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The *In Vitro* and *In Vivo* Efficacy of Hen IgY Against *Vibrio parahaemolyticus* and *Vibrio vulnificus*

Kassim, Neema[1], Adelard B. Mtenga[1,2], Won-Bo Shim[3], and Duck-Hwa Chung[1]*

[1]*Division of Applied Life Science (BK 21 Program), Graduate School of Gyeongsang National University, Jinju 660-701, Korea*
[2]*Tanzania Food and Drugs Authority, Epi Mabibo, Mandela Road, Box 77150, Dar es Salaam, Tanzania*
[3]*The College of Human Sciences, Nutrition, Food and Exercise Sciences, 432 Sandels Building, P. O. Box 3061493, Florida State University, USA*

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The inhibitory effect of IgY against *Vibrio parahaemolyticus* and *Vibrio vulnificus* responsible for seafood-borne diseases was investigated in this study. Water-soluble fractions (WSF) of protein containing IgYs were isolated from the egg yolk of hens initially immunized with formalin-inactivated *V. parahaemolyticus* or *V. vulnificus*. Protein, total and specific IgY contents of the WSF were determined. The inhibitory and protective effects of IgYs on the growth of *V. parahaemolyticus* and *V. vulnificus* were assayed in liquid medium and in mice. IgYs showed high affinity to their corresponding antigens with high titer from day 28 onwards. Protein contents and total IgY concentrations remained stable throughout the immunization period, whereas specific IgY concentrations increased steadily and reached a plateau at day 49. Specific IgY powder (150 mg/ml) significantly inhibited further multiplication of both *V. parahaemolyticus* and *V. vulnificus* in liquid medium as compared with the control IgY. The bacteria count in mice feces was lower in mice pretreated with specific IgYs than in those pretreated with PBS or control IgY. Higher survival of mice was observed in the experimental groups pretreated with either anti-*V. parahaemolyticus* (75% survival) or anti-*V. vulnificus* (87% survival) IgYs, compared with those in the control groups pretreated with PBS or nonspecific IgY. All mice in the control groups died within three days after bacteria inoculation; hence, the protective effect of specific IgYs against infection caused by *V. parahaemolyticus* and *V. vulnificus* was demonstrated.

Keywords: *V. parahaemolyticus*, *V. vulnificus*, immunoglobulin Y, *in vitro*, *in vivo*, efficacy

*Corresponding author
Phone: +82 55 772 1903; Fax: +82 55 757 5485;
E-mail: dhchung@gnu.ac.kr

Microbial contamination is one of the major challenges facing the seafood industry worldwide. *Vibrio* species, which occur naturally in the estuarine and marine environment, have been frequently isolated from seafood, seawater [16], and vegetables [41]. They have been associated with foodborne illnesses resulting from consumption of contaminated raw or half-cooked seafoods such as oysters, raw fish, shell fish, shrimps, mussels [30], and raw vegetables [41]. *Vibrio parahaemolyticus* was identified and documented by the Korea Food and Drug Administration (KFDA) in 2006 as one of the leading causes of foodborne diseases in Korea [24]. The increase in ocean surface temperatures due to global warming has resulted in a higher prevalence of *Vibrio* spp., which in return increases the risk of *Vibrio* foodborne illnesses [16]. Among 12 species of *Vibrio* recognized as human pathogens [33], 8 species have been directly associated with food contamination [35]. *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholera* are the main principle and responsible for seafood-borne illnesses in humans [3, 5], resulting in severe intestinal diseases [20, 34].

Immunoglobulin Y (IgY) is the major low molecular weight serum immunoglobulin in oviparous animals with an overall structure similar to that of mammalian immunoglobulins composed of 2 light chains and 2 heavy chains [31]. Chicken egg yolk antibodies (IgYs) have been applied successfully in scientific fields such as immunodiagnostics [11, 12], food analyses in detection of toxic materials and pathogenic microorganisms [9, 40], as well as in prophylactic and therapeutic purposes [9, 17, 23, 25]. IgY as a natural and food-grade antimicrobial compound can be applied in the food industry to replace chemical preservatives to prevent foodborne infections and enterotoxicity, thus improving food safety. Several studies successfully elucidated the effect of chicken IgY on the growth of various food pathogens such as *Salmonella* [7, 23], *Escherichia coli* [42, 27, 38], *Escherichia coli* O157:H7 [39], vegetative and spore forms of *Clostridium perfringens* [31], and *Staphylococcus*

aureus [15]. However, the *in vitro* and *in vivo* effects of IgY on *Vibrio* species originating from an estuarine environment have not been addressed. Therefore, the objective of this study was to investigate the inhibitory effect of IgY against two *Vibrio* species responsible for seafood-borne diseases.

MATERIALS AND METHODS

Bacteria and Culture Conditions

Bacteria strains used included *V. parahaemolyticus* ATCC 17802, *V. vulnificus* KCTC 2959, *V. alginolyticus* KCCM 40513, *V. cincinnatiensis* KCCM 41683, *V. fluvialis* KCCM 40827, *V. mimicus* KCCM 42257, *Listeria monocytogenes* ATCC 19111, *Salmonella typhimurium* ATCC 19586, *Staphylococcus aureus* ATCC 14458, *Bacillus cereus* ATCC 40136, and *Escherichia coli* ATCC 25922. Bacteria stock suspensions in 50% glycerol and tryptic soy broth (TSB) (Becton & Dickinson, Sparks, MD, USA) in the ratio of 1:2 were stored at −70°C until use.

