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Production of Mouse Anti-Quail IgY and Subsequent Labeling with Horseradish Peroxidase Using Cyanuric Chloride

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Polyclonal antibodies labeled with a tracer have been commonly used as secondary antibodies in immunochemical assays to quantify the concentration of antibody–antigen complexes. The majority of these antibodies conjugated with a tracer are commercially available, with the exception of few untouched targets. This study focused on the production and application of mouse anti-quail IgY as an intermediate antibody to link between quail egg yolk IgY and goat anti-mouse IgG–HRP as primary and secondary antibodies, respectively. Subsequently, the produced mouse anti-quail IgY was labeled with horseradish peroxidase (HRP) and its efficiency on enzyme linked immunosorbent assay (ELISA) was compared with that of commercial rabbit anti-chicken IgY–HRP. As an intermediate antibody, mouse anti-quail IgY was successfully produced with good affinity and sensitivity (1:10,000) to the primary and secondary antibodies. Subsequently, mouse anti-quail IgY was effectively conjugated with HRP enzyme, resulting in a secondary antibody with good sensitivity (1:10,000) to quail anti-*V. parahaemolyticus* and *V. vulnificus* IgY. The detection limit was 10^5 CFU/ml for both *V. parahaemolyticus* and *V. vulnificus*. The efficiency of the produced conjugate to detect quail IgY on ELISA was comparable to that of the commercial rabbit anti-chicken IgY–HRP, and hence the produced and labeled mouse anti-quail IgY–HRP can be used as a secondary antibody to detect any antibody produced in quail.

Key words: Mouse anti-quail IgY, HRP labeling, cyanuric chloride, ELISA, SDS-PAGE

Antibodies coupled with tracers such as enzymes have been widely used in the presence of commercially available synthetic chromogenic and fluorescent substrates to quantify the concentrations of antibody–antigen complexes. Varieties of enzymes are commercially available or can be synthesized in laboratories; however, their relevance as tracers depends highly on the chemical structure, source, and stability [2]. Owing to this reason, very few enzymes such as horseradish peroxidase (HRP), alkaline phospatase, and β-D-galacosidase [6, 13, 16, 22, 27] have revealed practical use as tracers. Several methods for coupling HRP to antibodies and antigens have been described including the sodium periodate oxidation [21, 24, 33, 34, 36], glutaraldehyde reagent [25], and dihydrazide methods [5, 30, 31], bifunctional reagent procedures [10, 23, 26, 38] such as meleimide-sulfhydryl methods using heterobifunctional coupling reagents [3, 12, 14, 28], and the use of lysine residues in peptide chains [8, 9] and oligobucleotides [20] for signal amplification. HRP enzyme, a glycohemoprotein which is stable and widely available in the roots of the horseradish plant [29, 35] is commonly used as a labeling agent in immunochemical techniques [2, 37]. The enzyme is covalently coupled to the signaling secondary antibody, primary antibody, antigen, or haptein *via* bifunctional bridging molecules such as cyanuric chloride [1, 2]. The chlorine atoms in the cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) molecule react with nucleophilic groups (thiol, amino, imino, and hydroxyl functions) to form stable linkages. The reactions take place at specific temperatures and pH, a phenomenon that makes the molecule an efficient heterobifunctional coupling reagent for linking hydroxyl, amino, and thiol groups [1].

