

## Production of Egg Yolk Immunoglobulin Against *Escherichia coli* From White Leghorn and Lohmann Chickens

<sup>1,2</sup>Jenn-Fa Liou, <sup>1</sup>Jen-Wen Shiau, <sup>3</sup>Chein Tai and <sup>1,3</sup>Lih-Ren Chen

<sup>1</sup>Division of Physiology, Livestock Research Institute, Council of Agriculture, 712 Tainan, R.O.C. Taiwan

<sup>2</sup>National Cheng Kung University, 701 Tainan, R.O.C. Taiwan

<sup>3</sup>Southern Taiwan University of Technology, 710 Tainan, R.O.C. Taiwan

**Abstract:** This study investigates the immunization response of different breeds of chickens to *Escherichia coli* (*E. coli*) antigen and the function of specific Immunoglobulin in Yolk (IgY) produced. The antibody was raised by the intramuscular immunization of White Leghorn hens and Lohmann hens with inactivated *E. coli* antigen either from a Commercial vaccine (CM) or isolated from a local farm (AF). Anti-*E. coli* IgY antibodies were isolated using the water extraction method and their *E. coli* inhibitory effects were then determined. The anti-*E. coli* IgY production levels of White Leghorn chickens/Lohmann chickens immunized with the CM and AF antigens were 65.28/55.78  $\mu\text{g mL}^{-1}$  WSF and 48.31/14.4  $\mu\text{g mL}^{-1}$  WSF, respectively. The specific IgY from White Leghorn chickens immunized with CM antigen exhibited a superior inhibitory effect on the growth of activated *E. coli*. The least effective concentration to inhibit the growth of activate *E. coli* for this specific IgY was 63  $\mu\text{g mL}^{-1}$  WSF. The passive protective effect of egg-yolk antibodies against *E. coli* K99 infection in neonatal piglets indicates that piglets treated with antibodies from the hens immunized against Enterotoxigenic *E. coli* (ETEC) were protected against the deleterious effects of this organism.

**Key words:** IgY, *E. coli*, chicken, immunized, antibodies, egg-yolk

---

### INTRODUCTION

Immunoglobulin in Yolk (IgY) antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibians and are transferred from the serum to the egg yolk in the females to confer passive immunity to their embryos and neonates (Leslie and Clem, 1969). Researchers have studied the potential of orally administered IgY for the prevention and treatment of many pathogens including *Escherichia coli* (Yokoyama *et al.*, 1992; Amaral *et al.*, 2002), *Helicobacter pylori* (Shin *et al.*, 2002), Salmonella (Yokoyama *et al.*, 1998), Rotavirus (Hatta *et al.*, 1993), Staphylococcus (LeClaire *et al.*, 2002), *Streptococcus mutans* (Hatta *et al.*, 1997), Yersinia (Lee *et al.*, 2002), Coronavirus (Ikemori *et al.*, 1997), Porcine epidemic diarrhea virus (Kweon *et al.*, 2000) and Enterovirus (Liou *et al.*, 2010). *E. coli* (*Escherichia coli*) infection causes serious problems and economic losses in pig farms in tropical areas. Piglets are easily attacked by this pathogen, especially during their weaning period; this pathogen results in acute diarrhea and even death. The younger piglets are infected, the more severe the syndrome becomes. Even if the piglets recover, their sub-sequent

development will be retarded. The diarrhea caused by *E. coli* used to be treated with antibiotics. However, prohibition of the use of antibiotics in the animal industry is a developing trend around the world. Thus, it is worthwhile to develop a new approach to prevent or cure *E. coli* infection in pigs.

Previous research shows that IgY is effective in preventing bacteria shedding or infection *in vivo* (Ikemori *et al.*, 1992; Lee *et al.*, 2002). Thus, this study evaluates the production of IgY in different chicken breeds and their protective efficiency against *E. coli*.

### MATERIALS AND METHODS

**Preparation of *E. coli* antigens:** Two inactivated *E. coli* antigens were used in this study. One is a commercial four-in-one vaccine containing *E. coli* pilus antigens of strains K88, K99, 987P and F41 (CM antigen). The other antigen was isolated from a piglet suffering from *E. coli* diarrhea in a local pig farm.

The isolated *E. coli* was cultured in Trypticase Soy Broth (TSB) at 37°C overnight. After adjusting to a concentration of about  $2 \times 10^{10}$  cfu mL<sup>-1</sup>, the *E. coli* solution was centrifuged at 2,000×g for 15 min. The

resulting pellet was then resuspended with 0.4% formaldehyde in Phosphate Buffered Solution (PBS, Invitrogen, USA) and incubated overnight at 37°C for inactivation. Removal of formaldehyde was performed by centrifugation at 2,000×g for 15 min. The pellet was resuspended in the same volume of PBS and the resulting solution was stored at -80°C and used as *E. coli* antigen (AF antigen).

