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*Full Length Research Paper*

# Characterization of egg yolk immunoglobulin (IgY) against enterotoxigenic *Escherichia coli* and evaluation of its effects on bovine intestinal cells

**Romina Valeria Bellingeri*, Lucila Busso, Fabrisio Eduardo Alustiza, Natalia Yanina Picco, Daniela Paola Molinero, María Carolina Grosso, Carlos Eugenio Motta and Adriana Beatriz Vivas**

Departamento de Anatomía Animal, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, Río Cuarto (5800) Córdoba, Argentina.

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**Enterotoxigenic *Escherichia coli* (ETEC) infection is common in calves. Egg yolk antibodies (IgY) have been used to treat gastrointestinal infectious diseases. This study aimed to characterize IgY against bovine ETEC and to evaluate its effects on bovine intestinal cell culture challenged with a bovine ETEC strain. IgY was isolated from the egg yolks of hens immunized with ETEC. The characteristics of IgY were determined by Bradford, ELISA, gel electrophoresis and immunoblotting. Significant differences in anti-ETEC activity between anti-ETEC IgY and non-specific IgY were found in lyophilized fractions. In the bacterial growth assay, anti-ETEC IgY (40 mg/mL) showed growth inhibition of ETEC after 2 h of incubation ($p<0.05$). The difference in bacterial growth between anti-ETEC IgY and non-specific IgY groups was 0.51 log CFU/ml after an 8 h incubation ($p<0.05$). The bacterial adhesion assay indicated that anti-ETEC IgY (40 mg/ml) significantly decreased the adhesion of ETEC to bovine intestinal epithelial cells within 4 h (about 1.36 log units compared with the control group; $p<0.05$). This study demonstrates that anti-ETEC IgY inhibits the growth and adherence of ETEC to bovine intestinal cells and is a potential alternative to traditional treatments of *E. coli* infections.**

**Key words:** Immunoglobulin Y (IgY), neonatal calf diarrhea, enterotoxigenic *Escherichia coli*, bacterial adherence, bacterial growth.

## INTRODUCTION

Neonatal calf diarrhea is a leading cause of morbidity and mortality in pre-weaned dairy calves. It is estimated that diarrhea accounts for greater than 50% of pre-weaned calf deaths (USDA, 2008). Thus, research about prevention and therapies for this disease is certainly needed.

Pathogenic *Escherichia coli* (*E. coli*) are one of the most common and serious bacterial causes of neonatal calf diarrhea. Enterotoxigenic *Escherichia coli* (ETEC) produce, at least, one member of the two defined groups of enterotoxins: termostable (ST) and termolabile (LT). One of the main colonization factors or surface proteins described in pathogenic bovine *Escherichia coli* is F17 fimbriae (Nguyen et al., 2011).

Considering the present awareness of the problems associated with the excessive use of antibiotics and the lack of success of specific vaccines for the post-weaned calf, alternatives for preventing infectious diarrheal diseases are required.

*Corresponding author. E-mail: rbellingeri@ayv.unrc.edu.ar. Tel/Fax: +54-358-4676210.

Egg yolk immunoglobulin (IgY) technology is a highly innovative and growing biotechnology area, offering numerous advantages including low cost, effectiveness, efficiency and the minimization of animal suffering (Pauly et al., 2009).

Previous studies have observed that the success of IgY is associated with its capacity to block bacterial binding to the host cell (Cook and Trott, 2010). While there are some published reports that IgY inhibited the adhesion of *E. coli* to piglet intestinal cells (Deneke et al., 1984; Jungling et al., 1991; Yokoyama et al., 1992) and intestinal pig mucus (Jin et al., 1998), to the best of our knowledge, no studies have been done in bovine intestinal cells.

Therefore, the aim of the present study was to characterize anti-ETEC IgY and evaluate its effects on bovine intestinal cells challenged with a bovine ETEC strain.

## MATERIALS AND METHODS

### Bacterial strain and culture conditions

The ETEC strain used in this study was isolated from a neonatal calf belonging to a dairy farm in Córdoba Province (Argentina) and cultured on MacConkey agar (Britania®, Argentina) at 37°C for 48 h. The strain was $F17^+$ and $STa^+$, as indicated by molecular analysis of PCR (Picco et al., 2011). The presence of F17 antigen was assayed with bacteria grown on Minca solid media (Guinée et al., 1977) by slide agglutination using specifically adsorbed antiserum.

### Animals

Twelve (12) Lohmann Brown-Classic hens (19-week-old) were obtained from a poultry farm (Cabaña Avicola Jorju SACIFyA, Buenos Aires-Argentina). The animals were kept in individual cages with food and water *ad libitum*, without contact with any other animals. The food was based on a balanced food for laying hens (Nutriarte, Córdoba-Argentina). A cycle of light/dark of 16/8 h was used. The room temperature was 20 ± 2°C and relative humidity between 55 to 60%.

This study received formal approval from the ethics committee of the Universidad Nacional de Río Cuarto. In order to guarantee a safe, correct and careful use and handling of experimental animals, the investigators proceeded according to the ethical guidelines of animal welfare committee (Olfert et al., 1993).

### Immunization of hens

Eight hens were immunized to obtain anti-ETEC IgY loaded eggs. To elaborate the immunogen, bacteria were inactivated with 0.5% formaldehyde and incubated overnight 37°C. After incubation, bacteria were pelleted by centrifugation (2,500 rpm for 5 min) and washed 3 times with PBS. A total amount of 1 ml of inactivated bovine ETEC whole cells (1 x $10^9$ CFU/ml) was mixed with 7% aluminum hydroxide and injected intramuscularly in breast muscles. The remaining four hens only received 1 ml sterile PBS with 7% aluminum hydroxide to obtain non-specific IgY. Booster immunizations were given at 2 and 4 weeks after the initial immunization in the same manner. Eggs were collected daily from day 40 since 175 post-first immunization and stored at 4°C until used.

