive blood (Hale, 1995; Hohenhaus, 2004). Dogs that are negative for DEA1.1 can give blood to dogs that are DEA1.1 negative or positive but dogs that are DEA1.1 positive can only give blood safely to dogs that are DEA1.1 positive. If a DEA1.1 positive dog is used as a blood donor, the erythrocyte should only be given to a DEA1.1 positive recipient to prevent sensitization of the recipient to the DEA1.1 antigen. The frequencies of blood types and their combinations in dog populations should be considered when selecting a blood donor to avoid sensitization to antigens that could possibly cause transfusion reaction. Dogs do not have performed antibodies to other blood types therefore they are highly unlikely to have a transfusion reaction on the first transfusion (Hohenhaus, 2004).

A transfusion from a dog with a different blood type will sensitize the recipient's immune system due to the risk with subsequent transfusions. Cross-matching should be carried out for subsequent transfusions in dogs even if the transfusions occur many years later. Multiple transfusions can also be a problem. This may occur even when compatible cross-matched blood is given to a patient but where, over the subsequent few days, the recipient develops antibodies to the transfused cells. This results in erythrocyte destruction of the transfusion which may manifest as hemoglobinuria. The reaction is rarely as serious as an acute reaction (Giger *et al*., 1995; Goy-Thollot *et al*., 2017). Immunoglobulins are glycoproteins called antibodies, which are secreted by plasma cells in response to antigen exposure and are considered a product that significantly affects humoral immunity. The property of antibodies is the recognition of small specific structures on other molecules which make them an indispensable tool in laboratory in various applications such as research, diagnostics and therapy. Antibodies presently available for these purposes are mostly mammalian monoclonal or polyclonal antibodies. The production of these antibodies normally requires the use of laboratory animals. Most classically chosen mammals for polyclonal and monoclonal antibodies are rabbits and mice, respectively (Hanly *et al*., 1995). The procedure involves two steps, each of which causes distress to the animals: the immunization itself and repeated bleeding or sacrifice for spleen removal, which is a prerequisite for antibody preparation (Lipman *et al*., 2005). Commercially available immunoglobulins play a critical role in diagnostic assays, therapy and the purification of specific target compounds (Chalghoumi *et al*., 2009).

Specific IgY development and production can be achieved by immunizing laying hens with the target antigen. Immunized hens with a specific antigen such as canine ghost cells can give a specific IgY produced in the egg yolk against the given antigen. The aim of this study was to produce and purify the IgY antibodies against DEA1.1 from chicken egg yolk for canine blood group detection.

Materials and Methods

Immunization of the chickens and egg collection: For the immunization, 0.301 mg/mL of DEA 1.1-positive canine ghost cells were used. The three inoculations were administered at intervals of one week, intramuscular in the *Musculus pectorals* of the chickens (*Gallus gallus domesticus*) with a final volume of 1.0 mL, distributed at two points. After an interval of 7 days from the last inoculation, the eggs were collected daily over two weeks and stored at 4 °C until the next step, which was the extraction of IgY from the immunized egg yolk. The procedures were approved by the Institutional Animal Care and Use Committee Kasetsart University with approved protocol number ACKU04060, and care was taken to comply with the 3R concept.

Extraction of total IgY: The extraction of total IgY from egg yolk was carried out using precipitation by polyethylene glycol 6000 (PEG 6000) (Pauly *et al*., 2011). The egg shell was carefully cracked and the egg yolk was transferred to a filter paper to remove as much egg white as possible, then the yolk skin was cut with a pipette tip. The yolk was poured into a 50 ml tube and the egg volume was registered. Twice the egg yolk volume of phosphate buffer saline (PBS), containing 10 mM phosphate buffer, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4 (Amresco, USA), was mixed with the yolk and, thereafter, 3.5% PEG 6000 (w/v) (Ajax-Finechem, Thermo Fisher Scientific, USA) of the total volume was added and mixed, for 10 mins. The extraction procedure separated the suspension in two phases. One phase consisted of yolk solids and fatty substances and a watery phase containing IgY and other proteins. The tubes were centrifuged at 4°C for 20 mins (10,900 rpm according to 13,000×g, Thermo Scientific™ Sorvall™ Legend™ XTR, USA). The supernatant was poured through a folded filter and transferred to a new tube. 8.5% PEG 6000 in gram (calculated according to the new volume) was added to the tube, mixed and centrifuged (10,900 rpm) at 4°C for 20 mins. The supernatant was discarded. The pellet was carefully dissolved in 1 ml PBS by means of a vortex. PBS was added to the final volume of 10 ml. The solution was mixed with 12% PEG 6000 (w/v, 1.2 gram) and mixed. The extract was dialyzed overnight in 0.1 % saline (1,600 ml) and gently stirred by means of a magnetic stirrer. The extract was dialyzed using dialysis tubing cellulose membrane (MWCO 14,000 Da, Sigma aldrich, USA). The next morning, the saline was replaced by PBS and dialyzed for another three hours. After dialysis, 3 ml of polyclonal antibody