**Immunization of chickens:** Six White Leghorn laying hens and six Lohmann hens at 6 months of age were used for the production of anti-*E. coli* IgY. All the animals were obtained from the Livestock Research Institute, Council of Agriculture, Executive Yuan, Taiwan. Either 1 mL of CM or AF antigen was emulsified with an equal volume of Freund's complete adjuvant and intramuscularly injected to 4 sites in the breast muscle of the chicken. Four booster injections of antigen with Freund's incomplete adjuvant were given at 2, 6, 12 and 16 weeks after the 1st immunization. Before each injection, a 2 mL blood sample was collected from each chicken for serum IgG determination. Eggs were collected daily from the week before immunization to 2 weeks after the last booster immunization and stored at 4°C. The egg yolk was separated, pooled and kept at -20°C prior to the purification of IgY. All egg yolks from each chicken in each week were pooled as an analysis sample.

**Isolation and purification of IgY:** The isolation of IgY was performed as described by Akita and Nakai (1993) with some modifications. The yolk sample was mixed with cold distilled water (acidified with 0.1 N HCl to a pH of 5.0) at a ratio of 1:9 and stirred overnight in an agitator at 4°C. The mixture was then centrifuged at 6139×g, 4°C for 1 h to obtain a Water Soluble Fraction (WSF). The WSF was collected and filtered with No. 1 filter paper to remove remaining lipids and then stored at -20°C for further analysis.

**Antibody activity determination for serum IgG and total IgY specific anti-*E. coli* IgY:** The antibody activity of anti-*E. coli* IgY was determined using the Enzyme-Linked Immuno Sorbent Assay (ELISA) described by Lee *et al.* (2002) with some modifications. Microtiter plates were coated with 100 µL inactivated *E. coli* antigen (10 µg well<sup>-1</sup>) while control wells were coated with rabbit anti-chicken IgY antibody (10 µg mL<sup>-1</sup>, Sigma-aldrich) instead. The plate was incubated overnight at 4°C. After washing with PBS-Tween 20 buffer, 2% BSA blocking proceeded overnight at 4°C. The wells were then washed twice with PBS-Tween 20 buffer and once with PBS. Thereafter, 100-fold diluted WSF was added to the sample

wells (100 µL well<sup>-1</sup>). Control WSF from the same chicken before immunization was used as a background. Wells for standard curve were filled with 100 µL serial-diluted pure chicken IgY at concentrations ranging from 0.015-1 µg mL<sup>-1</sup> (Promega, G116A) and incubated overnight at 4°C.

After washing with PBS-Tween 20 buffer, 100 µL of alkaline phosphate-conjugated goat anti-chicken IgY (Promega, G115A) was added to the wells and incubated for 2 h at 37°C. After washing with PBS-Tween 20 buffer, 100 µL disodium p-nitrophenyl phosphate as substrate (Sigma, N9389) was added to each well and allowed to react for 10 min at 37°C. The absorbance was then measured at 405 nm using a microplate reader (Multiskan MS: Thermo Lab systems). The absorbance of standard curves provided a relative measurement of anti-*E. coli* IgY concentration.

To determine the serum IgG and total IgY each well of the microtiter plate was coated with 100 µL of commercial monoclonal anti-chicken IgG and rabbit anti-chicken IgY antibody (10 µg mL<sup>-1</sup>, Sigma-aldrich), respectively and 100 µL of 10000-fold diluted WSF was then added. The following protocols were performed as described before.

**Determination of *in vitro* inhibitory efficiency for anti-*E. coli* IgY:** The *in vitro* *E. coli* inhibition efficiency of IgY antibody was determined by Co-culture test with active *E. coli* K99. The IgY was purified using ultra filtration with an Amicon Ultra-15 filter (PL-100, Millipore) to condense 50 mL of IgY-containing WSF into 8 mL. These WSF concentrates were subjected to an *in vitro* Inhibition test for *E. coli*. An activated *E. coli* stock solution was prepared by mixing 10 mL of TSB with 5 µL of active *E. coli* isolated from a farm and then cultured overnight at 37°C. The concentration of resulting *E. coli* K99 solution was measured in terms of OD value by a spectrophotometer and then diluted to 2.6×10<sup>7</sup> cfu mL<sup>-1</sup> with TSB. For the Co-culture test, 100 µL of purified anti-*E. coli* IgY obtained from immunization with CM and FA antigen, respectively was added and mixed well with the same volume of diluted *E. coli* K99 solution and then co-cultured for 1 h at 37°C. The mixture was then centrifuged at 167×g for 5 min. Each 170 µL sample of the supernatant was diluted to 10,000 and 100,000-fold and smeared on a plate then cultured overnight at 37°C. The number of *E. coli* colonies formed was counted under a microscope. Purified IgY obtained from the egg of a non-immunized chicken served as a control.

**Immunofluorescence microscopy analyses:** Total 100 µL of *E. coli* K99 cells suspended in PBS (2.6×10<sup>7</sup> cfu mL<sup>-1</sup>)

were incubated with the same volume of specific IgY or non-specific IgY (100 µg of IgY powder mL<sup>-1</sup> PBS) or without IgY for 1 h at 37°C. After washing with PBS two times, Fluorescein Isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG (sigma) diluted 1:250 in PBS was added. The mixture was then incubated for 1 h at 37°C. The samples were washed as before and suspended in 50 µL of PBS. A (10 µL) cell suspension was smeared on a microscope slide which was then air-dried. Immunofluorescent staining of specimens was detected by an immunofluorescence microscope (ZEISS, AXioskop2 plus, Japan) (Sunwoo *et al.*, 2002).