Experimental Animals

Twelve-weeks-old single comb brown leghorns were obtained from Sangolnongjang, Sancheong-gun, Gyeongsangnam-do, and seven-week-old BALB/c female mice were from Hyochang Science, Daegu, South Korea. Separately, hen and mice were maintained in broad well-ventilated cages with wire mesh and provided with full access to water and feed *ad libitum*. The room temperature was maintained at 27±2°C and the photoperiodicity was controlled at 14 h lighting and 10 h darkness. Both animals were allowed to adapt to the new environment for 2 weeks prior immunization. Animal care was approved and complied with the guidelines of, the Division of Animal Laboratories of Gyeongsang National University Policy of Animal Care and Use.

Preparation of Immunogens

Formalin-inactivated *V. parahaemolyticus* and *V. vulnificus*, both as immunogens for immunization and antigens for ELISA experiment, were prepared according to a previously reported method [19]. Briefly, *V. parahaemolyticus* and *V. vulnificus* were cultured in alkaline peptone water (APW; pH 8.6) (Becton & Dickinson, Co.) at 37°C for 24 h, and then subcultured in fresh broth and incubated at 37°C for 18 h. Portions of bacteria cells in each subculture were serially diluted in sterile phosphate-buffered saline (PBS, pH 7.2) and plated on tryptic soy agar (TSA) (Becton & Dickinson, Co.) to determine bacteria concentrations. Immediately, the rest of the cells were treated with 1% formalin at 37°C for 12 h. Viability of the formalin-treated cells was checked on TSA to ensure no viable cells persisted. Cells were harvested by centrifugation at 9,300 ×*g*, 4°C for 20 min, and the pellets were washed three times with sterile PBS. The cells concentrations were adjusted to 1.0×10^9 CFU/ml with sterile PBS and stored at −20°C as ready-to-use immunogens. Other *Vibrio* species and selected pathogenic bacteria used for cross-reactivity studies were prepared in APW and TSB, respectively, as described above.

Immunization of Hens

A total of 12 hens were divided into 3 groups containing 4 hens each. The three groups were immunized as follows; the first group with formalin-inactivated *V. parahaemolyticus*, the second group with formalin-inactivated *V. vulnificus*, and the third (control) group with sterile PBS. Hens were immunized according to a method previously described [39] with slight modifications. Immunogens (1.0×10^9 CFU/ml) were individually emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, USA) in the first immunization and 0.25 ml per site was intramuscularly immunized at four different sites (each upper and lower parts of the right and left sides) of the major pectoralis (breast muscles). From the second to the fourth immunizations, Freund's complete adjuvant was replaced with the Freund's incomplete adjuvant. The fifth immunization (booster) was performed in the same way, except that immunogens were used alone without adjuvant. Immunizations were carried out in 2-week intervals. Eggs from each group were collected daily, labeled accordingly, and kept at 4°C. IgY isolation was performed weekly.

Isolation of WSF Containing IgY and Preparation of IgY Powder

The water-soluble fraction (WSF) containing IgY was isolated from the egg yolk using a water dilution method [1]. The egg yolks were carefully separated from the albumin and then filtered through sterile gauze to remove the yolk membrane. The yolks were homogenized well, diluted 2 times with double-distilled deonized water, and then 8 volumes of 3 mM HCl (pH 3.0) was added to make a final dilution of 1:10. The mixture was mixed well, the pH was adjusted to 5.0 using 10% acetic acid, and then incubated at 4°C for 12 h. After incubation, the mixture was centrifuged at 9,300 ×*g*, 4°C for 20 min to obtain the supernatant containing the water-soluble fraction of protein, which was neutralized to pH 7.0 by 0.1 M NaOH, and stored at 4°C for further experiments. The quality of the WSF of protein containing IgY was analyzed on Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 12% resolving and 5% stacking acrylamide gels. The gel was stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) staining solution. The WSFs were analyzed for activity, protein content, total and specific IgYs, and the fraction that contained specific IgY with higher activity or nonspecific (control) IgY was lyophilized to obtain IgY powder.

Specific Activity and Cross-Reactivity of IgYs

The activity of IgY was monitored by the enzyme linked immunosorbent assay (ELISA) procedure optimized in a previous study [19]. The wells of 96-well microtiter plates were coated with 100 µl of formalin-inactivated *V. parahaemolyticus* or *V. vulnificus* at a concentration of 10^8 CFU/ml in carbonate buffer (pH 9.6). Two hundred microliter of 1% skimmed milk in PBS was used to block the uncoated surface. One hundred microliter of anti-*V. parahaemolyticus* or anti-*V. vulfinicus* IgYs diluted 1:1,000 in PBS was used as primary antibody. One hundred microliter of rabbit anti-chicken IgG conjugated with horseradish peroxidase (Sigma-Aldrich) diluted 1:10,000 in PBS was used as a secondary antibody. One hundred microliter of 0.1 M citrate buffer (pH 4.0) containing 0.025% ABTS and 0.03% H_2O_2 was used for color development. The absorbance was measured at 405 nm using a Thermo Scientific Multiscan FC microplate reader (Thermo Fisher Scientific, Vantaa, Finland).