against DEA1.1 was concentrated by Amicon Ultra-15 centrifugal filter units (Merck Millipore, USA). The sample was pipetted from the dialysis bag and stored at -20°C.

Protein quantitation: The protein content (mg/ml) of the samples was measured photometrically at 280 nm (1:50 diluted with PBS) using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and calculated according to the Lambert-Beer law with an extinction coefficient of 1.33 for IgY.

IgY characterization by SDS-PAGE: After the isolation and purification procedures, IgY was characterized by SDS PAGE gel, using a Protean II electrophoresis system (Bio-Rad) with the discontinuous buffer system of Laemmli (1970). The IgY was diluted to 1:10 and examined in 15% SDS-PAGE under reduction conditions. The electrophoretic run was performed at 120 volts for 80 mins. For visualization, Coomassie Blue staining solution was used.

Hemagglutination: The hemagglutination test in microtitration plates was carried out. 0.1 ml of the IgY (anti DEA1.1) and concentrated IgY (anti DEA1.1) were diluted at two-fold dilution with PBS pH7.4. Fifty microliters of 5% canine DEA1.1 positive RBC was added to serially diluted antibody. The mixture was stored at room temperature for 1 hour and at 4°C overnight.

Statistical analysis: The presented results were the means ± standard deviation of three replicate trials. The effect of PEG6000 on IgY purification steps was examined by Kruskal-Wallis test. Analyses were carried out using GraphPAD.

Results

Precipitation by polyethylene glycol (PEG) 6000 was carried out to extract the total IgY from the egg yolk. The egg weight was 57.12 ± 7.15 g while the yolk volume was 14.17 ± 1.44 ml. Measurements of the purity and yield for each stage of purification from immunized egg yolk are shown (Table 1 and Figure 1). After dialysis, the purified protein and yield of IgY samples were 102.52 ± 0.13 mg/mL and 25.13 ± 2.45% with 9.75 %CV.

Table 1 Measurements of the purity and yield for each stage of purification from immunized egg yolk

	Measurements	Total volume (ml)	Protein concentration(mg/ml)
Egg weight (g)	57.12 ± 7.15		
Yolk volume (ml)	14.17 ± 1.44		
3.5% PEG 6000 precipitate	22.5 ml	22.5 ml	408.76 ± 23.20
Pellet precipitate	1 ml		
Pellet dissolved in PBS	10 ml		
After dialysis ((A280/1.33)/ml)	mg/ml	10 ml	102.52 ± 0.13