It has been previously reported that quail IgY and chicken IgY are similar in several aspects including sedimentation coefficients, molecular weights, and yielding

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pattern of their heavy and light chains under reduced conditions [4, 11, 19]. The use of quail to produce specific antibodies and their application in immunoassays such as ELISA were reported to have similar advantages as that of antibodies produced in chicken [32]. Despite the small amount of IgY in quail egg yolk compared with that of chicken [4], quail may be useful in producing small-scale antibodies or virus-specific antibodies in serological experiments owing to the less immunogens required, which is a big advantage as viruses can only be purified in a very low yield [32]. However, the major shortcoming on the use of specific IgYs produced from quail is the lack of signal-developing (biotin or enzyme labeled) antibodies directed against quail IgY. As a result, it requires the use of three kinds of antibodies including primary, intermediate, and secondary (labeled) antibodies in an assay such as indirect ELISA [32] or the use of anti-chicken antibodies labeled with enzyme such as rabbit anti-chicken IgY–HRP (secondary antibody) to detect the specific quail IgY [15]. Moreover, a conjugation of both antigen and antibodies with an accompanying molecule, such as digoxigenin (DIG), is later detected by the signal-developing antibody previously conjugated to the same accompanying molecule and an enzyme [4]. However, most of these routes are much longer and/or may result in a reduced affinity and performance of the assay. To overcome these shortcomings, this study developed mouse antibodies directed against quail IgY, following our previous work [15], and the developed antibodies were subsequently conjugated with horseradish peroxidase enzyme as a tracer. The produced antibodies affinity, antibody−HRP conjugation efficiency, and the use of the resultant conjugates in immunoassays were demonstrated by ELISA and SDS-PAGE.

Materials and Methods

Experimental Animals

Five-week-old BALB/c female mice in broad well-ventilated cages with wire mesh were provided with full access to water and feed *ad libitum*. The room temperature was maintained at 27 ± 2^oC and the photoperiodicity was controlled at 14 h lighting and 10 h darkness. Mice were left to adapt to the new environment for two weeks prior to immunization. Animal care and use for experiment were approved and complied with the guidelines of the Division of Animal Laboratories of Gyeongsang National University Policy of Animal Care and Use.

Production of Antibodies

Quail immunoglobulin Y (IgY) previously purified from non-experimental control quail egg yolk [15] using a water dilution / ammonium sulfate precipitation method was used as the immunogen to produce anti-quail IgY antibodies from mice. Immunogens at a concentration of 1 mg of water-soluble fraction of protein containing IgY in 1 ml of phosphate buffered saline (PBS) were emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) in the first immunization. Each of the three mice was individually immunized with 0.2 ml intraperitoneally. In the second and third immunizations, the Freund's complete adjuvant was replaced with the incomplete form. Booster immunization was administered in the same way and amount, except that only immunogens were used in the absence of the adjuvant. Control mice were immunized with sterile PBS, and all immunizations were performed in 2-week intervals. Two days after booster immunization, the blood serum was collected individually from each mouse through the caudal vein and the antisera titers were determined by ELISA. The mice with higher antisera titer were sacrificed, the entire blood serum was collected, and antibodies were harvested by the ammonium sulfate precipitation method.

The collected blood serum was centrifuged for 10 min at 15,500 $\times g$, 4^oC. The supernatant was collected in sterile glass vials, which were then placed into a water bath at 56^oC for 30 min. The contents were 2-fold diluted with sterile PBS and equal volumes of saturated ammonium sulfate were added drop-wise while stirring for 30 min. The contents were centrifuged for 10 min at 15,500 $\times g$, 4^oC. The supernatant was discarded and the pellets were resuspended in sterile PBS. The same precipitation procedures with ammonium sulfate were repeated. After centrifuging the contents for 30 min at 15,500 $\times g$, 4^oC, the pellets were resuspended in sterile PBS and stored at -20^oC as a ready-to-use mouse anti-quail IgY, in this experiment named as an intermediate antibody.