For cross-reactivity studies, the anti- *V. parahaemolyticus* or anti-*V. vulfinicus* IgYs were prepared as described above, except that the microtiter plates were coated with formalin-inactivated cells of *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. mimicus*, *L.*

monocytogenes, *S. typhimurium*, *S. aureus*, *B. cereus*, and *E. coli* as antigens to determine any binding possibilities. The cross-reactivity was assessed by comparing the optical density obtained when the produced IgYs were used with non-corresponding antigen coated, with those of corresponding antigens, which were considered as 100%.

Determination of Protein, Total and Specific IgY Contents

Total protein content in the WSF and IgY powder were determined based on the Bradford method for protein assay [6] using bovine serum albumin (BSA) (Bio-Rad Laboratories) as a reference protein and Bio-Rad Protein Assay Dye Reagent Concentrate.

Based on the ELISA conditions described above and the previous reported method [39], total and specific IgY contents were determined using rabbit anti-chicken IgG (2.4 mg/ml) (Thermo Scientific, Rockford, IL, USA) as a standard plate coating antigen and purified normal chicken IgY (200 µg/0.5 ml) (Santa Cruz Biotechnology, CA, USA) as a reference antibody. The standard curves obtained by the titration between rabbit anti-chicken IgG and reference purified normal chicken IgG were used to calculate the relative concentrations of the total and specific IgYs individually.

Growth Inhibition Assay

The inhibitory activity of the produced chicken IgY on the growth of the same strains of *V. parahaemolyticus* / *V. vulnificus* in liquid medium was performed according to a method reported [38]. Bacteria were grown in APW at 37°C, 150 rpm for 18 h, and the optical density at 600 nm using UV spectrophotometer (UV-1601, SHIMADZU Corporation, Kyoto, Japan) was adjusted to 0.07 and 0.20 for *V. parahaemolyticus* and *V. vulnificus*, respectively, which corresponded to 1.0×10^6 CFU/ml. The same volume of 20% glycerol in APW was added and stored at −70°C until used.

Two milliliters of the above-prepared bacteria suspension was mixed with an equal volume of APW and incubated at 37°C, 150 rpm, and the absorbance of the culture was measured at 30 min intervals for the first 5 h and then at 1 h intervals to attain 12 h and at 2 h intervals to attain 24 h. The growth curve was plotted and used to determine the sampling time for the growth inhibition assay.

IgY powders were reconstituted in sterile APW to a final concentrations of 100, 200, and 300 mg/ml. After a thorough mixing, the contents were centrifuged at 1,600 ×*g* and then the supernatant was aseptically filtered using a 0.20 µm membrane filter (Sartorius Stedim Biotech, Goettingen, Germany). Two milliliters of the sterile specific or nonspecific IgY solutions was separately mixed with 2 ml of the prepared bacteria culture, and the mixture was incubated at 37°C, 150 rpm. Aliquots of 100 µl were taken after every 2 h for 12 h of incubation for viable bacteria count, performed in duplicate on TSA plates. TSA plates were incubated at 37°C overnight to determine the actual bacteria count.

Protective Effect of IgY Against *V. parahaemolyticus* and *V. vulnificus* Infection in Mice

Specific and nonspecific IgY powders were dissolved in sterile PBS to a final concentration of 300 mg/ml, centrifuged at 1,600 ×*g*, and then the supernatant was aseptically filtered using a 0.20 µm membrane filter. BALB/c mice were divided into two groups, and from each of the two groups, subgroups of eight mice were orally administered with 0.5 ml of sterile PBS, control (nonspecific) IgY, anti-*V. parahaemolyticus* IgY, or anti-*V. vulnificus* IgY solutions, respectively. IgY solutions and PBS were administered *via* oral gavages using a blunt round-ended flexible stainless steel needle connected to a syringe. After an hour, mice in the first group were orally inoculated with 50 µl (1.0×10^7 CFU/ml) of *V. parahaemolyticus* and those in the second group with *V. vulnificus* cells suspensions in PBS. All mice were transferred to clean cages and fecal shedding from each group was separately collected within 12 h after inoculation. One gram of fecal samples was suspended in 9 ml of sterile PBS and viable bacteria count was determined by the spread plate technique on heart infusion (HI) agar (Becton, Dickinson, Co.) as previously described [32]. Plates were incubated at 37°C for 18 h. Furthermore, mice survival was monitored for 7 days following bacteria inoculation.