**Passive protection of piglets:** Total 21 days old healthy LYD piglets were randomly divided into control and treatment groups of 10 piglets each. All piglets were challenged with 5 mL of *E. coli* K99 at a dose of 2×10<sup>10</sup> cfu mL<sup>-1</sup> per piglet twice a day for 2 days. The suspension of *E. coli* K99 was administered at 0 and 6 h of the experiment. After the challenge, the piglets in the treatment group were treated with 2 g of egg-yolk antibodies at a concentration of 65.28 µg mL<sup>-1</sup> WSF, two times a day (9:00 a.m. and 4:00 p.m.) for 2 consecutive days after the 1st *E. coli* challenge whereas the control groups received a placebo treatment. The clinical response of each piglet was monitored throughout the experiment in terms of occurrence of diarrhea, fecal consistency score and mortality was performed when diarrhea occurred.

**Statistical analysis:** The data obtained in this study were analyzed using the general linear model of the SAS program and the differences among groups were determined by Duncan's multiple range test (SAS, 1996). Probability values of *p*<0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

The major components of an egg include the egg yolk, egg white and egg shell. The total solid content of an egg yolk is approximately, 50% and the major components of the yolk protein include apovitellenin, phosvitin, lipovitellin, apoproteins and livetins. The water-soluble fraction of yolk plasma consists of 3 kinds of lipoproteins: α, β and γ-livetin. The γ-livetin proved to be an IgG-like immunoglobulin (Polson and von Wechmar, 1980), referred to as IgY. IgY is functionally equal to the IgG of mammals. It is the immunoglobulin transferred from the blood to the egg yolk and plays an important role in the passive protection of embryos in avian, reptile and amphibian species (Leslie and Clem, 1969; Polson and

von Wechmar, 1980). A chicken can lay 280 eggs each year and an egg yolk contains 100-200 mg of IgY antibodies. Antigen specific IgY generally account for 2-10% of the total IgY (Wang *et al.*, 1999). Comparing the production of antibody from traditional immunization of rabbits to the chicken IgY suggests that an immunized hen could yield antibody equivalent to that from around 40 rabbits (Marquardt *et al.*, 1999). The non-invasive system of producing IgY antibody in chickens combined with their high egg production capacity holds great promise for the therapeutic use of IgY. Researchers have thoroughly studied the separation of IgY from the egg yolk along with methods of purifying IgY (Akita and Nakai, 1993; Kim and Nakai, 1998; Narat, 2003). Chicken eggs provide a more cost-efficient, convenient and plentiful source of antibodies, making the large-scale production of specific IgY for immunotherapeutic purpose feasible.

Researchers have successfully applied IgY to the passive protection of disease in the treatment of gastrointestinal pathogens in domestic animals including the protection of diarrhea in neonatal piglets caused by *E. coli* (ETEC) (Yokoyama *et al.*, 1992; Marquardt *et al.*, 1999) and the diarrhea of neonatal calves caused by bovine rotavirus (Kuroki *et al.*, 1994) or bovine coronavirus (Ikemori *et al.*, 1997).

IgY is a constituent of the plasma protein of the yolk. As a water-soluble protein, IgY can easily be extracted from the yolk by centrifugation and filtration (Polson and von Wechmar, 1980). The pH value and caliber of dilution are the two major factors influencing the extraction efficiency of IgY. A low pH of 5.0-5.2 increases the recovery rate of IgY and decreases the LDL residue in WSF (Akita and Nakai, 1992). As for the dilution caliber, extended dilution decreases the recovery and purity of IgY. A dilution caliber of 8-10 fold can increase the efficiency of fat removal and achieve a IgY recovery rate of 60% (Kwan *et al.*, 1991). In addition to the water extraction method, different methods have also been developed for IgY isolation such as extracentrifugation (Mc Bee and Cotterill, 1979), extraction using polyethylene glycol (Polson *et al.*, 1985), polyacryl acid resins (Hamada *et al.*, 1991), sodium dextran sulfate (Jensenius *et al.*, 1981) and carrageenan-xanthan gum (Hatta *et al.*, 1990). Akita and Nakai (1993) compared the purity and production of IgY extracted with water, polyethylene glycol or sodium dextran sulfate and found similar results. However, the water extraction method prevents IgY from possible damage and the residue problems of various solvents and chemical reagents. The water extraction method is a safe, simple and economical method of extraction IgY. Therefore, this study uses the

water extraction method. Figure 1 and 2 show the total IgY extracted from the egg WSF of the White Leghorn chickens and Lohmann chickens after immunization. For White Leghorn chickens, the total IgY produced after immunization with CM and AF antigen averaged 4.09 and 4.28 mg mL<sup>-1</sup> in the yolk WSF, respectively. When the CM and AF antigen was given, the total IgY content in the yolk WSF of Lohmann chickens was 5.33 and 4.68 mg mL<sup>-1</sup> on average, respectively. Sunwoo *et al.* (2002) used *E. coli* strain O157:H7 as an antigen to produce anti-*E. coli* O157:H7 IgY. The resulting total IgY in WSF was 12.58 mg mL<sup>-1</sup> on average. This total IgY yield was much higher than that reported in the current study. Total IgY production has reportedly ranged from 6-25 mg mL<sup>-1</sup> in WSF and demonstrated that the yield could be affected by the methods of IgY extraction and measurement (Rose *et al.*, 1974; Shimizu *et al.*, 1988; Li-Chan *et al.*, 1998). Kariyawasam *et al.* (2004) compared the IgY production efficiency of *E. coli* strains

FimH, IutA, PapG and LPS and indicated that the serum IgG titers induced by these four antigens all significantly increased after the 3rd immunization. The peak level occurred after 4 weeks, lasted for 5 weeks and then declined. This study also determines the serum IgG levels of the White Leghorn and Lohmann chickens after immunization with *E. coli* antigens. Figure 3 shows the changes of serum IgG concentration in the hens after immunization. The serum IgG level of the White Leghorn and Lohmann chickens increased 2 weeks after the 1st injection.