Determination of Antibody Activity

To determine the activity of the intermediate antibody, microtiter plates (Nunc-Immunoplates, Denmark) were coated with 2-fold serial dilutions (1:100−1:102400 corresponding to 10−0.01 μg) of 1 mg of water-soluble fraction of protein containing control quail IgYs in 1 ml of carbonate buffer (pH 9.6) and incubated for 1 h at 37^oC. The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) by using the Nunc-Immuno Wash 8 microplate washer (Nalge Nunc International). After washing, the uncoated surface of the wells were blocked with 200 μl of 1% skimmed milk in PBS and incubated at 37^oC for 1 h. After washing the plates 4 times with PBST, 100 μl of mouse anti-quail IgY diluted 1:10,000 in PBS was added and incubated at 37^oC for 1 h. The plates were washed 5 times with PBST and 100 μl of goat anti-mouse IgG-HRP (Sigma Chemical Co.) diluted 1:10,000 in PBS was added and incubated at 37^oC for 1 h. The plates were washed 6 times with PBST and incubated with 100 μl of 0.1 M citrate buffer (pH 4.0) containing 0.025% ABTS [2,2'-azinobis (3-ethylbenz-thiazoline-6-sulfonic acid)] and 0.03% H_2O_2 at 37^oC for 30 min. The absorbance was measured at 405 nm using a Thermo Scientific Multiscan FC microplate reader (Thermo Fisher Scientific, Vantaa, Finland).

Determination of the Affinity of the Intermediate Antibodies

The affinity of the produced intermediate antibodies when coupled to the specific antibodies produced in quail and commercial goat anti-mouse IgG-HRP was evaluated using indirect ELISA. The ELISA procedures were performed as described above, except the microtiter plates were coated with formalin-inactivated cells of either *Vibrio parahaemolyticus* or *Vibrio vulnificus* prepared in our previous experiment [15] at a concentration range from 10^{10} to 10^4 CFU/ml. Specific IgYs against *V. parahaemolyticus* or *V. vulnificus* previously produced in quail were added to the wells as primary antibody. The intermediate antibody produced in this work against quail IgY was diluted 1:10,000, and 100 μl was added to the wells.

The secondary antibody, goat anti-mouse IgG-HRP, substrate, and the measurement of absorbance were performed as described above. For comparison purposes, the same procedures were performed in the absence of intermediate antibody and the goat anti-mouse IgG-HRP was replaced by rabbit anti-chicken IgY-HRP (Sigma Chemical Co.) as a secondary antibody, and the optical density readings reflecting the sensitivity of the assay were compared.

HRP Labeling of Mouse Anti-Quail IgY

To reduce the long experimental steps of using three kinds of antibodies when intermediate antibody was used as a bridge between the primary and secondary antibodies, we carried out an experiment to label the intermediate antibody with a commonly used tracer, a horseradish peroxidase enzyme (Sigma-Aldrich, Co.) using cyanuric chloride (Sigma-Aldrich, Co.) based on slight modifications of the methods reported by Abuknesha *et al*. [1, 2]. Briefly, mouse anti-quail IgY in sterile PBS was centrifuged at 15,500 $\times g$, 4°C for 10 min and the supernatant was discarded. The pellets were redissolved in 50 mM sodium bicarbonate buffer adjusted to pH 9.4 using sodium carbonate and then the antibody solution was dialyzed against 2 L of the same buffer for 16 h at 4°C. The antibody was collected into sterile glass vials on ice and the protein concentration in the sample was estimated by the Bradford method for protein assay [7] using bovine serum albumin (BSA) as the reference protein and the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules CA, USA).

An aliquot of cyanuric chloride (3.5 mg) previously dissolved in cold acetone was transferred into a glass vial and the solvent was evaporated under a stream of nitrogen at 4°C. Six milligrams of HRP powder was dissolved in 1 ml of cold bicarbonate buffer (pH 9.4) and immediately added to the glass vial containing the dry cyanuric chloride. The contents were stirred at 4°C for 4 h to allow coupling of the HRP molecules to cyanuric chloride in the presence of sodium bicarbonate buffer (pH 9.4) as a conjugation buffer. Care was taken in amber-colored glass vials to protect the HRP from light. The final solution was dialyzed against 2 L of the same buffer for 16 h at 4°C to remove excess cyanuric chloride. The HRP–cyanuric chloride conjugate was collected into an amber-colored glass vial and an aliquot of the antibody preparation corresponding to 6 mg was added. After mixing, the contents were incubated in a water bath at 37°C for 16 h with several mixing during the incubation period to facilitate interactions of the molecules and enhance the coupling between the antibodies and the HRP–cyanuric chloride complex. The contents were then freeze dried and the powder was redissolved in 0.5 ml of 50 mM sodium phosphate dibasic (adjusted to pH 5.5 using citric acid) solution. An equal volume of glycerol was added, mixed well, and stored at –20°C as a ready-to-use mouse anti-quail IgY-HRP, a secondary antibody.