Statistical Analysis

Analysis of variance (ANOVA), a component of Statistics to use [21], was used to analyze the results. Student's *t*-tests were utilized to test the significance differences between the antibody titer of the control and experimental groups, cross-reactivity of specific antibodies to other pathogenic bacteria and other *Vibrio* species, protein contents, total and specific IgY concentration. Data were presented as means ± standard deviations. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

Production of Specific IgY in Hens

Formalin-inactivated *V. parahaemolyticus* and *V. vulnificus* immunogens were used for immunization of hens. Both immunogens produced high immugenicity to the immunized hens. The WSF obtained from the egg yolks of both *V. parahaemolyticus* and *V. vulnificus* immunized hens resulted into a short lag phase seen in the first week of immunization, followed by a rapid response shown by an increase in specific IgY activity at the second week. This exponential increase of specific IgY in the second week was maintained for a further two weeks, the peak was reached at the fourth week of immunization and the titer

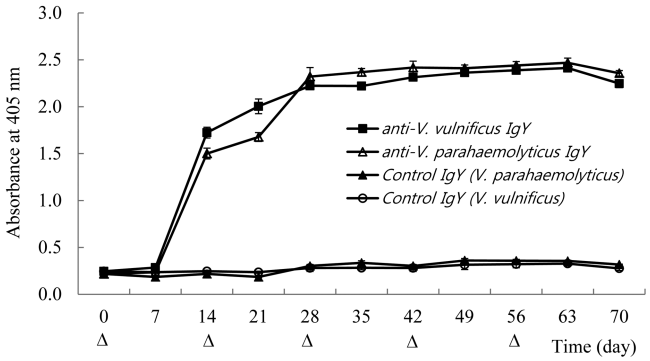

Fig. 1. Profile of IgY activity (1:1,000) from hens immunized with either formalin-inactivated *V. parahaemolyticus* or *V. vulnificus* in comparison with the control group immunized with sterile PBS.

For control IgY on the graph legend, the organisms in parentheses indicate coating antigen used. Immunization days are marked with Δ.

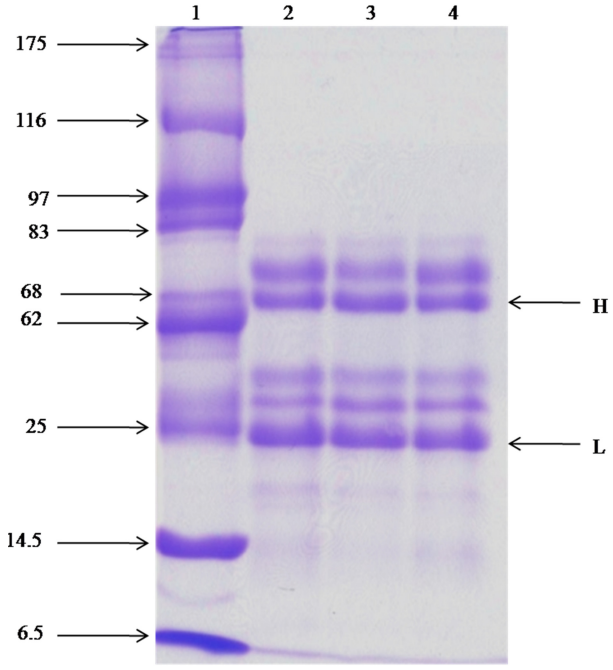

Fig. 2. SDS-PAGE results of WSF of protein containing IgY from chicken egg yolk.
Protein marker (lane 1); WSF obtained from control chicken (lane 2), chicken immunized with *V. parahaemolyticus* (lane 3), and those immunized with *V. vulnificus* (lane 4), formalin-inactivated cells.

was maintained more or less constant for about six weeks regardless of the booster immunization given at the 8th week. The antibody levels of the immunized hens were significantly higher than those of the controls ($p < 0.05$) for both *V. parahaemolyticus* and *V. vulnificus* immunized hens (Fig. 1). There was no significant difference in the IgY titer ($p > 0.05$) of the WSF obtained from the egg yolks of hens immunized with *V. parahaemolyticus* or *V. vulnificus* immunogens.

The quality of the isolated WSF of protein containing IgY is presented with two major bands at about 68 and 25 kDa as the heavy and light chains of IgY, respectively, for both fractions obtained from immunized (specific IgYs) and control (nonspecific IgYs) hens (Fig. 2). Minor bands imply other proteins contained in the WSF, which can be omitted by further purification of IgY from the fraction.

Determination of Protein, Total and Specific IgY Contents in the WSF

The isolated WSF of protein from egg yolk throughout the immunization period was characterized by the determination of protein content, total and specific IgYs. The protein and total IgY concentrations remained more or less stable throughout the immunization period. There was no significant increase in protein and total IgY contents ($p > 0.05$) in egg yolk throughout the immunization period for hens immunized with *V. parahaemolyticus* or *V. vulnificus* immunogens.

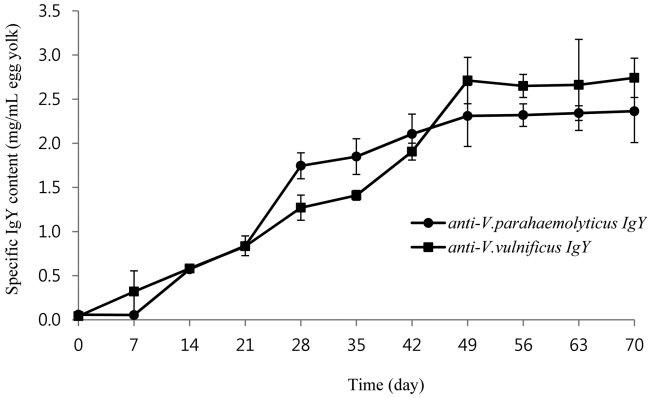

Fig. 3. Specific IgYs concentrations in the WSF obtained from egg yolks of hens immunized with *V. parahaemolyticus* and *V. vulnificus* immunogens throughout the immunization period.

