Growth Inhibition of *Escherichia coli* 987P by Neutralizing IgY Antibodies

Hoon H. Sunwoo*, Eun N. Lee, Naiyana Gujral and Mavanur R. Suresh

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, 114 St - 89 Ave, Edmonton, Alberta T6G 2N8, Canada

Abstract: Egg yolk antibodies, called IgY, were isolated from the egg yolks of chickens immunized with enterotoxigenic *Escherichia coli* (ETEC) 987P. Specific binding activity of IgY against ETEC 987P was found. Whole cell titers remained relatively high during the immunization period (up to 19 weeks). The IgY antibodies cross-reacted with other members of Enterobacteriaceae, *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *S. typhimurium* by 28.3%, 21.1% and 19.7%, respectively. Specific IgY with a concentration higher than 0.54 mg/ml was found to inhibit the growth of ETEC 987P in a liquid medium. The difference in bacterial growth between the specific and the non-specific IgY was 6.1 log CFU/ml after 8 hr incubation. The specific binding activity of IgY to bacteria was further evaluated by immunofluorescence and immunoelectron microscopy, showing the binding of specific IgY to the bacterial surface. The immunoelectron microscopic observation revealed structural alterations on the bacterial surface bound by specific IgY. This proves that specific binding activity of IgY against ETEC 987P whole cells resulted in growth inhibition of effect ETEC 987P *in vitro*.

Keywords: Egg yolk antibody (IgY), *Escherichia coli* 987P, specific binding activity, growth inhibition, immunofluorescence, immunoelectron microscopy.

INTRODUCTION

Chickens are known to be effective antibody producers that produce immunoglobulin Y (IgY) in the egg yolk [1]. The IgY present in chicken egg yolk can be produced in large quantities and is easily purified compared to mammalian IgG. The IgY is also relatively stable under various conditions, including heat, pressure, acid, alkali, and proteolytic enzyme [2]. Furthermore, IgY does not bind to protein A or G and mammalian Fc receptors, whereas mammalian IgG binds strongly, possibly leading to false positives in samples tested using mammalian IgG [2]. The binding activity of antibodies against antigens is the main immune function, which results in the immobilization or neutralization of antigens. As a polyclonal antibody, IgY has the capability to bind a number of different antigenic epitopes and has more possibilities to react with antigens than monoclonal antibodies, and is thus most commonly used as immunological tools. These advantageous characteristics of IgY allow to achieve a wide range of application such as passive immunization, diagnostic test and protein isolation [3-6].

The binding activity of antibodies may be considered as an important factor for various applications where antibodies can function in capturing or reacting with antigens. Antibodies, in this regard, should be characterized to show the degree of specific activities they have and how they immobilize antigens, mainly from pathogenic bacteria and viruses. In terms of avidity and specificity, anti-human interleukin-6 IgY antibodies have values that are similar to the ones produced in sheep [7]. Although there have been several reports on the effectiveness of IgY in the prevention and control of infectious diseases caused by pathogenic bacteria such as Enterotoxigenic *Escherichia coli* (ETEC), the mechanism of passive immunization using antibodies is not fully understood. Oral administration of specific IgY against ETEC protected piglets from fatal enteric colibacillosis [8]. The inhibition of ETEC adhesion to piglet intestinal cells or mucosa by IgY was suggested as one of the potential reasons for the reduction in pathogenicity of this bacterium [9]. However, more studies are needed to demonstrate anti-bacterial activities of IgY.

Therefore, the present study was conducted to characterize IgY specific against ETEC 987P in terms of productivity, specificity, and activity using ELISA, growth inhibition assay, and microscopic analysis. ETEC 987P was chosen as the antigen to produce IgY since it is commonly responsible for enteric enterotoxic collibacillosis in newborn or post weaning pigs [10, 11].

MATERIALS AND METHODS

Bacteria and Culture Conditions

A local strain of ETEC 987P (Em 88-1604) was obtained from Animal Health Laboratories Branch, Alberta Agriculture, Food and Rural Development, Edmonton, Alberta, Canada. *Salmonella enteritidis* (ESO 9325-92), *Salmonella typhimurium* (ATCC 14028) and *E. coli* O157:H7 (ATCC 43895) were cultured in E-media broth (0.1 mM $MgSO_47H_2O$, 0.01 M Citric acid, 0.06 M K_2HPO_4, 0.02 M $NaNH_4HPO_4H_2O$, 0.002% (v/v) chloroform, 0.5% (v/v) dextrose) at 37°C for 48 hr with shaking. After

*Address correspondence to this author at the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, 114 St - 89 Ave, Edmonton, Alberta T6G 2N8, Canada; Tel: 780-492-0547; Fax: 780-492-1217;
E-mail: hsunwoo@ualberta.ca

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incubation, cells were harvested by centrifugation at 5,000 × *g* for 15 min and were treated with 3.7% formalin overnight. The inactivated cells were washed three times, suspended in a sterile saline and then freeze-dried. Lyophilized whole cell cultures were stored at -20°C until use.