The White Leghorn chickens responded better than the Lohmann chickens after immunization with either CM or AF antigen. The Leghorn chickens exhibited a peak IgG titer of 2,500 and 1,000 µg mL<sup>-1</sup> in the 16 and 20th week after the 1st immunization, respectively. However, the Lohmann chicken group exhibited no significant increase of serum IgG between 2-14 weeks after immunization. The elevation of IgY titer in the Lohmann chickens only appeared from 16 weeks after immunization with the CM antigen but not the AF antigen. These results suggest that the White Leghorn chickens were much more sensitive to the *E. coli* antigens used in this study than the Lohmann chickens. In addition, the antigens of different *E. coli* strains seemed to affect the induction of serum IgG in the Lohmann chickens.

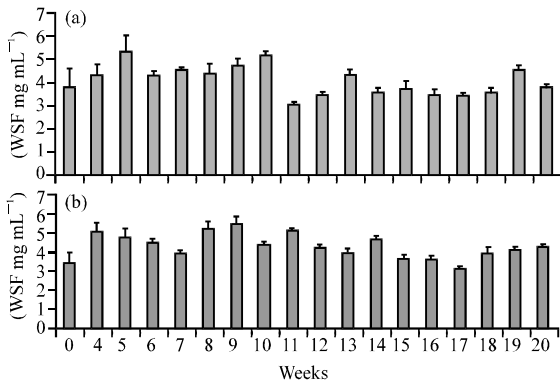


Fig. 1: The concentration of total IgY content for White Leghorn chickens (WSF mg mL<sup>-1</sup>) 0-20 weeks post immunization with CM and AF antigen: a) CM and b) AF

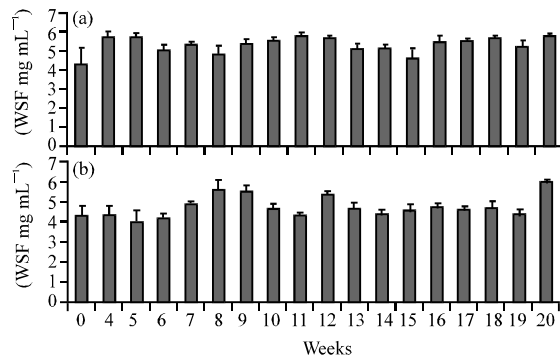


Fig. 2: The concentration of total IgY content for Lohmann chickens (WSF mg mL<sup>-1</sup>) 0-20 weeks post immunization with CM and AF antigen: a) CM and b) AF

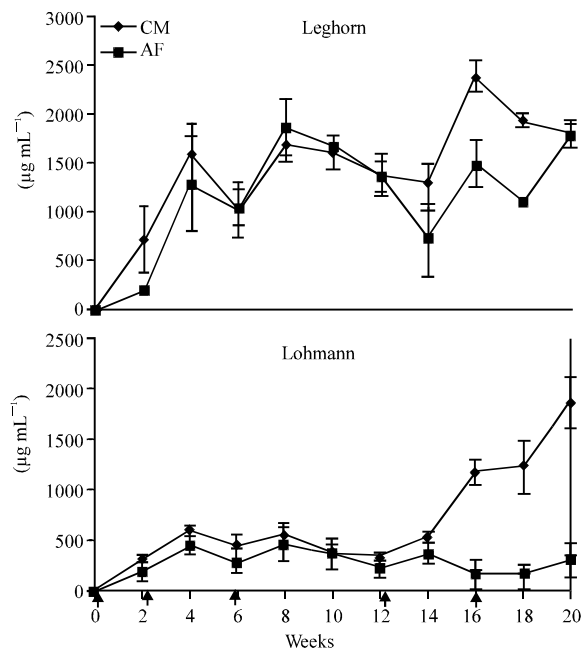


Fig. 3: The serum specific IgY concentration (µg mL<sup>-1</sup> serum) of White Leghorn and Lohmann chickens 0-20 weeks after immunization with CM and AF antigen. ↑: Time of antigen immunization

Figure 4 shows the changes in the anti-*E. coli* IgY concentrations in WSF for both White Leghorn and Lohmann chickens after immunization. For the White Leghorn chickens, the curve patterns of specific IgY production either immunized with CM or AF antigen were similar to those of their serum IgG counterparts. The peak contents of anti-*E. coli* IgY in the eggs of White Leghorn hens treated with CM and AF antigen were 96.28 and 84.05  $\mu\text{g mL}^{-1}$ , respectively and both occurred in the 16th week after immunization.