The protein and total IgY contents of unimmunized hens was also similar to those of the immunized hens. The average protein contents were found to be 30.30 ± 0.61 and 32 ± 1.30 mg/ml of egg yolk for WSF obtained from the egg yolk of hens immunized with *V. parahaemolyticus* and *V. vulnificus*, respectively. The average total IgY contents were 25.11 ± 2.28 and 21.07 ± 2.18 mg/ml of egg yolk for WSF obtained from the egg yolk of hens immunized with *V. parahaemolyticus* and *V. vulnificus*, respectively.

The specific IgY content of the WSF significantly increased ($p < 0.05$) with the increase in immunization period for both *V. parahaemolyticus* and *V. vulnificus* immunized hens, with the highest concentration reached at day 49 from the first immunization and was maintained constant for a further three weeks of observation. The increase in concentration of the specific IgY was independent to the constant contents of protein and total IgY. The highest concentrations of specific IgY were 2.36 ± 0.36 and 2.74 ± 0.22 mg/ml of egg yolk for WSF obtained from the egg yolk of hens immunized with *V. parahaemolyticus* and *V. vulnificus*, respectively (Fig. 3). This may imply the point at which the maximum conversion of nonspecific IgY to specific IgY may have been reached.

Specific Activity and Cross Reactivity of IgY

To determine the immunoreactivity and specificity of the IgYs in the isolated WSF, indirect ELISA was used. Both anti-*V. parahaemolyticus* and anti-*V. vulnificus* IgYs resulted in high affinity to their corresponding plate-coated formalin-inactivated *V. parahaemolyticus* and *V. vulnificus* antigens. The WSF obtained by 1:10 dilution of the egg yolk presented significantly high sensitive IgYs at a dilution of 1:500. Therefore, both immunogens resulted in a highly sensitive IgY at a total dilution of 1:5,000 of the egg yolk.

The cross-reactivity studies showed a moderate cross reaction between both anti-*V. parahaemolyticus* and anti-*V. vulnificus* IgYs to other *Vibrio* species tested. In the case of anti-*V. parahaemolyticus* IgY, a cross-reactivity of 68.55%, 69.85%, 65.46%, 55.28%, and 54.92% was obtained with *V. vulnificus, V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, and *V. mimicus*, respectively, whereas for anti-*V. vulnificus* IgY, a cross reactivity of 46.17%, 50.46%, 52.42%, 35.81%, and 39.59% was obtained with *V. parahaemolyticus, V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, and *V. mimicus*, respectively. When the cross-reactivity of both IgYs was tested against the selected pathogenic bacteria, a relatively low cross-reactivity of less than 40% was observed for all bacteria tested.

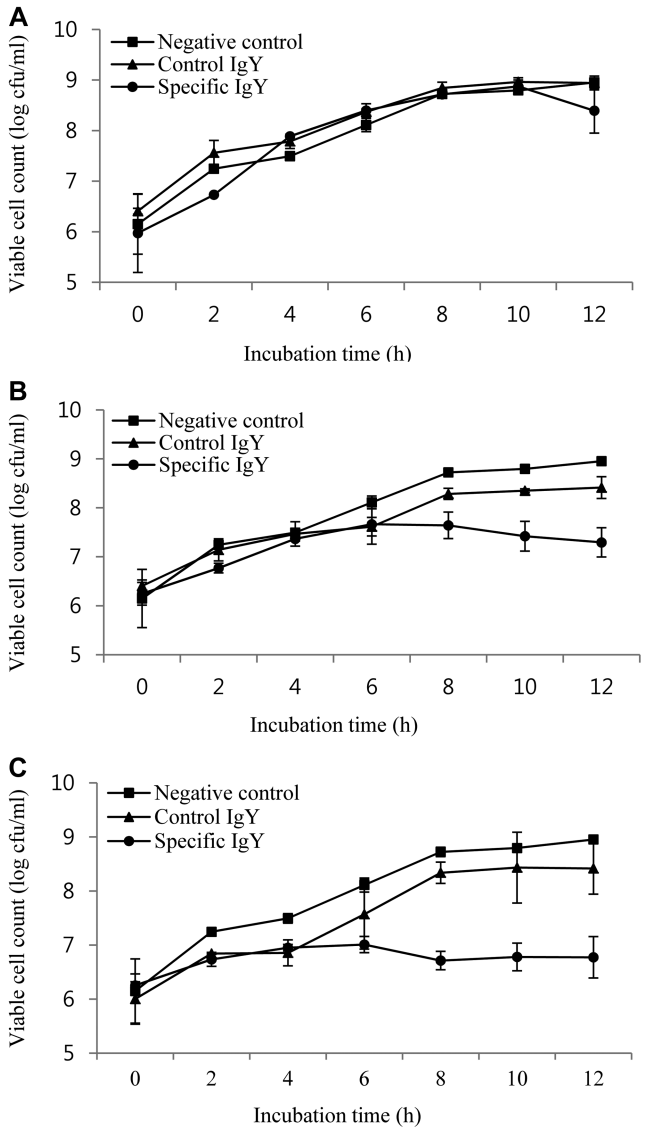

Fig. 4. Effects of different concentrations [(**A**) 50 mg/ml, (**B**) 100 mg/ml, and (**C**) 150 mg/ml] of anti-*V. parahaemolyticus* IgY powder on the growth of *V. parahaemolyticus* in liquid medium.
Negative control (no IgY used); control IgY (nonspecific IgY was used).