Determination of Mouse Anti-Quail IgY–HRP Complex Formation

The formation of anti-quail IgY–HRP conjugate was analyzed on Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving and 5% stacking acrylamide gels under nonreducing conditions. The electrophoresis of proteins was performed at 80 V for the first 10 min until the bromophenol blue reached the bottom of the stacking gel and then the voltage was increased and maintained at 100 V. The bromophenol blue was allowed to run off the gel for a further 10 min and then the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories). The resultant protein bands and their corresponding molecular weights were used to postulate the formation of the new conjugated complex.

Determination of Mouse Anti-Quail IgY–HRP Conjugation Efficiency

Antibody binding and its associated conjugation with HRP enzyme were assessed by two ELISA tests: the conjugate dilution response test and analyte dose response competitive (standard curve) assay [1]. For the conjugate dilution response test, the microtiter plates were coated with 2-fold serial dilutions of quail IgY as plate coating antigen. After incubation and blocking with 1% skimmed milk, the antibody–HRP conjugate was diluted in PBS at six different dilutions (100, 1,000, 10,000, 50,000, 100,000, and 150,000) and 100 µl of each dilution was added and incubated at 37°C for 1 h. After washing the plates with PBST, the substrate addition and measurement of absorbance were performed as described previously.

The analyte dose response competitive assay was performed as described above under subtitle “Determination of the affinity of the intermediate antibodies,” except that the intermediate antibody was not used and the conjugated mouse anti-quail IgY–HRP was used as a secondary antibody.

Statistical Analysis

Analysis of variance (ANOVA), a component of Statistics [17], was used to analyze the results. Student’s *t*-tests were utilized to test the significance differences between the antibody titer among the experimental groups, experimental and control groups, reactivity of the produced intermediate antibody, and the conjugated mouse anti-quail IgY–HRP and that of rabbit anti-chicken IgY–HRP to specific quail anti-*V. parahaemolyticus*/*V. vulnificus* IgYs. Data were presented as means ± standard deviations. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

Determination of Antibody Activity

The determination of the antisera activity was performed by indirect ELISA using 2-fold serial dilutions (1:100–

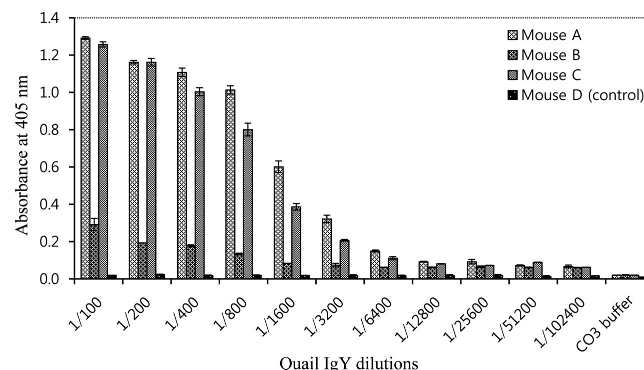

Fig. 1. Antisera activity profiles for experimental mice (A, B and C) immunized with quail IgYs and control mice (D) immunized with sterile PBS, as determined by ELISA.