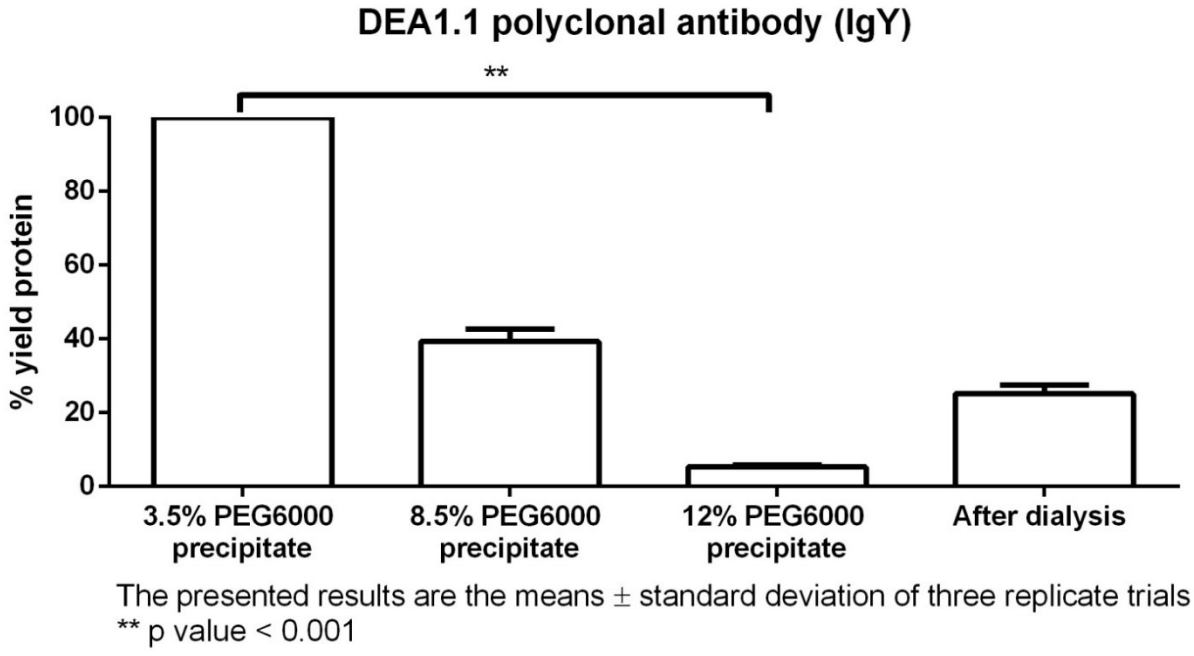

Figure 1 Measurements of the purity and % yield for each stage of purification from immunized egg yolk

SDS-PAGE gel electrophoresis was used to determine the molecular weight of the purified chicken IgY antibodies. Electrophoretic separation of egg yolk IgY antibodies in a 15% resolving SDS-PAGE gel is shown in Figure 2. Following precipitation with 3.5% PEG, different bands, apparently with high concentrations, then started to dilute with an increased concentration of PEG to 8.5%, then some of these bands totally disappeared when increasing the concentration to 12% PEG. This efficient method consisted of three PEG precipitations, starting with 3.5%, followed by 8.5%, to remove lipid substances, and then 12% PEG to precipitate the IgY. Other bands that appeared between the heavy and light chains of IgY were minor impurities corresponding to molecular weights between 65 and 27 kDa, and were removed by dialysis.

Hemagglutination was used to determine the ability of IgY against DEA1.1 to attach to antigens on the surface of red blood cells. Canine red blood cells with DEA1.1 positive showed agglutination with monoclonal antibody against DEA1.1 in a Rapid Vet H detection kit (fig 3A). Canine red blood cells with DEA1.1 positive which agglutinated with monoclonal antibody against DEA1.1 in a Rapid Vet H detection kit reacted with polyclonal antibody against DEA1.1. Hemagglutination of three sample-polyclonal antibodies against DEA1.1 and two samples of canine red blood cell with DEA1.1 positive was shown at titer 4. Antibody may be detected and measured by hemagglutination at lower concentrations than those detectable by other techniques. Hemagglutination of polyclonal antibody against DEA1.1 and canine red blood cell with DEA1.1 positive is shown in figure 3. Polyclonal antibody against DEA1.1 was diluted between titer 2 and 4096. The canine red blood cell with DEA1.1 positive reacted with polyclonal antibody against DEA1.1 at titer 4 at 4 °C, overnight. Meanwhile, concentrated polyclonal antibody against DEA1.1 by Amicon Ultra-15 centrifugal filter units (Merck Millipore) reacted with canine red blood cell and DEA1.1 positive at titer 4 at room temperature for 1 hour.