Immunization of Chickens

Laying hens were maintained in accordance to the guidelines of the Canadian Council on Animal Care. Immunization of hens was carried out as previously described [13]. Lyophilized ETEC 987P (500 µg of cell/ml; 15.6 µg of protein/ml) was suspended in sterile phosphate buffered saline (PBS, pH 7.2) and emulsified with an equal volume of a Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA). Eight 23-weeks-old Single Comb White Leghorn (SCWL) chickens were intramuscularly injected with the emulsified saline, with or without the cells at four different sites (0.25 ml per site) in the breast muscles (two sites per left or right breast muscle). A booster immunization was given after two weeks and four weeks of the initial immunization. Eggs were collected daily and stored at 4°C until the extraction of IgY antibody.

Isolation of Anti-ETEC 987P IgY

The water soluble fraction (WSF) containing IgY was prepared from egg yolk using the water dilution method [14]. The egg yolk was physically separated from egg white by mixing gently with 8 volumes of cold distilled water (acidified with 0.1 M HCl to give a pH of 4.0) to avoid possible disruptions of egg yolk granules due to the presence of high concentrations of acid. Cold acidified distilled water (pH 2.0) was then added to make the final dilution of 1:10. After mixing, the pH of the diluted egg yolk was adjusted to around 5.0 to 5.2 and then incubated at 4°C for 12 hr. The WSF was obtained by centrifugation (3,125 × g) at 4°C for 20 min. The supernatant was collected and considered to be the IgY enriched WSF and then titrated by indirect ELISA using lyophilized ETEC 987P as a coating antigen. The fractions found to have the higher titers were further neutralized with 0.1 N NaOH, lyophilized, and then analyzed for the concentrations of protein, total IgY and specific IgY.

Enzyme-Linked Immunosorbent Activity Assay (ELISA)

The WSF and the lyophilized WSF containing IgY was assayed by an ELISA procedure as previously described [12, 14] with the following modifications.

Specific Binding Activity of IgY

Microtiter plates (Costar, Corning, NY, USA) were coated with 150 µl of lyophilized ETEC 987P in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at a concentration of 15.6 µg of protein/ml (0.5 mg of cell/ml) and incubated at 37°C for 90 min. The plates were then washed four times with PBS containing 0.05% Tween 20 (PBS-Tween). After washing, 200 µl of 1% BSA solution (w/v) in PBS-Tween (0.05%) was added to each well and the plates were again incubated at 37°C for 45 min. The BSA solution was then discarded and the wells were washed four times with PBS-Tween. The WSF (diluted 1:1,000 in PBS-Tween) containing specific IgY or non-specific IgY as a control was added to each wells (150 µl per well) and the plates were further incubated at 37°C for 1 hr. After washing 100 µl of rabbit anti-chicken IgY conjugated with horseradish peroxidase (HRPO) (diluted 1:3,000 in PBS-Tween, Sigma, St. Louis, MO, USA) were added to each well and incubated at 37°C for 90 min. The plates were washed and then 100 µl of freshly prepared substrate solution, 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO, USA) in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide was added to each well. After 30 min, optical density was read at 405 nm (OD_{405}) using an ELISA V_{max} kinetic microplate reader (Molecular Devices Corp, Sunnyvale, CA, USA). The ELISA value of antibody binding activity was determined by subtracting the value of the control antibody from that of specific antibody.

Cross-Reactivity of IgY

The cross-reactivity of IgY between ETEC 987P and *E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium* was determined by using the above ELISA method. All bacteria, except ETEC 987P, were obtained from ATCC. Wells of the microtiter plate were coated with 150 µl of four different lyophilized bacteria in carbonate-bicarbonate buffer (ETEC 987P, 0.5 mg/ml; *E. coli* O157:H7, 0.31 mg/ml; *S. enteritidis,* 1.67 mg/ml; and *S. typhimurium*, 1.67 mg/ml). The anti-ETEC 987P IgY powder was reconstituted in PBS-Tween at a dilution of 1:1,000 (0.16 to 0.04 mg/ml). Reconstituted IgY solution (150 µl per well) was added to each well to react with the coated antigens. The cross-reactivity of IgY against selected bacteria was determined by comparing optical density values from the bacteria to the values from ETEC 987P.

Total IgY Concentration

To quantify the total IgY concentrsation in the lyophilized WSF, ELISA method was performed as described above, except the microtiter plates were coated with 150 µl of rabbit anti-chicken IgY at a final concentration of 3.75 µg/ml. Specific and non-specific IgY powder were reconstituted and serially diluted in PBS (2 to 0.125 µg/ml). Two-fold serial dilutions of purified chicken IgY (Sigma, St. Louis, MO, USA) in PBS (0.5 to 0.031 µg/ml) were used as the reference antibody to make a standard curve. The standard curve was then used to estimate the total IgY concentration in the samples.