The content of specific IgY in the eggs of White Leghorn chickens increased 2 weeks after the 1st injection and exhibited an obvious increase 2 weeks after each injection with CM antigen. The same phenomena appeared in the White Leghorn chickens immunized with AF antigen except the last booster injection which did not rescue the afterward declined tendency of specific IgY production. For the Lohmann chickens immunized with CM and AF antigen, the content of anti-*E. coli* IgY in the eggs also increased 2 weeks after each injection.

However, all the antibody levels declined afterward, exhibiting a different pattern than the White Leghorn chickens. In addition, the anti-*E. coli* IgY contents in the eggs of Lohmann chickens were much lower than those treated with CM antigen. The anti-*E. coli* IgY induced by AF-antigen injection was  $<20 \mu\text{g mL}^{-1}$  throughout the experimental period. Yokoyama *et al.* (1992) used an enterotovigenic *E. coli* antigen to produce anti-*E. coli* IgY.

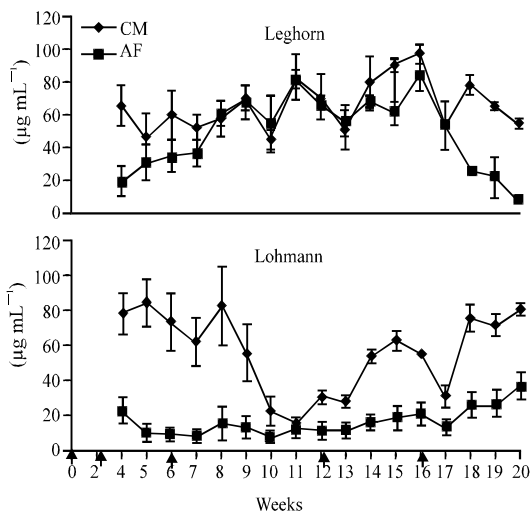


Fig. 4: The production of anti-*E. coli* IgY (WSF  $\mu\text{g mL}^{-1}$ ) in White Leghorn and Lohmann chickens immunized with CM and AF antigen. Values are represented as the average of the eggs collected from 3 chickens in each week 4-20 weeks after immunization. ↑: Time of antigen immunization

They found that the specific antibody increased after 2 weeks of immunization but decreased 3~4 weeks later. The same phenomena appeared in the current study and that reported by Marquardt *et al.* (1999) who used *E. coli* K88 antigen.

Figure 5 shows the yield of specific IgY from the chickens after immunization. The average anti-*E. coli* IgY contents in the WSF egg<sup>-1</sup> was 65.28 and 48.31  $\mu\text{g mL}^{-1}$  in the Leghorn chickens immunized with CM and AF antigen, respectively. The eggs of Lohmann chickens treated with CM and AF exhibited average anti-*E. coli* IgY contents of 55.78 and 14.4  $\mu\text{g mL}^{-1}$ , respectively in the WSF.

The different responses of these two chickens might result from their different genetic backgrounds or the interactions between breeds and antigen origins. The most important factor determining the immunization response in birds may be the antigen origin. In addition, the antigen itself has many antigen determinant sites with different immunodominance factors, so that the antigen preparation can affect immune response. Previous research on Staphylococcus, Salmonella and *E. coli* for IgY production shows that the antibody titers raised using cell membrane proteins and fimbrial adhesions were different (Yokoyama *et al.*, 1992). To determine the inhibitory efficiency of the anti-*E. coli* IgY, purified WSF of higher and lower specific IgY titer from the White Leghorn and Lohmann eggs collected at the 9 and 13th week after immunization, respectively was used in the Co-culture test. The specific antibody contents  $\text{mL}^{-1}$  SWF at the 9 and 13th week were 67.15/63.12  $\mu\text{g}$  and 44.08/18.27  $\mu\text{g}$  for White Leghorn/Lohmann chickens immunized with CM and AF antigen, respectively.

Table 1 and 2 shows the results of the Co-culture test. The number of *E. coli* colonies formed after overnight co-culture with anti-*E. coli* IgY in the 9th week and

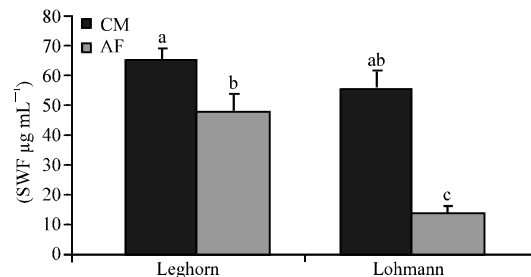


Fig. 5: The average anti-*E. coli* IgY content (SWF  $\mu\text{g mL}^{-1}$ ) in eggs collected from white leghorn and lohmann browns chickens 4-20 weeks after immunization with CM and AF antigen; <sup>ab</sup>values in the column with different superscripts are significantly different ( $p < 0.05$ )

Table 1: The *E. coli* inhibitory effects of the anti-*E. coli* IgY produced from White Leghorn and Lohmann chickens in the 9th week after immunization with antigen of commercial four-in-one *E. coli* vaccine (CM) and inactivated *E. coli* from a local farm (AF)