1:102,400 corresponding to 10–0.01 μg/ml) of quail IgY (1 mg/ml) as plate-coated antigen and was expressed as the mean ± standard deviations of the triplicate optical densities. From three female BALB/c mice immunized with quail IgY, the serum obtained through the caudal vein for testing or drained as a whole from the sacrificed animal for antibody harvesting revealed that the antisera obtained from experimental mice showed significantly higher activity than those of the control ($p < 0.05$). Moreover, the antisera from mice A and C showed a significantly higher activity than the antisera obtained from mouse B ($p < 0.05$). There was no significant difference in the antisera activity obtained from mice A and C ($p > 0.05$) (Fig. 1). Generally, except for mouse B, all other mice immunized with immunogens showed a relatively strong immune response to quail IgY.

Determination of the Affinity of the Intermediate Antibody

In an effort to assess the usefulness of the produced mouse anti-quail intermediate antibody, the ability of the antibody to serve as a linkage between specific antibodies to *V. parahaemolyticus* and *V. vulnificus* previously produced in quail and goat anti-mouse IgG–HRP was evaluated by indirect ELISA. The produced intermediate antibody (1:10,000) showed high affinity to both quail anti-*V. parahaemolyticus* and *V. vulnificus* antibodies bound to their corresponding antigens. Additionally, the intermediate antibody could also be detected by goat anti-mouse IgG-HRP, (Fig. 2A). In comparison with the use of rabbit anti-chicken IgY–HRP for the direct detection of quail anti-*V. parahaemolyticus* and *V. vulnificus* antibodies, the two scenarios were not significantly different ($p > 0.05$) in affinity and sensitivity (Figs. 2A and 2B). However, utilization of the intermediate antibody prolonged the experiment time as a result of the use of three antibodies.

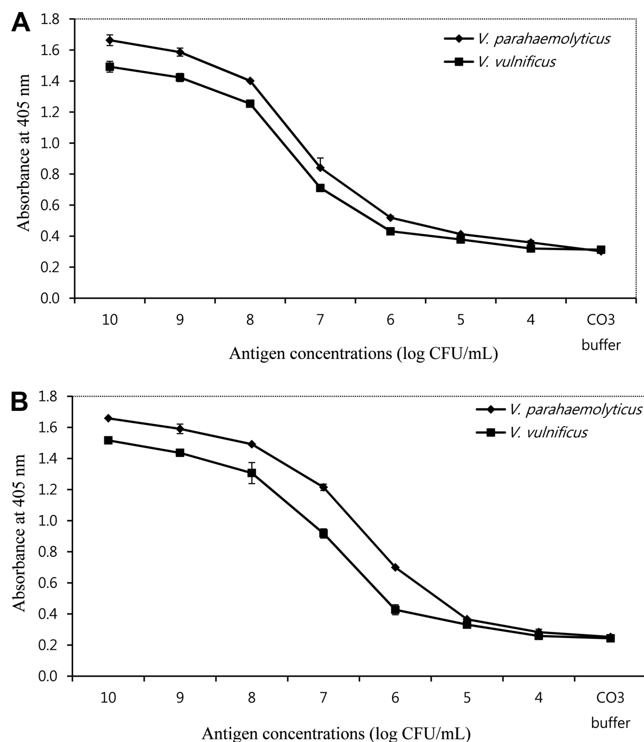

Fig. 2. Affinity of (**A**) mouse anti-quail IgY (intermediate antibody, 1:10,000) and (**B**) rabbit anti-chicken IgG-HRP (1:10,000) to quail specific antibodies for the detection of *V. parahaemolyticus* and *V. vulnificus*.