Antigen Specific IgY Concentration

The concentration of ETEC 987P-specific IgY was determined using the ELISA method as previously described [14, 15]. Wells of microtiter plate were coated with 150 µl of rabbit anti-chicken IgY or lyophilized ETEC 987P at a concentration of 3.75 µg/ml and 0.5 mg/ml in carbonate-bicarbonate buffer (0.05 M, pH 9.6) respectively. After incubation at 37°C for 90 min, the plates were washed four times with PBS-Tween. Two hundred µl of 1% BSA solution (w/v) in PBS-Tween were then added to each well and incubated at 37°C for 45 min. Following four washing steps with PBS-Tween, ETEC 987P specific IgY (25, 12.5 and 6.25 µg/ml) or non-specific IgY (4.5, 2.25, and 1.13 mg/ml) in PBS were added to wells (150 µl per well) coated with ETEC 987P. Wells coated with rabbit anti-chicken IgY were filled with 2-fold serial dilutions of purified chicken IgY (Sigma, St. Louis, MO, USA) in PBS-Tween (0.5 to

0.008 μg/ml). The plates were again incubated at 37°C for 90 min and then washed. Other procedures, including addition of secondary antibody and substrate, and the measurement of absorbance, were described above. The OD_{405} was converted to μg of specific IgY/mg of IgY powder by using a quantitative standard curve (titration curve of rabbit anti-chicken IgY and purified chicken IgY).

Protein Assay

Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), based on the method of Bradford (1976) was performed using purified chicken IgY (1 mg of protein/ml, Sigma, St. Louis, MO, USA) as the reference protein. A serial dilution of the IgY powder and reference protein in PBS (0.5 to 0.0625 mg/ml) was assayed on the microtiter plate. Absorbance of OD_{595} was taken after 5 min using an ELISA V_{max} kinetic microplate reader. The protein concentration of the lyophilized WSF containing specific or non-specific IgY was also measured using the same procedures.

Growth Inhibition Assay

Preparation of Bacteria

The strain of ETEC 987P, used as an antigen for immunizing the chickens, was subcultured on a blood agar plate (5% defibrinated sheep blood in Columbia Agar; Oxoid, Basingstoke, Hampshire, England) at 37°C overnight and then suspended in E-media broth. The suspension was adjusted to an OD_{600} of 0.05, corresponding to a cell density of approximately 1×10^5 CFU/ml. The same volume of 20% (v/v) of glycerol in E-media broth was added and stored at -70°C until used.

Bacterial Growth Curve

Two ml of prepared bacteria culture were mixed with 2 ml of E-media broth and incubated at 37°C with shaking. The turbidity of the culture (OD_{600}) was measured using a spectrophotometer (Bausch and Lomb Spectronic 20, Rochester, NY, USA) at 1 hr interval. The growth curve was plotted until stationary phase was reached. The curve was used to determine the time of sampling for the growth inhibition assay.

Preparation of IgY Solution

To evaluate the effect of the amount of IgY on the growth inhibition of the bacteria, different concentrations of IgY solution were considered. Lyophilized WSFs containing specific or non-specific IgY were reconstituted to 90, 180, and 360 mg/ml (equivalent to 0.045 mg/ml, 0.09 mg/ml, 0.18 mg/ml of specific or non-specific IgY) with E-media broth and then centrifuged at $1,500 \times g$ at 4°C for 20 min. The supernatant was sterilized by using a 0.22 μm-pore-size membrane filter (Millipore, Bedford, MA, USA).

Growth of ETEC 987P with IgY

Two ml of specific or non-specific IgY solution were added to the same volume of the prepared ETEC 987P culture. The bacteria and IgY were incubated at 37°C with shaking. Aliquots of samples (100 μl) were taken at 0, 2, 4, 8 and 24 hr of incubation time. Plate counts were performed by the spread plate method on TSB agar plates (Difco, Detroit, MI, USA) in duplicate. The inoculated plates were incubated at 37°C overnight. The number of colony-forming units (CFU) per plate was counted to determine the total number of bacteria in CFU per ml of sample.

Microscopic Analyses

Immunofluorescence Microscopy

A 100 μl of ETEC 987P cells suspended in PBS (1.7×10^7 CFU/ml) was incubated with the same volume of specific IgY or non-specific IgY (100 μg of the lyophilized WSF/ml in PBS) or without IgY at 37°C for 1 hr. After washing with PBS two times, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgY (Sigma, St. Louis, MO, USA) diluted 1:250 in PBS was added and then incubated at 37°C for 1 hr. The samples were washed as described before and resuspended in 50 μl of PBS. A cell suspension (10 μl) was smeared onto a microscope slide and air dried, then the cover slip was mounted by using a drop of mounting buffer (Glycerol-PBS, pH 7.2). Immunofluorescent staining of specimens was detected by using a 2001 confocal laser scanning microscope (Molecular Dynamics, Uppsala, Sweden).

Immunoelectron Microscopy

One ml of ETEC 987P cells suspended in PBS (1.7×10^7 CFU/ml) was centrifuged at $12,800 \times g$ for 10 min. An equal volume of specific IgY or non-specific IgY (500 μg of the lyophilized WSF/ml of 1% BSA in PBS) was also mixed with the cell pellet. After incubation at 37°C for 1 hr, samples were washed with 1% BSA in PBS two times and then mixed with 100 μl of rabbit anti-chicken IgY (Sigma, St. Louis, MO, USA; diluted 1:14 in 1% BSA in PBS), followed by incubation at 37°C for 1 hr. After washing, samples were incubated with 300 μl of goat anti-rabbit IgY gold conjugate (Sigma, St. Louis, MO, USA; diluted 1:25 in 1% BSA in PBS). The suspended cells were used for negative staining and ultrathin sectioning.