Growth Inhibitory Activity of IgYs

The growth inhibitory effects of IgYs on *V. parahaemolyticus* and *V. vulnificus* were assessed in liquid medium. Under the same conditions as those used to obtain growth curve, which was used to determine the sampling time, *V. parahaemolyticus* and *V. vulnificus* were cultured and incubated separately with either their corresponding specific or nonspecific IgYs powder at concentrations of

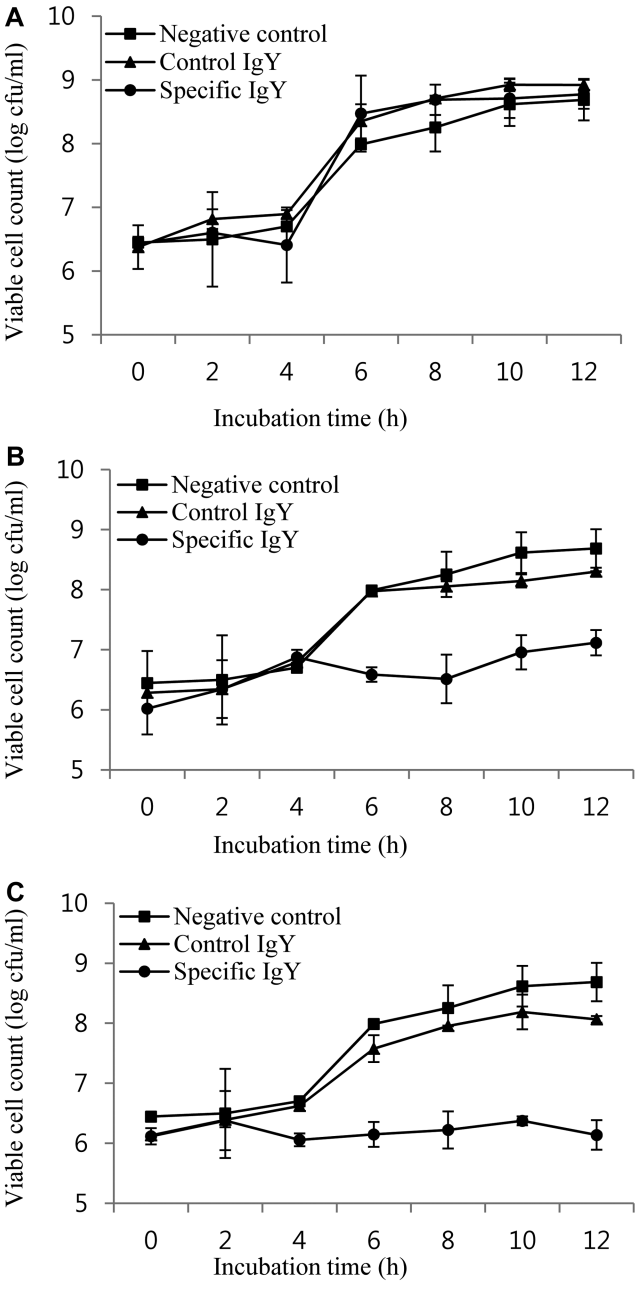

Fig. 5. Effects of different concentrations [(**A**) 50 mg/ml, (**B**) 100 mg/ml, and (**C**) 150 mg/ml] of anti-*V. vulnificus* IgY powder on the growth of *V. vulnificus* in liquid medium.
Negative control (no IgY used); control IgY (nonspecific IgY was used).

50, 100, and 150 mg/ml. As shown in Fig. 4 and 5, the incubation of bacteria with 50 mg/ml IgY solution did not inhibit the growth of both *V. parahaemolyticus* and *V. vulnificus* as the number of bacteria count in the control and experimental groups were similar. Increasing the concentration of IgY powder to 100 mg/ml resulted in a decrease in viable cell count of the experimental group after 6 h of incubation for *V. parahaemolyticus* and after 4 h for *V. vulnificus* and no effect was observed in the control group throughout the incubation time. A further increase in IgY concentration to 150 mg/ml in the incubation mixture resulted in a further decrease in viable cell count of the experimental group, and no effect was observed in the control group for both *V. parahaemolyticus* and *V. vulnificus*. Therefore, the inhibitory effect of specific IgY powder on the growth of *V. parahaemolyticus* and *V. vulnificus* was dose-dependent. The presence of different concentrations (50, 100, and 150 mg/ml) of nonspecific IgY powder in the incubation mixture did not have any effect on the growth of bacteria, as the growth pattern in these groups was similar to those incubated in the absence of IgY.