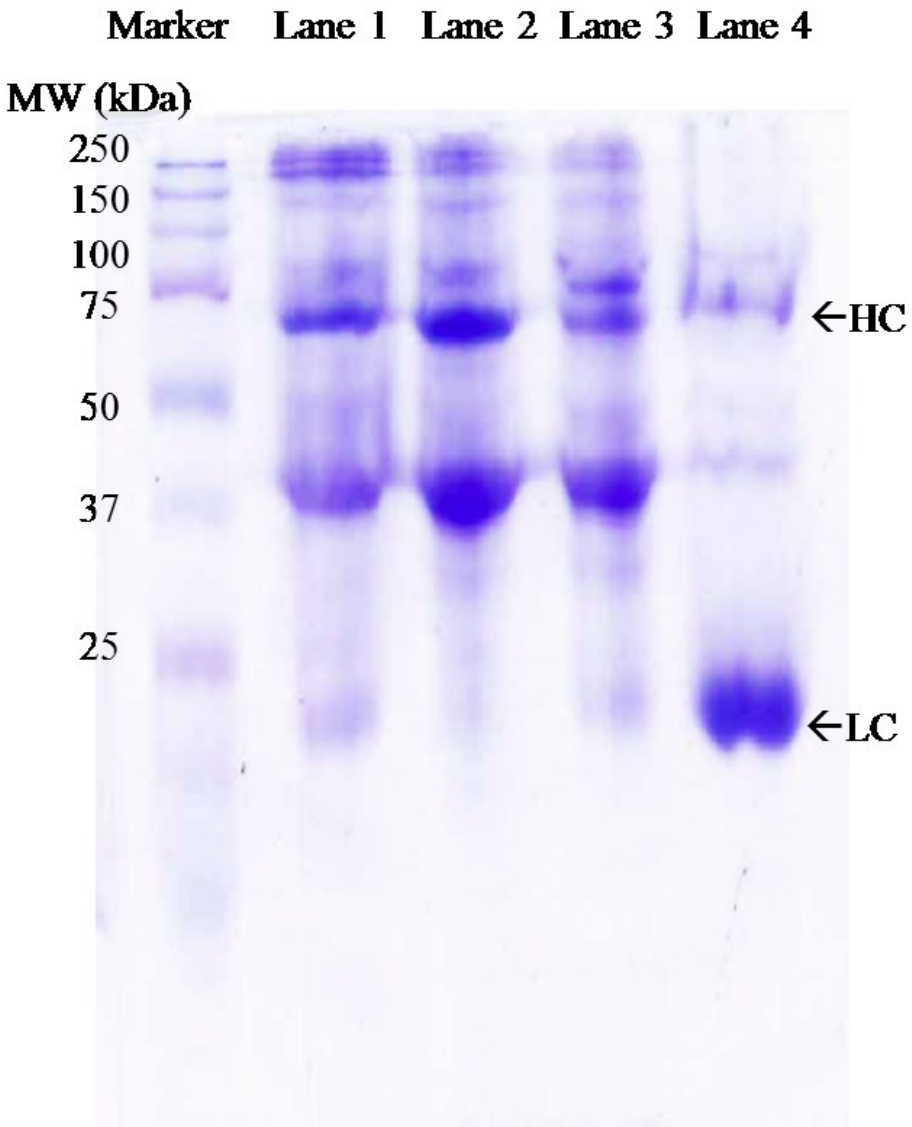

Figure 2 The SDS-PAGE profile of IgY. The two IgY chains appeared on the SDS PAGE using 15% resolving SDS-PAGE gel. Marker = pre-stained Protein Ladder, lane 1 = purified IgY after 3.5% PEG 6000 precipitation, lane 2 = purified IgY after 8.5% PEG 6000 precipitation, lane 3 = purified IgY after 12% PEG 6000 precipitation, lane 4 = purified IgY after dialysis, HC - heavy chains (65 kDa), LC - light chains (27kDa)