For negative staining, bacterial cells were washed with distilled water two times and subsequently mounted on a 300 mesh copper grid. Grid-mounted samples were stained with 2% uranyl acetate. After washing and drying, specimens were observed with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

To prepare ultrathin sections, bacteria treated with IgY antibodies were washed with 1% BSA in PBS two times, fixed with 2.5% glutaraldehyde for 1 hr, and post fixed with 1% osmium tetroxide for 1 hr. The fixed samples were dehydrated in a graded series of ethanol and embedded in Spurr's medium. After infiltration with Spurr's medium, polymerization was accomplished at 70°C for 12 hr. The specimens were then thin sectioned with an ultramicrotome (Ultracut E model, Reichert-Jung, Austria). Ultrathin sections were mounted on the 200 mesh copper grid and stained with 2% uranyl acetate and then with lead citrate. The specimens were examined with a transmission electron microscope (Hitachi H-7,000 TEM, Tokyo, Japan).

Statistical Analyses

Data were analyzed by a *t-test*. All statistical analyses were accomplished using the general linear model procedure of SAS® software (SAS Institute, 1991). Data are presented

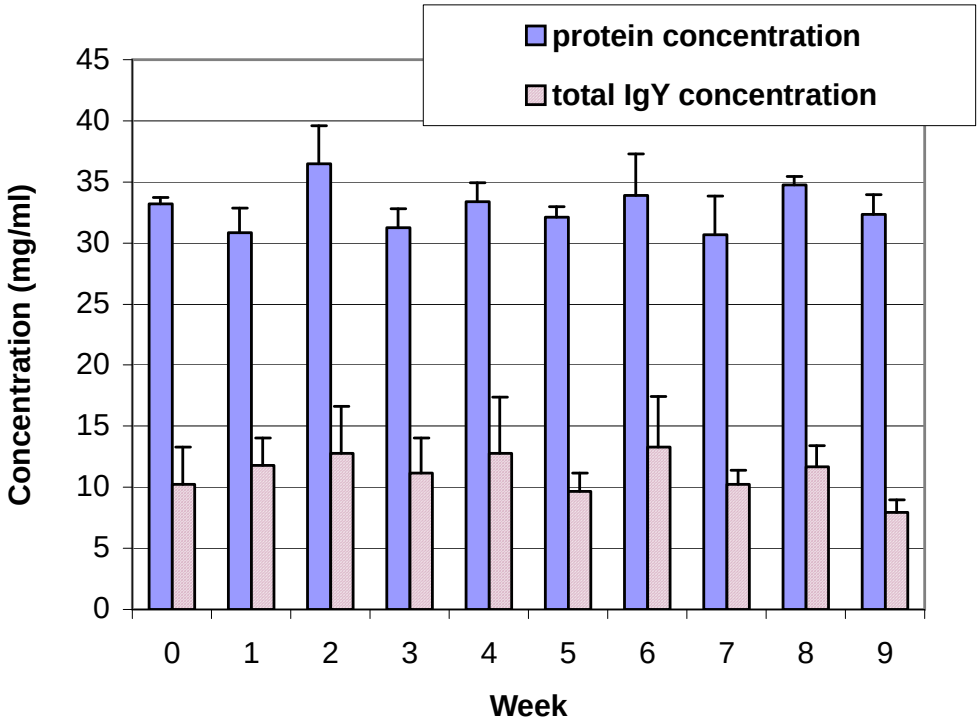

Fig. (1). The concentrations of protein and total IgY in the water-soluble fraction obtained from chickens immunized with ETEC 987P during the immunization period. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation.

as means ± standard deviation. A probability level of $p < 0.05$ was statistically considered as a significant difference.

RESULTS

Concentrations of Protein, Total and Specific IgY in the WSF

The WSF obtained every week during the immunization period (up to 9 weeks) was analyzed for the concentration of protein and total IgY. The average (± standard deviation) concentrations of protein and total IgY were 32.90 ± 1.84 and 11.16 ± 1.66 mg/ml, respectively. The purity of IgY was 33.9%, which was based on the amount of total IgY in protein content. Both protein and total IgY concentrations were relatively constant ($p > 0.05$) as shown in Fig. (**1**).

The WSF was prepared from eggs collected everyday during the 5 to 9 weeks and was used to determine the specific binding activities against ETEC 987P (Fig. **2**). The lyophilized WSF contained 540 ± 40, 90 ± 26, and 6 ± 0.7 mg/g of protein, total IgY, and specific IgY respectively (Table **1**). The protein and the total IgY concentrations of the control sample were comparable to the above experimental sample ($p < 0.05$). The antigen specific IgY concentration of the control sample was significantly lower than the experimental sample containing antigen specific IgY ($p < 0.05$). The proportion of ETEC 987P-specific IgY in total IgY concentration was 6.7%.