Formation of Mouse Anti-Quail IgY–HRP Complex

The SDS-PAGE analysis of mouse anti-quail IgY and the conjugated product under nonreducing conditions is presented in Fig. 3. The whole mouse IgG (lane 1) was obtained at about 150 kDa, and the HRP enzyme at about 40 kDa (lane 2), which is close to its apparent molecular mass of 44 kDa and that of the presumptive conjugated mouse anti-quail IgY-HRP at a higher molecular mass (lane 3). The top first band at lane 3 suggested the formation of antibody–cyanuric chloride–HRP complex of high molecular mass than that of the whole mouse IgG in lane 1. This may imply the presence of addition of enzyme on the whole mouse IgG molecule. A similar molecular mass of the unconjugated whole mouse IgG under nonreducing conditions was reported by Kritratanasak *et al*. [18]. The conjugated product (lane 3) resulted in proportions of unconjugated enzyme, which may suggest a need for individual optimization of the appropriate proportions of antibody, enzyme, and cyanuric chloride to be used for the conjugation procedure.

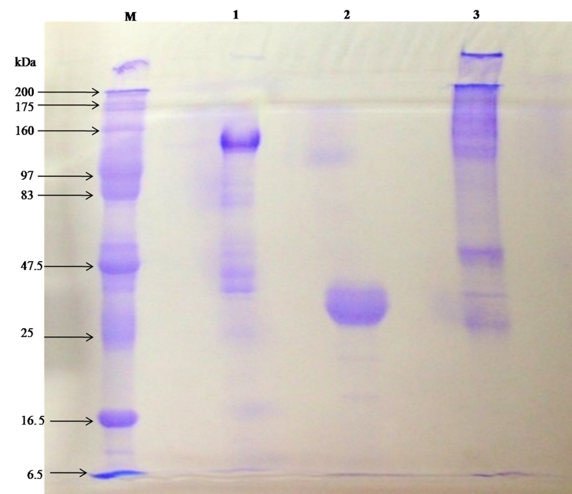

Fig. 3. SDS-PAGE analysis of mouse anti-quail IgY isolated from serum by the ammonium sulfate precipitation method (lane 1), and horseradish peroxidase enzyme (lane 2) and mouse anti-quail IgY conjugated to HRP *via* cyanuric chloride (lane 3) under nonreducing conditions.

Mouse Anti-Quail IgY–HRP Conjugation Efficiency

The conjugation efficiency of the produced mouse ant-quail IgY to horseradish peroxidase *via* cyanuric chloride was determined using 2-fold serial dilutions (1:100–1:102,400 corresponding to 10–0.01 μg/ml) of quail IgY (1 mg/ml) coated on ELISA plates. This test was performed using different dilutions of the conjugate and the coated antibody, and the conjugate dilution response test is presented in Fig. 4. At the dilutions of 1,000 and below of the conjugated product, high and similar optical densities were obtained between the dilutions of 100 to 1600 of the coated antibody, implying the presence of excessive conjugated complexes that were available to react with the substrate. Then, the conjugate response was proportional to the concentration of the coated quail IgY antibody. Additionally, in the presence of coating buffer and absence of coating quail IgY, the low dilutions (1:100 and 1:1,000) of conjugate resulted in an absorbance signal implying false-positive as a result of excessive conjugated complex containing enzyme that reacts with substrate ABTS. At a dilution of 1:10,000, the conjugate resulted in a good response curve with respect to the concentration of the coated quail IgY. This dilution was found to be suitable with an optimal optical density of about 1.6, and no signal was observed in the presence of the coating buffer alone, implying the absence of a false-positive. Extended dilutions of 1:50,000 to 1:150,000 of the conjugate resulted in very low signals.

The analyte dose response competitive (standard curve) assay using the conjugated mouse anti-quail IgY–HRP *via* the cyanuric chloride bridging compound was performed on ELISA using conditions deduced from the conjugation dilution response (Fig. 4) and the previously optimized procedures [15]. The conjugated complex as a secondary antibody bound to quail anti-*V. parahaemolyticus*/*V. vulnificus* IgY resulted in optimal standard curves and binding percent for both *V. parahaemolyticus* and *V. vulnificus* plate-coating antigens (Figs. 5A and 5B). The results of this assay are similar to those obtained with the previously optimized ELISA conditions using rabbit anti-chicken IgY–HRP as the secondary antibody [15]. However, in this case, the detections limit for both *V. parahaemolyticus* and *V. vulnificus* was 10^5 CFU/ml compared with the 10^6 CFU/ml of *V. vulnificus* obtained previously, suggesting an efficient coupling and usefulness of the conjugate as a secondary antibody to detect any antibody produced in quail.