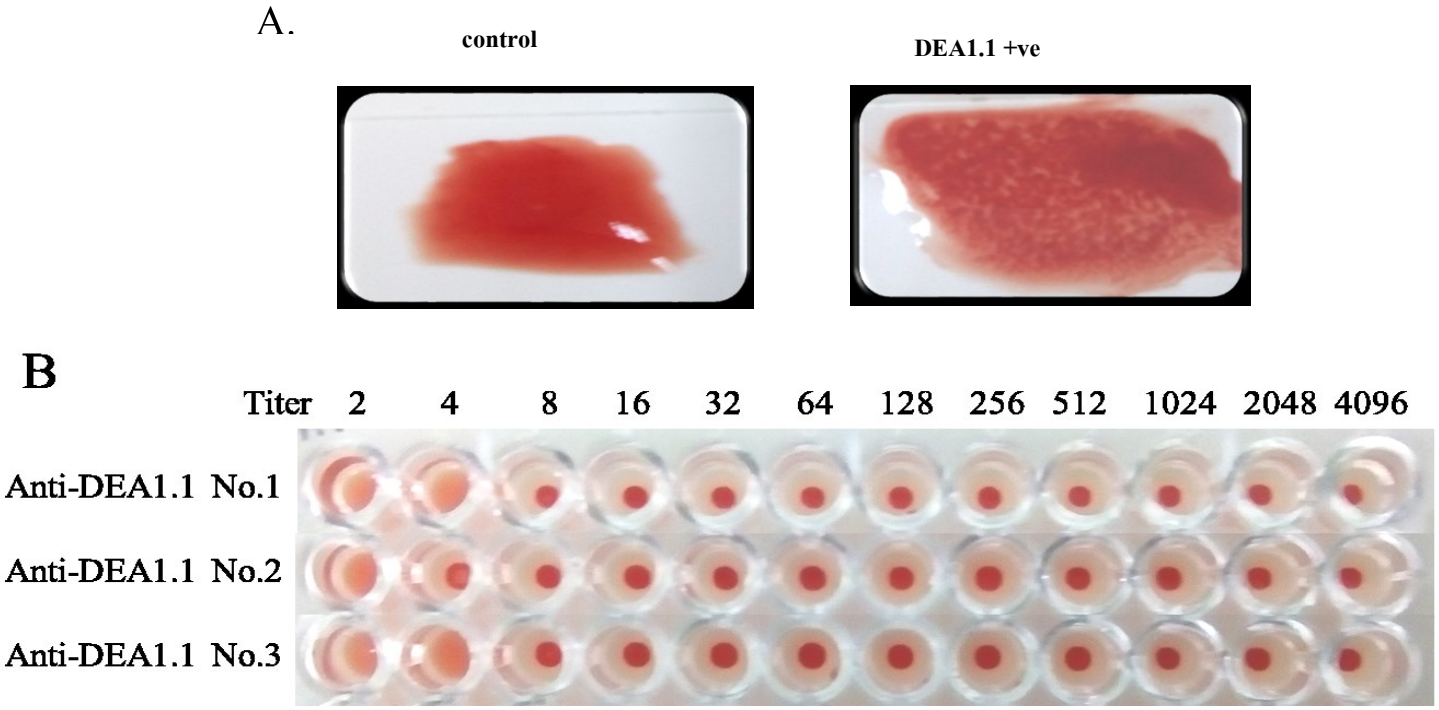

Figure 3 Hemagglutination of canine red blood cells
A. Hemagglutination of monoclonal antibody against DEA1.1 and canine red blood cells with DEA1.1 positive in a Rapid Vet H detection kit
B. Hemagglutination of polyclonal antibody against DEA1.1 and canine red blood cells with DEA1.1 positive

Discussion

Monoclonal antibodies are not generally useful for assays that depend on antigen cross-linking (e.g., hemagglutination) unless dimeric or multimeric antigens or antigens bound to a solid phase are used. (Lipman *et al*., 2005). Hens egg yolk immunoglobulins or IgY have been studied intensively due to their importance. Serum IgG of the hen is transferred from the mother hen to the offspring egg yolk to acquire immunity. Antibodies are transferred from hen to chick via the latent stage of the egg, and play an important role in the immunological function for the relatively immunoincompetent chick to resist various infectious diseases. Immunoglobulin is called IgY because it is present in the egg yolk and due to the differences in protein nature compared to that of the mammalian immunoglobulins (Chalghoumi *et al*., 2009). The greater molecular mass of IgY was due to an increased number of heavy-chain constant domains and carbohydrate chains. In addition, the hinge region of IgY was much less flexible compared to that of mammalian IgG. It suggests that IgY is a more hydrophobic molecule than IgG (Davalos-Pantoja *et al*., 2000). Therefore, IgY has been applied successfully in scientific, diagnostic, prophylactic and therapeutic purposes, for immunochemical reagents and in food formulation or supplements due to the stability of IgY under food processing conditions (Raj *et al*., 2004). IgY is an excellent antibody for use in immunological assays involving mammalian sera, due to the discriminative properties of IgY compared to mammalian IgG, as IgY does not react with the rheumatoid factor and human anti-mouse IgG antibodies do not activate the complement system and do not bind to Fc receptors (Larsson *et al*., 1991). Also, they have poor cross-reactivity to mammalian IgG due to immunological differences. IgY is usually low-cost and can be generated through convenient production processes that make it an attractive antibody for research and diagnosis (Hodek *et al*., 2013). The advantages can be concluded as; (1) IgY is produced in egg yolk; so there is no need to puncture animal blood, (2) considerable amounts of antibodies can be obtained at a fairly low cost (3) with a usually rapid production process, (4) IgY can be stored in eggs at 4 °C for at least one year, (5) it is achievable to produce a specific antibodies to small amounts of antigen that is poorly immunogenic in mammals (Hodek *et al*., 2013). There are several IgY isolation methods available but mostly based on using polyethylene glycol (such as PEG 6000) for precipitation from the supernatant extracts, which usually yields protein impurities (Polson *et al*., 1980; Fischer and Hlinak.1996).