	No. of <i>E. coli</i> colonies formed after co-culture with IgY of antigen p-values					
	CM	AF	Control	Breed	Antigen	Breed* antigen
Leghorn chicken	82±21.6 <sup>c</sup>	107±38.7 <sup>a</sup>	111±47.8 <sup>a</sup>	0.8152	<0.0001	0.5659
Lohmann chicken	78±7.5 <sup>b</sup>	114±15.3 <sup>a</sup>	112±11.9 <sup>a</sup>	-	-	-

Table 2: The *E. coli* inhibitory effects of the anti-*E. coli* IgY produced from White Leghorn and Lohmann chickens in the 13th week after immunization with antigen of commercial four-in-one *E. coli* vaccine (CM) and inactivated *E. coli* from a local farm (AF)

	No. of <i>E. coli</i> colonies formed after co-culture with IgY of antigen p-values					
	CM	AF	Control	Breed	Antigen	Breed* antigen
Leghorn chicken	89±19 <sup>b</sup>	126±42 <sup>a</sup>	113±28 <sup>ab</sup>	0.575	0.0007	0.847
Lohmann chicken	98±7 <sup>ab</sup>	133±15 <sup>a</sup>	110±18 <sup>ab</sup>	-	-	-

Means±SE (n = 6); <sup>a</sup>b values in the same row with different superscripts are significantly different (p<0.05)

control IgY from the White Leghorn chickens were 82±21, 107±38 and 111±47 and 78±7, 114±15 and 112±11 for the Lohmann chickens. On the other hand, the number of *E. coli* colonies formed after overnight co-culture with anti-*E. coli* IgY in the 13th week and control IgY from the White Leghorn chickens were 89±19, 126±42 and 113±28 and 98±7, 133±15 and 110±18 for the Lohmann chickens. The specific IgY antibodies produced from the Lohmann chickens had no inhibitory effects on growth of activated *E. coli* even when the IgY concentration obtained from CM antigen injection was as high as 63.12 µg mL<sup>-1</sup> WSF. The specific IgY produced in the 9 and 13th week from the injection of CM antigen had a better inhibitory effect on activated *E. coli* than the specific IgY produced from the injection of AF antigen and the control IgY (p<0.001). There was no effect of breed difference and breed x antigen on the inhibitory effects of anti-*E. coli* IgY.

In a study on the inhibitory effect determination for specific IgY against *E. coli* strain O157:H7, Sunwoo *et al.* (2002) used WSF containing 45, 90 and 180 µg mL<sup>-1</sup> of specific IgY in the Co-culture test. Their results indicate that a WSF with a specific IgY concentration exceeding 90 µg mL<sup>-1</sup> significantly decreased the formation of *E. coli* colonies after 2 h of incubation. This result agrees with the current study in which the anti-*E. coli* IgY obtained from White Leghorn chickens immunized with CM antigen decreased the growth of activated *E. coli* at a concentration >60 µg mL<sup>-1</sup> WSF.

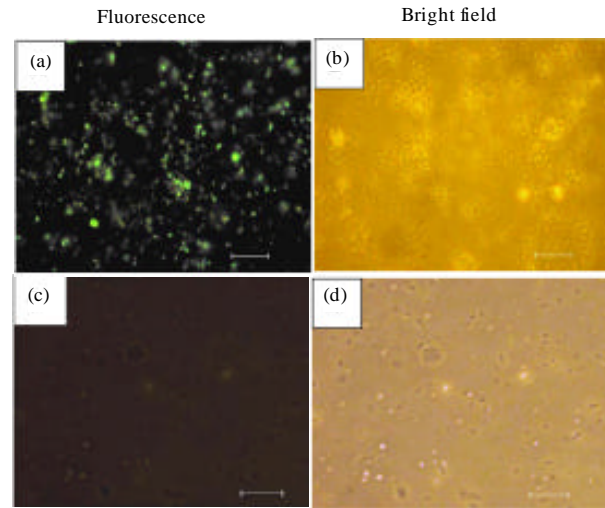


Fig. 6: Immunofluorescence micro-graphs of *E. coli* K99 incubated with: a) fluorescence; b) bright field specific IgY; c) fluorescence and d) bright field non-specific IgY. Scale slit shows 20 µm at ×400

The specific-binding activity of IgY against *E. coli* K99 which resulted in inhibiting bacterial growth was proven by ELISA and the growth-inhibition assay presented above. To visualize *E. coli* K99 with bound specific IgY, additional evaluation of IgY binding activity was conducted using immunofluorescence microscopy. The FITC used as a fluorescent dye or fluorochrome in this study absorbs blue light at one wavelength (490 nm) and emits an intense yellow-green fluorescence at longer wavelength (517 nm). Specific IgY binding to cells was visualized by tagging antibodies with FITC that is FITC conjugated anti-IgY.

The *E. coli* K99 reacted with CM specific IgY and fluoresced (Fig. 6a-d). On the contrary, fluorescence was not observed in bacteria incubated with nonspecific IgY (Fig. 5).