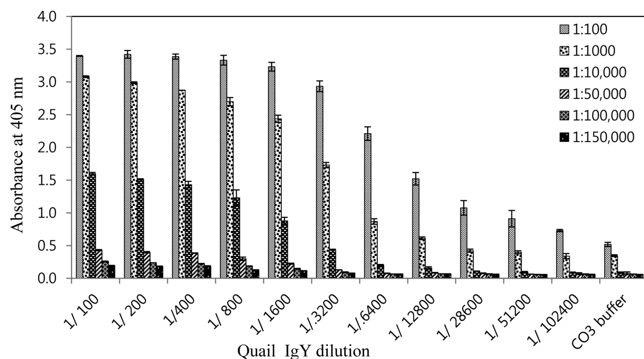

Fig. 4. Efficiency of mouse anti-quail IgY–HRP conjugation and dilution response with respect to coated quail IgY.

DISCUSSION

The use of horseradish peroxidase enzyme as a tracer in immunochemical analyses has been extensively studied [1, 2, 37] and its applicability has been justified. The main advantages of this enzyme include its wide availability in the roots of horseradish plants, suitability due to its small molecular weight and stability to chemical modification [1], efficiency and the revealed practical use as a labeling reagent [1, 2, 37], convenience in terms of use, and being environmental friendly as it is non-toxic. To date, there is a continuous increase in the number of commercially available HRP-labeled immunological reagents and this reflects the advantage of using HRP as a labeling reagent. However, depending on the source and target of the immunological

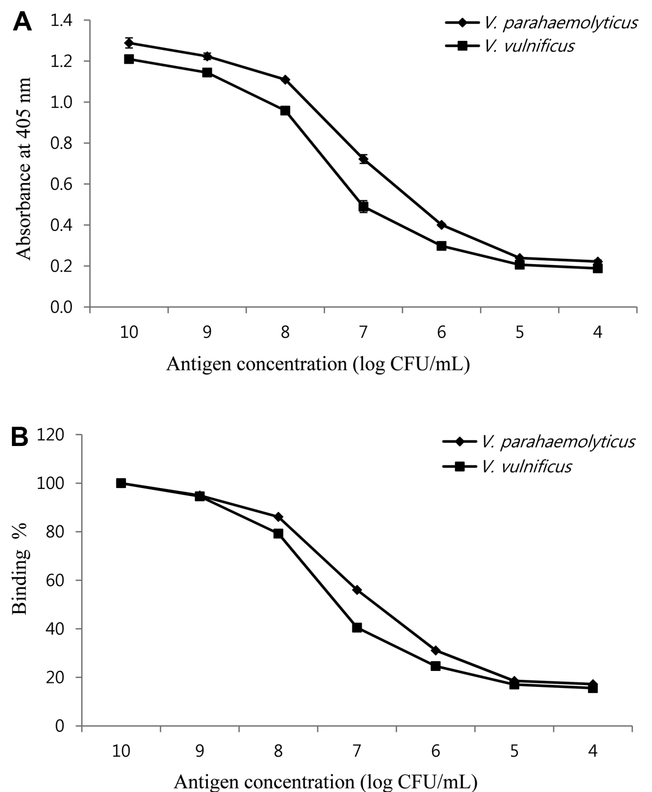

Fig. 5. Analyte dose response competitive (standard curves) assay using the conjugated mouse anti-quail IgG–HRP as a secondary antibody to quail IgYs directed to *V. parahaemolyticus* and *V. vulnificus*.