Binding Activity of IgY Against ETEC 987P

The specific binding activity of IgY in the WSF was determined by the ELISA method every week during the immunization period (Fig. **2**). The level of binding activity increased a week after the first immunization followed by an exponential rise. The level increased continuously to a maximum of OD_{405} 0.855 three weeks after the second booster immunization. IgY activity, thereafter, fell to a minimum of 0.564, which was a decrease of 34% in comparison to the maximum value. Subsequently, the level of IgY activity remained relatively high showing no considerable decline throughout the period that the eggs were monitored (up to 19 weeks).

Cross Reactivity of IgY

The cross-reactivity of IgY against other members of the Enterobacteriaceae family (*E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium*) was measured as presented in Fig. (**3**). Anti-ETEC 987P IgY cross-reacted with *E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium* by 28.3%, 21.1% and 19.7%, respectively, when the activity of IgY against ETEC 987P is considered to be 100%.

Growth Inhibition Assay of IgY

The growth curve of ETEC 987P consisted of lag phase (0 to 2 hr of incubation time), followed by exponential growth and then stationary phase (data not shown). The growth inhibition assay designed and performed on the basis of the previous growth curve, showed different results depending on the concentration of IgY. There was no significant difference ($p > 0.05$) between the growth of ETEC 987P with specific IgY (specific group) and that of bacteria with non-specific IgY (control group) in the experiment using 0.045 mg/ml of IgY (Fig. **4a**). In contrast, bacterial growth was different between the two groups in the

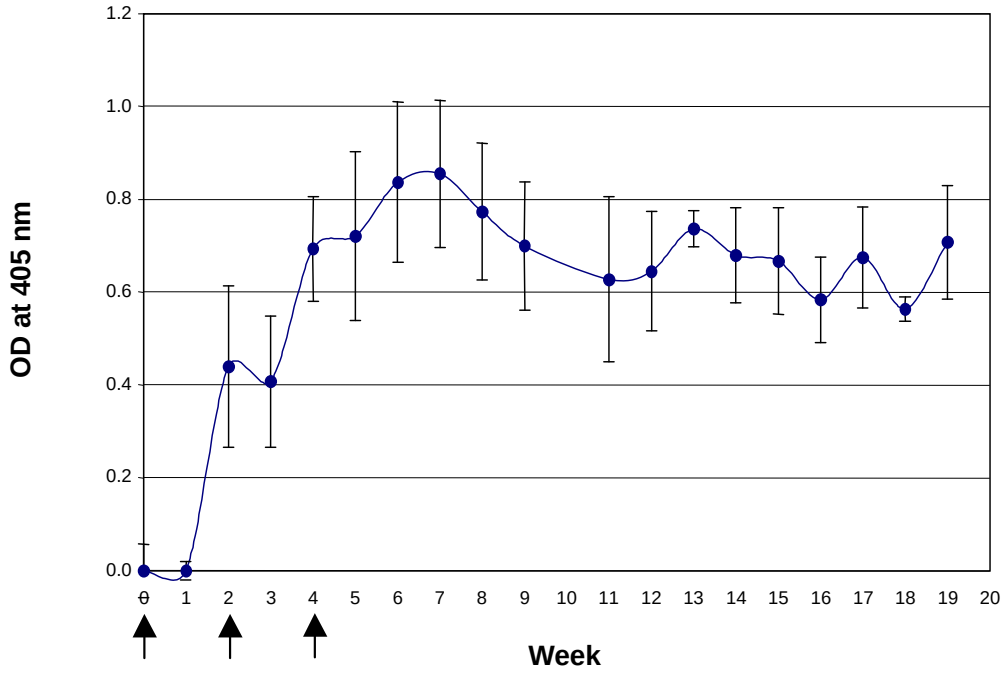

Fig. (2). The change in IgY specific binding activity in the WSF obtained from chickens immunized with ETEC 987P during the immunization period measured by ELISA method. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. Arrows indicate weeks of immunization.

Table 1. The Concentrations of Protein, Total IgY and Specific IgY in the Lyophilized WSF Containing ETEC 987P-Specific or Non-Specific IgY. Values are the Mean ± Standard Deviation

	Concentration (mg/g)		
Lyophilized WSF	**Protein**	**Total IgY**	**Specific IgY**
ETEC 987P-specific	540 ± 40	90 ± 26	6 ± 0.7
Non-specific	468 ± 62	93 ± 21	< 0.0001

presence of a higher concentration of IgY. As shown in Fig. (**4b**), bacteria were in lag phase during 2 hr incubation. During the exponential phase, the increase in number of ETEC 987P incubated with specific IgY was less than that of the control group. Cell counts of the control group increased by 1.9 log CFU/ml during 2-4 hr of incubation time while those of the specific group increased by 1.0 log CFU/ml. During 4-8 hr of incubation time, bacterial cell counts of the control group increased by 1.9 log CFU/ml while those of the specific group decreased by 0.8 log CFU/ml. After 8 hr incubation, there was 3.0 log CFU/ml increase and 0.4 log CFU/ml increase in the CFU of the control and specific group, respectively. The difference in bacterial growth between the two groups was 6.0 log CFU/ml at 24 hr of incubation time. The concentration of specific IgY, which was effective in the inhibition of ETEC 987P growth, was 0.54 mg/ml without considering losses of specific IgY during the preparation of IgY solution. A two-fold greater concentration of IgY (0.18 g/ml) also had an inhibitory effect on the growth of bacteria. The number of bacteria grown with specific IgY was reduced by 6.1 log CFU/ml when compared to that of the control group after 24 hr incubation (Fig. **4c**). As such, the growth of ETEC 987P in a liquid medium was inhibited by the presence of specific IgY raised against the homologous strain.