As a result, the observation of fluorescence from bacteria reacted with CM specific IgY demonstrated the binding of CM specific IgY to *E. coli* K99. The mechanism through which egg-yolk antibodies protect pigs against ETEC-induced diarrhea may involve antibodies reacting with the receptor binding component of the ETEC (fimbriae). This in turn prevents them from attaching and adhering to the mucosa of the intestines of piglets. Sunwoo *et al.* (2002) suggested that the role of specific-binding activity of IgY against bacterial surface components in inhibiting bacterial growth is merited. The passive protective effect of egg-yolk antibodies against *E. coli* K99 infection in neonatal piglets the results show

Table 3: Clinical response of 21 days old pigs after challenge with *E. coli* K99 and treatment with egg-yolk antibodies

Treatment	No. of pigs	No. of pigs with diarrhea (FC score) after time (h)			
		24	48	72	96
Control	8	5.8±1.62	8.8±2.50	7.7±2.30	6.6±2.50
Treatment	8	4.8±1.25	2.8±1.87	0.8±1.12	0.8±0.62

\*FC score is the mean fecal consistency score: 0 = normal; 1 = soft feces; 2 = mild diarrhea; 3 = severe diarrhea. Pigs with a fecal score of ≤ 1 were considered not to have diarrhea

of the developed severe diarrhea within 24 h and were dehydrated. About 25% of the pigs died of severe diarrhea. In contrast, the pigs treated with egg-yolk antibodies from the immunized hens exhibited no signs of diarrhea 48 or 72 h after treatment (Table 3). These results are consistent with the findings of other researchers (Imberechts *et al.*, 1997; Wiedemann *et al.*, 1991; Yokoyama *et al.*, 1992). These results indicate that the piglets treated with antibodies from the hens immunized against Enterotoxigenic *E. coli* (ETEC) were protected against the deleterious effects of this organism.

**CONCLUSION**

This study describe the production of the effective IgY antibody specific to *E. coli* from White Leghorn chickens by immunization with antigen prepared from a commercial four-in-one vaccine containing *E. coli* pilus antigens of strains K88, K99, 987P and F41. This specific IgY can inhibit the growth of activated *E. coli* at a concentration above 63 µg mL<sup>-1</sup> WSF. The chicken egg-yolk antibodies in this study were able to prevent the diarrhea experimentally induced by ETEC in 21 days old weaned piglets.

**REFERENCES**

Akita, E.M. and S. Nakai, 1992. Immunoglobulins from egg yolk: Isolation and purification. *J. Food Sci.*, 57: 629-634.  
 Akita, E.M. and S. Nakai, 1993. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. *J. Immunol. Methods*, 160: 207-214.  
 Amaral, J.A., M.T. de Franco, M.M. Carneiro-Sampaio and S.B. Carbonare, 2002. Anti-enteropathogenic *Escherichia coli* immunoglobulin Y isolated from eggs laid by immunised Leghorn chickens. *Res. Vet. Sci.*, 72: 229-234.  
 Hamada, S., T. Horikoshi, T. Minami, S. Kawabata, J. Hiraoka, T. Fujiwara and T. Ooshima, 1991. Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of *Streptococcus mutans*. *Infect Immun.*, 59: 4161-4167.

Hatta, H., M. Kim and T. Yamamoto, 1990. A novel isolation method for hen egg yolk antibody, IgY. *Agric. Biol. Chem.*, 54: 2531-2535.  
 Hatta, H., K. Tsuda, S. Akachi, M. Kim, T. Yamamoto and T. Ebina, 1993. Oral passive immunization effect of anti-human rotavirus IgY and its behavior against proteolytic enzymes. *Biosci. Biotechnol. Biochem.*, 57: 1077-1081.  
 Hatta, H., K. Tsuda, M. Ozeki, M. Kim and T. Yamamoto *et al.*, 1997. Passive immunization against dental plaque formation in humans: Effect of a mouth rinse containing egg yolk antibodies (IgY) specific to *Streptococcus mutans*. *Caries Res.*, 31: 268-274.  
 Ikemori, Y., M. Kuroki, R.C. Peralta, H. Yokoyama and Y. Kodama, 1992. Protection of neonatal calves against fatal enteric colibacillosis by administration of egg yolk powder from hens immunized with K99-piliated enterotoxigenic *Escherichia coli*. *Am. J. Vet. Res.*, 53: 2005-2008.  
 Ikemori, Y., M. Ohta, K. Umeda, F.C. Icatlo, Jr., M. Kuroki, H. Yokoyama and Y. Kodama, 1997. Passive protection of neonatal calves against bovine coronavirus-induced diarrhea by administration of egg yolk or colostrum antibody powder. *Vet. Microbiol.*, 58: 105-111.  
 Imberechts, H., P. Deprez, E. van Driessche and P. Pohl, 1997. Chicken egg yolk antibodies against F18ab fimbriae of *Escherichia coli* inhibit shedding of F18 positive *E. coli* by experimentally infected pigs. *Vet. Microbiol.*, 54: 329-341.  
 Jensenius, J.C., I. Andersen, J. Hau, M. Crone and C. Koch, 1981. Eggs: Conveniently packaged antibodies. *Methods for purification of yolk IgG*. *J. Immunol. Methods*, 46: 63-68.  
 Kariyawasam, S., B.N. Wilkie and C.L. Gyles, 2004. Resistance of broiler chickens to *Escherichia coli* respiratory tract infection induced by passively transferred egg-yolk antibodies. *Vet. Microbiol.*, 98: 273-284.  
 Kim, H. and S. Nakai, 1998. Simple separation of immunoglobulin from egg yolk by ultrafiltration. *J. Food Sci.*, 63: 485-490.  
 Kuroki, M., M. Ohta, Y. Ikemori, R.C. Peralta, H. Yokoyama and Y. Kodama, 1994. Passive protection against bovine rotavirus in calves by specific immunoglobulins from chicken egg yolk. *Arch. Virol.*, 138: 143-148.  
 Kwan, L., E. Li-Chan, N. Helbig and S. Nakai, 1991. Fractionation of water-soluble and -insoluble components from egg yolk with minimum use of organic solvents. *J. Food Sci.*, 56: 1537-1541.