(**A**) standard curve using O.D value, (**B**) standard curve using binding %.

reagent, few cases are yet to be studied, and are commercially unavailable, and hence have been prepared in laboratories for research and in-house experimental purposes.

This study produced mouse immunoglobulin G as an intermediate antibody targeting quail immunoglobulin Y. The intermediate antibody could also bind to the commercial goat anti-mouse IgG–HRP, allowing the detection and quantification of the coated antibody. This method utilized three antibodies in an assay procedure where the produced mouse anti-quail IgY acts as a bridge between quail IgY and goat anti-mouse IgG–HRP. The assay was comparable to the direct use of commercial rabbit anti-chicken IgY–HRP for the detection of quail IgY, suggesting the equivalence in affinity and sensitivity of the produced antibody to this commercial product. The use of intermediate antibodies in an assay for the detection of quail IgY due to unavailability of its counterpart anti-quail IgY–HRP labeled was also described by Somoriyarjo *et al.* [32] when determining the activity of the specific *Coturnix* quail immunoglobulin Y directed to three plant potyviruses.

Several methods of labeling immunological reagents with HRP enzyme have been postulated [5, 12, 21, 24, 31, 30, 38]. To reduce long experimental steps by using three kinds of antibodies in an assay, the study successfully labeled mouse anti-quail IgY with horseradish peroxidase using cyanuric chloride, a slight modification of the previously reported method [1, 2]. The cyanuric chloride acts as a bridge to link the horseradish peroxidase enzyme with mouse anti-quail IgY *via* chlorine atoms in the molecule and the nucleophilic groups of the antibody molecule [1, 2]. The resultant conjugate of mouse anti-quail IgY–HRP showed high affinity and sensitivity to quail IgY, allowing the quantitation of the corresponding antigen–antibody complex based on the intensity of the color. Subsequently, the conjugate had good reactivity with the substrate ABTS, reflected by the color development, which implies the proper conjugation of HRP enzyme to mouse anti-quail IgY. The conjugated mouse anti-quail IgY–HRP used as a secondary antibody showed high sensitivity (1:10,000) to quail IgY directed against *V. parahaemolyticus* and *V. vulnificus*. The use of the conjugated mouse anti-quail IgY–HRP as a secondary antibody that binds to specific or non-specific quail IgY resulted in a comparable affinity and sensitivity to that of commercial rabbit anti-chicken IgY–HRP. These similarities and interbinding phenomenon among antibodies developed in different host animals targeting these avians are due to the existing similarities between quail IgY and chicken IgY in several aspects of sedimentation coefficients, molecular weights, and the yielding pattern of heavy and light chains [4]. This may also suggest the possible use of the current conjugated mouse anti-quail IgY–HRP as a tracer to chicken immunoglobulin Y directed to various antigens. As the efficient use of anti-chicken IgY–HRP to detect quail IgY has been previously demonstrated [15], the speculated improvement that could result from this comparative use of anti-quail IgY–HRP was not to the seriously prominent level.

Conclusively, our current study produced mouse anti-quail IgY directed against quail immunoglobulin Y. The mouse anti-quail IgY was successfully used as an intermediate antibody to link between quail IgY and goat anti-mouse IgG–HRP. The use of specific quail IgY with the mouse anti-quail IgY as an intermediate antibody followed by goat anti-mouse IgG–HRP enabled the detection of *V. parahaemolyticus* and *V. vulnificus*. A subsequent labeling of the produced mouse anti-quail IgY with horseradish peroxidase resulted in a useful secondary antibody with relatively high titer, affinity, and sensitivity to quail IgY. The use of the conjugated mouse anti-quail IgY–HRP to detect specific or nonspecific quail IgY reduced the assay steps of using intermediate antibody and was comparable to the use of the commercial rabbit anti-chicken IgY–HRP.

Acknowledgments

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