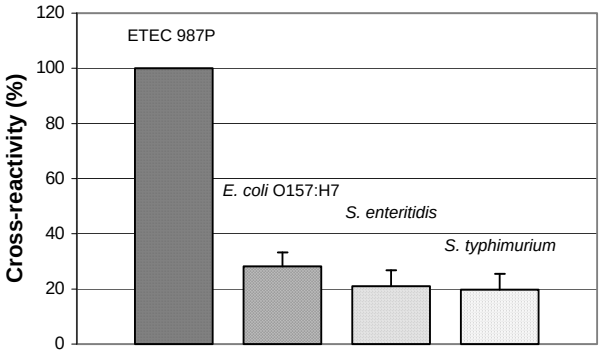

Fig. (3). The cross-reactivity of anti-ETEC 987P IgY with other members of Enterobacteriaceae, including *E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium*. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation.

Microscopic Observation of IgY Binding to ETEC 987P

The binding of IgY to ETEC 987P was observed using immunofluorescence microscopy. Bacteria reacting with specific IgY showed fluorescence, whereas bacteria in both control groups showed no fluorescence (data not shown). The presence of fluorescence was indicative of the binding

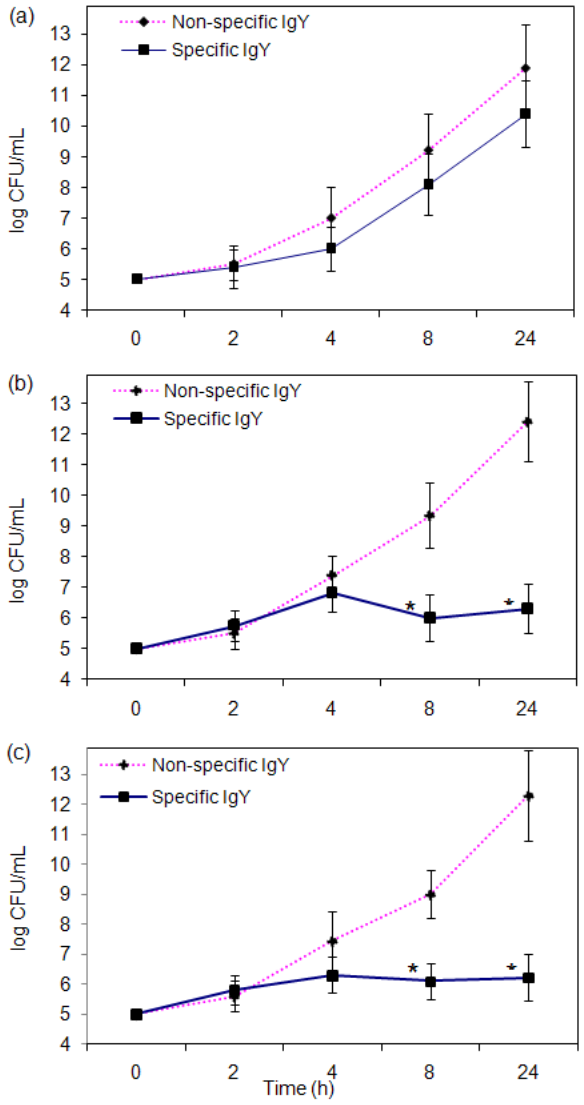

Fig. (4). The effect of IgY on the growth of ETEC 987P in a liquid medium. ETEC 987P (approximately 1×10^5 CFU/ml) were grown in E-media broth mixed with different concentrations of ETEC 987P-specific or non-specific IgY at 37°C with shaking: (**a**) 0.045 mg/ml; (**b**) 0.09 mg/ml; (**c**) 0.18 mg/ml. The viable cells were counted by the plate count method. Values are the mean of duplicate samples. Vertical bars indicate the standard deviation. The solid line shows the growth pattern of ETEC 987P with specific IgY. The dotted line shows that of ETEC 987P with non-specific IgY as a control. *Means differ significantly ($P < 0.05$).

of specific IgY to the bacteria. Immunoelectron micrographs presents the distribution of immunogold particles around ETEC 987P that reacted with specific IgY (Figs. **5a**, **6a**). In contrast, there was no immunogold in the observation of bacteria incubated with non-specific IgY (Figs. **5b**, **6b**). The presence of gold particles implied that the bacteria were bound specifically by IgY, as well as, roughly indicated the IgY binding sites or epitopes on the bacteria. It was found that the bacterial surface including the projection was labeled with gold particles and was structurally altered in the observation of bacteria reacting with specific IgY.