- Kweon, C.H., B.J. Kwon, S.R. Woo, J.M. Kim and G.H. Woo *et al.*, 2000. Immunoprophylactic effect of chicken egg yolk immunoglobulin (Ig Y) against porcine epidemic diarrhea virus (PEDV) in piglets. *J. Vet. Med. Sci.*, 62: 961-964.
- LeClaire, R.D., R.E. Hunt and S. Bavari, 2002. Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. *Infect Immun.*, 70: 2278-2281.
- Lee, E.N., H.H. Sunwoo, K. Menninen and J.S. Sim, 2002. *In vitro* studies of chicken egg yolk antibody (IgY) against *Salmonella enteritidis* and *Salmonella typhimurium*. *Poult. Sci.*, 81: 632-641.
- Leslie, G.A. and W.L. Clem, 1969. Phylogeny of immunoglobulin structure and function. 3. Immunoglobulins of the chicken. *J. Exp. Med.*, 130: 1337-1352.
- Li-Chan, E.C.Y., S.S. Ler, A. Kummer and E.M. Akita, 1998. Isolation of lactoferrin by immunoaffinity chromatography using yolk antibodies. *J. Food Biochem.*, 22: 179-195.
- Liou, J.F., C.W. Chang, J.J. Tailiu, C.K. Yu, H.Y. Lei, L.R. Chen and C. Tai, 2010. Passive protection effect of chicken egg yolk immunoglobulins on enterovirus 71 infected mice. *Vavvine*, 28: 8189-8196.
- Marquardt, R.R., L.Z. Jin, J.W. Kim, L. Fang, A.A. Frohlich and S.K. Baidoo, 1999. Passive protective effect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88+ infection in neonatal and early-weaned piglets. *FEMS Immunol. Med. Microbiol.*, 23: 283-288.
- Mc Bee, L.E. and O.J. Cotterill, 1979. Ion-exchange chromatography and electrophoresis of egg yolk proteins. *J. Food Sci.*, 44: 656-667.
- Narat, M., 2003. Production of antibodies in chickens. *Food Technol. Biotechnol.*, 41: 259-267.
- Polson, A. and M.B. von Wechmar, 1980. Isolation of viral IgY antibodies from yolks of immunized hens. *Immunol. Commun.*, 9: 476-493.
- Polson, A., T. Coetzer, J. Kruger, E. von Maltzahn and K.J. van der Merwe, 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol. Invest.*, 14: 323-327.
- Rose, M.E., E. Orlans and N. Buttress, 1974. Immunoglobulin classes in the hen's egg: Their segregation in yolk and white. *Eur. J. Immunogenet.*, 4: 521-523.
- SAS, 1996. SAS/STAT User's Guide. Release 6.11, SAS Institute Inc., Cary, NC., USA.
- Shimizu, M., R.C. Fitzsimmons and S. Nakai, 1988. Anti-*E. coli* immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. *J. Food Sci.*, 53: 1360-1366.
- Shin, J.H., M. Yang, S.W. Nam, J.T. Kim, N.H. Myung, W.G. Bang and I.H. Roe, 2002. Use of egg yolk-derived immunoglobulin as an alternative to antibiotic treatment for control of *Helicobacter pylori* infection. *Clin. Diagn. Lab. Immunol.*, 9: 1061-1066.
- Sunwoo, H.H., E.N. Lee, K. Menninen, M.R. Suresh and J.S. Sim, 2002. Growth inhibitory effect of chicken egg yolk antibody (IgY) on *Escherichia coli* O157:H7. *J. Food Sci.*, 67: 1486-1494.
- Wang, S.M., C.C. Liu, H.W. Tseng, J.R. Wang and C.C. Huang *et al.*, 1999. Clinical spectrum of enterovirus 71 infection in children in southern Taiwan, with an emphasis on neurological complications. *Clin. Infect. Dis.*, 29: 184-190.
- Wiedemann, V., E. Linckh, R. Kuhlmann, P. Schmidt and U. Losch, 1991. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. V. *In vivo* studies on protective effects against *Escherichia coli* diarrhoea in pigs. *J. Vet. Med. Ser. B*, 39: 283-291.
- Yokoyama, H., R.C. Peralta, R. Diaz, S. Sendo, Y. Ikernori and Y. Kodama, 1992. Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli* infection in neonatal piglets. *Infect. Immunity*, 60: 998-1007.
- Yokoyama, H., K. Umeda, R.C. Peralta, T. Hashi, F.C. Jr. Icatlo *et al.*, 1998. Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for *Salmonella enteritidis* and *S. typhimurium*. *Vaccine*, 16: 388-393.