Effect of IgY on *V. parahaemolyticus* and *V. vulnificus* in Mice

When the protective effect of specific IgY against *V. parahaemolyticus* and *V. vulnificus* infection was examined in mice, fecal shedding of bacteria revealed a significant reduction in the number of bacterial count in mice pre-treated with specific IgYs compared with the control groups (Fig. 6A and 6B). Fecal shedding of bacteria in the control groups pre-treated with either PBS or nonspecific IgYs was found to be similar in both *V. parahaemolyticus* and *V. vulnificus* infected mice. A further monitoring of mice survival for seven days following bacteria inoculation showed higher survival of mice in the experimental groups pre-treated with either anti-*V. parahaemolyticus* (75% survival) or anti-*V. vulnificus* (87% survival) IgYs, compared with those in the control groups pre-treated with PBS or nonspecific IgY. High death rates were encountered in the control mice, and all were dead within three days after bacteria inoculation (Fig. 7A and 7B). This suggests that specific IgYs have a protective effect against infection caused by *V. parahaemolyticus* and *V. vulnificus*, and hence increase the survival of the host animal.

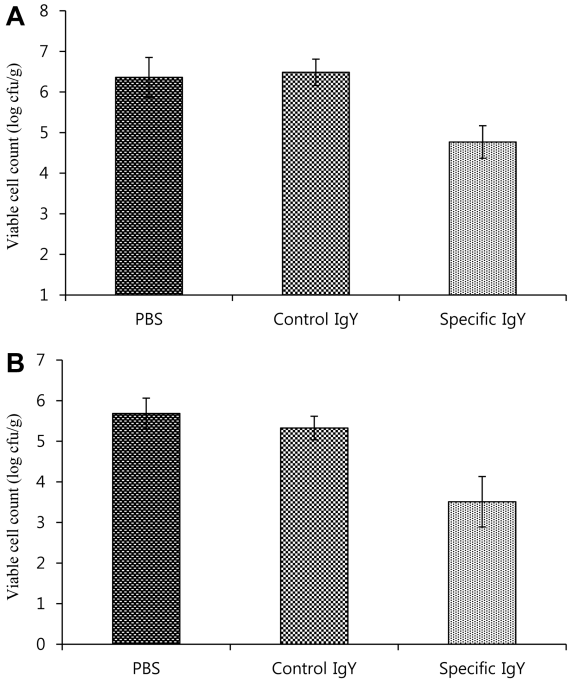

Fig. 6. Fecal shedding of (**A**) *V. parahaemolyticus* and (**B**) *V. vulnificus* in orally infected mice pre-treated with PBS, control, or specific IgYs.

DISCUSSION

Several studies have been done to explore the usefulness of avian immunoglobulin Y in food, drug, microbial, and

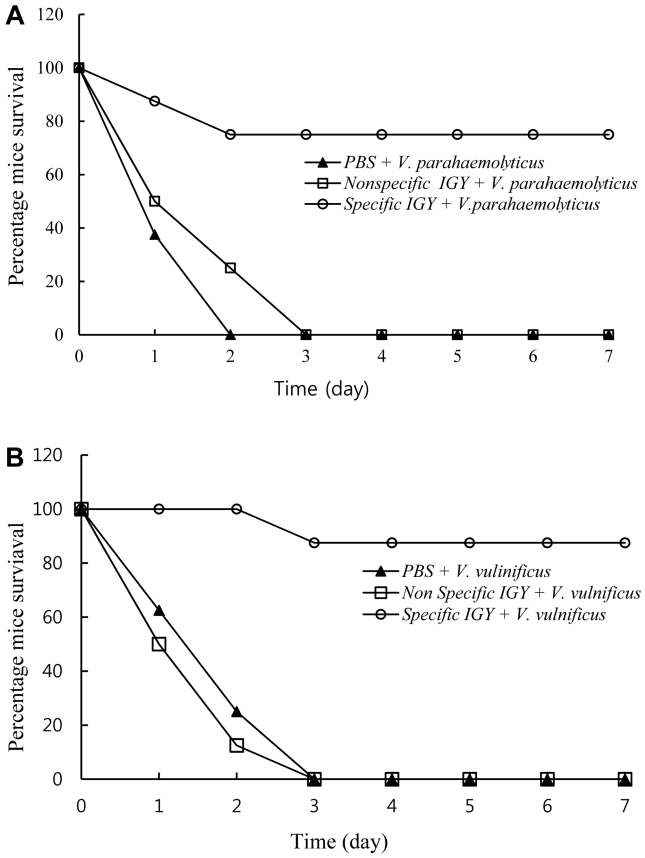

Fig. 7. Survival of mice orally inoculated with (**A**) *V. parahaemolyticus* or (**B**) *V. vulnificus* after pre-treatment with PBS, control, or specific IgYs.