The extraction procedure separated the suspension in two phases. One phase consisted of yolk solids and fatty substances and a watery phase containing IgY and other proteins. Dialysis of the extracted IgY against PBS gave pure extracted IgY. The results indicate that the combination between PEG and dialysis methods is quite important to improve purity.

Our results showed that it is possible to generate IgY antibodies from chicken eggs with chicken immune system boosted by vaccination with canine ghost cells. Compared to antibody production in rabbits, the IgY technology offered several advantages; no blood sampling, only eggs needed following immunization and low quantities of antigen were required to obtain high and long-lasting IgY titers in the yolk of immunized hen eggs (Hodek *et al*., 2013).

Therefore, the production of polyclonal antibodies through chicken immunization makes IgY an excellent alternative, producing the antibodies in large amounts and quality from simple methods of production without the need for invasive techniques.

In hemagglutination of polyclonal antibody against DEA1.1 and canine red blood cell with DEA1.1 positive, the canine red blood cell with DEA1.1 positively reacted with polyclonal antibody against DEA1.1 at titer 4. This relied on the ability of antibodies to cross-link red blood cells by interacting with the antigens on their surface. Hemagglutination is expressed as titer. In the zone of equivalence, the correct proportion of antibody to antigen occurs, resulting in a visible mat formed by Ag-Ab complex cross-linking (Armstrong, 2008). At high concentrations of antibodies, Ag-Ab complex cross-linking was prevented from occurring; each epitope on one antigen particle may bind to a single antibody molecule. At a higher dilution of serum, agglutination may occur and cross-linking is possible. The agglutination of an antigen, as a result of cross-linking by antibodies, was dependent on the correct proportion of antigen to antibody (Armstrong, 2008). The canine red blood cells with DEA1.1 positive reacted with polyclonal antibody against DEA1.1 at titer 4 at 4 °C, overnight. While, concentrated polyclonal antibody against DEA1.1 by Amicon Ultra-15 centrifugal filter units (Merck Millipore) reacted with canine red blood cells with DEA1.1 positive at titer 4 at room temperature for 1 hour. This indicated ultracentrifugation might decrease the impurity from extracted polyclonal antibody against DEA1.1.

We successfully purified chicken IgY antibody in egg yolks of immunized hens, which were used as a diagnostic reagent for canine blood group DEA1.1 detection. The benefits of IgY technology and its universal application in research and medicine is expected to expand on a large-scale. The purified chicken IgY antibody will become standardized and relatively simple to perform, compared with standardization for blood typing. For further study the purified IgY will be subjected to determined immunological activity and validation by immunoprecipitation and Western blot to demonstrate specificity with DEA1.1.

The production of polyclonal antibodies through chicken immunization proved to be an excellent alternative, producing antibodies in large amounts and quality from the simple methods of production without the need for invasive. IgY should be used as an alternative to mammalian antibodies and it is better to immunize chickens before they begin to produce eggs, since the stress induced by handling them could have an adverse effect on egg production. It is expected that IgY will play an increasing role in research, diagnosis, and immunotherapy in the future.

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