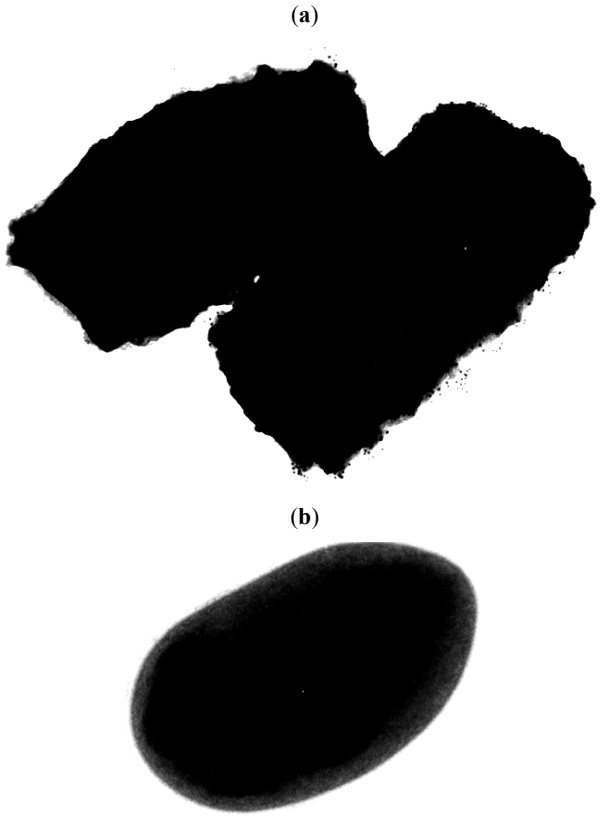

Fig. (5). Immunoelectron micrographs of negatively stained ETEC 987P incubated with (**a**) specific IgY (magnification 6,000 ×); (**b**) non-specific IgY (magnification 9,000 ×).

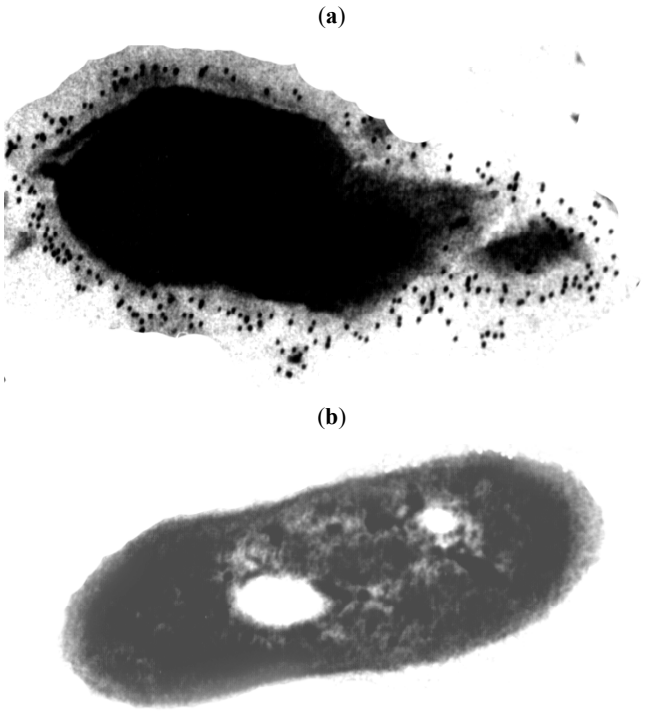

Fig. (6). Immunoelectron micrographs of ultrathin sectioned ETEC 987P incubated with (**a**) specific IgY; (**b**) non-specific IgY (magnification 9,000 ×).

DISCUSSION

Specific IgY against ETEC 987P was obtained from the egg yolk of SCWL chickens immunized with ETEC 987P whole cells. The water dilution method, as a simple and economical process, was used to extract the WSF containing IgY. IgY is known as γ-livetin and exists in egg yolk together with two other water-soluble proteins, α- and β-livetin, and lipoprotein [16]. Therefore, separation of IgY or γ-livetin requires extraction of the WSF from yolk lipoprotein, followed by purification from other livetins [17]. For the extraction of WSF from egg yolk with water, two factors are critical: the pH and the extent of egg yolk dilution [18]. The amount of protein recovered in the supernatant decreased with increased dilution; however, the purity of IgY recovered in the supernatant was higher when the dilution factor was increased. This is probably due to greater removal of other protein components into the pellet fraction. Eight to ten-fold dilutions would be preferred for more efficient separation of lipid (90-93%) from the WSF, with moderate (60%) recovery of IgY [19]. The amount of lipids in the WSF was affected by pH. The WSF was almost devoid of lipids at mild acidic conditions (pH 4.6 to 5.2) with increasing levels of lipids at pH below and above this range [18]. Accordingly, the WSF, in this study, was extracted from egg yolk under the conditions of 10-fold dilution and pH of 5.0-5.2.