residual analyses [2, 36, 40], immunodiagnostics [11, 12], passive immunization, and therapeutic functions [9, 17, 23, 25]. The advantages of using avians such as chicken and quails as a source of immunoglobulins have also been narrated [10, 13, 14, 18, 22]. Based on the previous study [19], chicken egg yolk antibodies targeting *V. parahaemolyticus* and *V. vulnificus* were produced. The analysis of the WSF of the egg yolk protein on SDS-PAGE resulted into two main bands as heavy and light chains of IgY, along with minor bands of other proteins contained in the fraction. These minor proteins could be omitted by further purification of IgY from the fraction. However, for the purpose of this work, the crude protein extract was sufficient for the subsequent experiments on the antibacterial effect of IgY, and hence averting further addition of chemicals in the mixture, which could have a negative impact on the *in vitro* and *in vivo* assays. In other related studies, 2 bands of heavy and light chains of both chicken and quail IgYs at the same molecular weights on SDS-PAGE analysis under reducing conditions were obtained [4, 19].

The titer of the specific antibody produced in immunized hens increase rapidly from the second week and reached a peak at the fourth week and was maintained stable for a further six weeks of observation, regardless of the booster immunization being given at the 8^{th} week. At this point, the conversion of nonspecific to specific IgY might have reached a maximum; however, the booster immunization might have supported the stability phenomenon observed from the 4^{th} to the 10^{th} weeks of observation. This rapid increase in antibody titer implies a quick immune response in immunized hens. The stability of IgY titer potentiates a longer period and subsequently a large quantity of antibody can be harvested from hens. When the same immunogens were used to produce IgY in quail, a relatively slow immuneresponse and instability of the IgY titer were observed [19], signifying that hens may be of more advantage if a large amount of antibody needs to be harvested for further applications, such as for passive immunization, therapeutic or prophylactic purposes.

The protein and total IgY contents of the WSF were found to be similar in both control and immunized hens, and it was maintained almost stable throughout the immunization period. The concentrations of protein and total IgY were not influenced by the immunization schedule, but rather by the isolation process, which may have resulted in minor differences from one isolation batch to another. These analogous findings of similar concentrations of protein and total IgY in control and experimental animals throughout the immunization period have also been reported [23, 38, 39]. Irrespective of the constant concentrations of protein and total IgY, specific IgY in the same WSF increased with the increase in immunization period to day 49, where the peak was reached and maintained constant. This signifies that the amounts of protein and total IgY are not influenced by immunization, whereas the specific IgY is initiated and increased with immunization.

The produced IgYs were highly sensitive to their corresponding antigens, demonstrated by a dilution of 1:5,000 on ELISA. A relatively high specificity was observed when the affinity of both IgYs was tested with other pathogenic bacteria including *L. monocytogenes*, *S. aureus*, *S. typhimurium*, *B. cereus*, and *E. coli*. However, when the specificity of the IgYs was tested against other *Vibrio* species including *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, and *V. mimicus*, anti-*V. parahaemolyticus* IgY demonstrated a cross-reactivity of up to 69.85%, whereas anti-*V. vulnificus* IgY showed a relatively low cross-reactivity of up to 52.42%. The observed cross-reactivity might have been attributed by similarities in the *V. parahaemolyticus* and *V. vulnificus* epitopes with those of other *Vibrio* spp. tested.

The bactericidal and bacteriostatic effects of specific IgYs has been associated with a mechanism of antibody to opsonize bacteria for phagocytosis or activation of compliment for lysis as well as the ability of antibody to inhibit growth or killing of bacteria [8, 15, 17, 27]. The prepared specific IgY powder showed a significant antibacterial activity on the growth of *V. parahaemolyticus* and *V. vulnificus*. The dose-dependent pattern of the antibacterial effect observed in this study is supported by similar findings on other pathogenic bacteria such as *E. coli* [38, 39, 42], *Salmonella* spp. [7, 39] *Staphylococcus aureus* [15], and *Streptococcus mitis* [28]. These findings substantiate the potential use of IgY in food applications, owing to its ability to inhibit or neutralize pathogenic activities associated with infectious diseases. The protective effect of specific IgY against *V. parahaemolyticus* and *V. vulnificus* infection was demonstrated in mice. A reduction in the number of colony forming units in experimental mice feces compared with those of the control groups was observed. Furthermore, mice survival was higher in the experimental group compared with the control for both *V. parahaemolyticus* and *V. vulnificus* infected mice. Similar findings on the protective effect of specific IgY against *V. cholerae* infection in suckling mice [17] and *Yersinia ruckeri* infection in rainbow trout [25] have been reported. These findings are supported by the previous findings that suggested the possible use of specific IgYs as alternative antibiotics [37] and preservatives.

In conclusion, the produced IgYs showed a good titer with high reactivity to their corresponding antigens. The protein and total IgYs contents were not influenced by the immunization schedule, whereas the specific IgYs increased with immunization. IgYs showed a significant inhibitory effect on the growth and multiplication of *V. parahaemolyticus* and *V. vulnificus* both in liquid medium and in the mice model. Having an essential antibacterial effect both *in vitro* and *in vivo*, IgYs can be used as an alternative antibiotic or an oral immunotherapeutic agent against *Vibrio*-associated

foodborne illnesses. Moreover, in food industries, IgYs can be used as an alternative to chemical preservatives to prevent further multiplication of *Vibrio* spp.

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