The WSF contained 11.16 ± 1.66 mg of total IgY, which could be converted to approximately 13.93 mg/ml, in egg yolk based on the extractability of 80.1% [13]. There have been other reports on the determination of the total IgY concentration in the egg yolk of immunized chicken, where the values differed ranging from 8 to 25 mg/ml. These differences are likely to result from the various methods to isolate and measure IgY since the total IgY concentrations were independent of the species of chicken, the nature of immunogen, and the types of adjuvant used [4, 9, 19, 21-23]. The lyophilized WSF, which was prepared by the freeze-drying for a concentrated form, showed a decrease in IgY purity by 17% compared to that of the WSF. This might result from the freeze-drying effect, which reduced the solubility and ELISA value of IgY. The use of ammonium sulfate or polyethylene glycol may be alternative methods for the concentration of the IgY.

As presented in Fig. (**2**), the immunization of SCWL chickens with ETEC 987P induced a relatively strong immune response for a long duration of time (up to 19 weeks). The immunization was simple, since ETEC 987P whole cells (250 μg of cell; 7.8 μg of protein) were sufficiently immunogenic to induce an immune response in the chickens after the initial immunization. This result indicates that the production of antigen-specific antibody can be efficiently elicited in SCWL chickens immunized intramuscularly with the whole ETEC 987P cells emulsified with Freund's incomplete adjuvant. The ELISA result also indicated that the WSF with IgY specific for ETEC 987P showed high levels of binding. Anti-ETEC 987P IgY showed specificity for ETEC 987P as well as some cross-reactivities with other Enterobacteriaceae (*E. coli* O157:H7, *S. enteritidis*, and *S. typhimurium*, Fig. **3**). Presumably those bacteria share some antigenic-epitopes with ETEC 987P. The common lipopolysaccharide (LPS) of gram-negative bacteria is likely to be one of the factors leading to the cross-reactivity [20, 24]. The IgY could also react with heterologous antigens as well, since IgY is a polyclonal antibody raised against large numbers of bacterial antigens [14, 25].

The binding activity of IgY against ETEC 987P resulted in the inhibition of ETEC 987P growth in a liquid medium (Fig. **4**). The growth inhibitory effect of specific IgY was a concentration dependent, requiring greater than 0.54 mg/ml of IgY. Since crude IgY (16.7% purity, data not shown) obtained by the simple extraction method without further purification was used in this study, a simple and practical quantitative ELISA was used to determine the amount of specific IgY, which was found to be 6.7% of specific IgY in total IgY (Table **1**). The mechanism of the growth inhibitory effect of IgY on ETEC 987P is not clearly understood. The binding of antibody to bacteria may be the main step necessary to cause the bacterial growth inhibition. Antibodies bound to particular components on the bacterial surface possibly block the function of the components that may be crucial for motility and growth of bacteria [14, 25]. IgY used in this study possesses binding activities against various epitopes derived from the bacterial surface due to the characteristic of a polyclonal antibody raised against whole bacterial cells. Therefore, binding activities of IgY against bacterial surface components, including fimbriae (pili), outer membrane protein, and LPS may cause the growth inhibition of bacteria.

Microscopic analyses of IgY binding activity to ETEC 987P support this hypothesis, providing detailed images of bacteria bound by specific IgY. Immunofluorescence microscopy may be suitable as a preliminary test for further study of immunoelectron microscopy due to its simple procedure. Positive results of immunofluorescence microscopy demonstrated the binding activity of IgY against bacteria (data not shown). Immunoelectron micrographs (Figs. **5a**, **6a**) presented immunogold particles distributed around ETEC 987P, proving bacteria bound to specific IgY. It was noted that there were gold particles specifically on the surface of bacteria. Furthermore, a structural alteration was observed in sectioned bacteria with bound specific IgY as displayed in Fig. (**6a**). These findings indicate that IgY binding to the surface components of bacteria may cause structural alterations. As speculated in the growth inhibition assay, particular components of the bacterial surface may be bound with IgY, causing bacteria morphological changes and resulting in the impairment of bacterial growth. The characterization of these components, which may be crucial for bacterial growth, is under investigation. The mechanism that leads to the growth inhibition of bacteria following the binding of IgY to bacteria needs to be elucidated as well.

In addition, the results might suggest the mechanism or additional information regarding to IgY specificity for other strains of ETEC [8, 9, 12]. We produced IgY against ETEC 987P and analyzed its characteristics, such as the content of total and specific IgY. The binding activities of IgY were studied on their effects on the bacterial growth and morphological changes. The results of these studies may provide further understanding of IgY immunological properties and support the potential use of IgY for immunological application.

CONCLUSION

SCWL chickens showed strong immune responses to whole ETEC 987P cells and produced IgY specific to the bacterial cell antigens. IgY could be obtained in large quantities by the water dilution method. Specific binding activity of IgY against ETEC 987P whole cells resulted in a growth inhibitory effect on ETEC 987P *in vitro*. Through microscopic observation, it was further found that the binding of specific IgY to ETEC 987P caused structural alterations of the bacterial surface.

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ABBREVIATIONS

ETEC	=	Enterotoxigenic *Escherichia coli*
SCWL	=	Single comb white leghorn
WSF	=	Water-soluble fraction
TSB	=	Tryptic soy broth
FITC	=	Fluorescein isothiocyanate.

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