### Purification of yolk antibodies

The water-soluble fraction (WSF) containing IgY was prepared from egg yolk using the water dilution method (WD) developed by Akita and Nakai (1992) with minor modifications. Briefly, egg yolk was physically separated from egg white and rolled on paper towels to remove the rest of adhered egg white. The membrane was punched and the yolk was allowed to flow into a graduated cylinder without the membrane. The egg yolk was mixed gently with six volumes of cold acidified distilled water (pH 2.5 adjusted with 0.1 M HCl). After mixing well, the mixture was adjusted to a pH ranging from 5.0 to 5.2 and incubated at 4°C for 12 h. The WSF was obtained by centrifugation (Beckman Coulter Avanti J-25) at 12,000 x *g* and 4°C for 20 min, the supernatant was considered as WSF and stored at -20°C, until use.

The avian immunoglobulins were precipitated by sodium sulfate as described previously for purification of IgY (Wooley and Landon, 1995). Sodium sulfate was added at a final concentration of 20% (w/v) and stirred for 20 min at room temperature and then the mixture was centrifuged at 2,000 x *g* for 30 min at 4°C. Finally, the pellet was resuspended in PBS to initial yolk volume and dialyzed against 0.1% NaCl solution.

After dialysis, IgY samples were frozen at -20°C in plastic containers and freeze-dried for 48 h (Labconco Stoppering Try Dryer lyophilizer).

### Electrophoresis and western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). The gels were stained with Coomassie Brillant Blue R-250 (Ultra-Pure™, Gibco BRL). Wide molecular weight marker ranging from 6.5 to 205 kDa (Sigma®, M-4038) was used as standard. The gel was electrophoretically transferred to polyvinylidene difluoride [(PVDF) (Millipore Immobilon™)] membranes. The membranes were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-chicken antibody (Sigma®, A9046) and placed in peroxidase chromogenic substrate solution (3,3'-diaminobenzidine tablets, Sigma® D4418) and then rinsed in excess distilled water to stop the color development.

### Protein quantification

The protein concentration was determined at 595 nm by Bradford method (Bradford, 1976) with a spectrophotometer (Jasco" modelo V-630 Bio).

### Total IgY concentration

Total concentration of IgY was evaluated by ELISA, it was performed as described by Sunwoo et al. (2012) with modifications carried out by our group. The wells of microtiter plate (Costar, Corning, N.Y., U.S.A.) were coated with 50 µL of serial dilutions of purified chicken IgG (1 mg/ml) (Sigma®, I-4881) in carbonate-bicarbonate buffer to prepare a standard curve. In the same plate, anti-ETEC IgY and non-specific IgY powders were reconstituted (2 mg/ml) and diluted in carbonate-bicarbonate buffer (0.05 M, pH 9.6). The plate was coated with these preparations and incubated over night at 4°C. The plate was washed 4 times with PBS containing 0.05% Tween 20 between each step. After washing, 150

µL of a 5% (w/v) non-fat dry milk were added to each well and incubated at 37 °C in humid chamber for 1 h. Rabbit anti-chicken IgG conjugated with horseradish peroxidase (diluted 1:1000 in PBS with 2% of non-fat dry milk) (Sigma®, A-9046) was added to each well and incubated at 37 °C for 2 h in humid chamber. After washing, 100 µl of freshly prepared substrate solution of 3,30,5,50-tetramethylbenzidine (TMB, Sigma®, T-3405) in 0.05 M phosphate citrate buffer (pH 5.0) (Sigma Chemical Co.) containing 6 µl of 30% hydrogen peroxide was added. (Sigma Chemical Co.). The reaction was stopped by 3 M $H_2SO_4$ and measured in an ELISA reader at 450 nm (Biolatin CPD Reader 212).

#### Specific IgY determination

Anti-ETEC activity was done using the ELISA method developed by our research group. Briefly, plates were coated with 50 µl of a suspension of concentrated and heat-inactivated ETEC strain (>$10^{10}$ UFC/ml) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) and incubated overnight at 4°C. Non-specific protein-binding sites were blocked with PBS-T (pH 7.2) containing 5% non-fat milk. Serial dilutions of anti-ETEC IgY and control IgY ($1/10^1$ to $1/10^{15}$) in PBS-T with 2% non-fat milk were then incubated at 37°C for 1 h in humid chamber. After washing with PBS-T, the plates were incubated for 1 h in humid chamber with 150 µl of rabbit anti-chicken IgG horseradish peroxidase-conjugated (diluted 1:1000 in PBS with 2% non-fat dry milk). After washing, the TMB solution was added and the reactions were stopped with $H_2SO_4$ (3 M). The absorbance was read at 450 nm (Biolatin CPD Reader 212). The titles of specific anti-ETEC IgY and control IgY were determined.

#### Bacterial growth assay

The bacterial growth assay was performed as described by Amaral et al. (2008). Lyophilized IgY was resuspended in 2 ml of brain hearth infusion (BHI), the solutions were filtered (0.22 µm) and mixed with 2 ml of bacterial suspension. The growth assay was carried out with four groups: control 1 (ETEC - 1.35 CFU/ml), control 2 (ETEC - 1.35 CFU/ml + non specific IgY- 40 mg/ml), treatment 1 (ETEC - 1.35 CFU/ml + anti-ETEC IgY 20 mg/ml) and treatment 2 (ETEC - 1.35 CFU/ml + anti-ETEC IgY 40 mg/ml). The cultures were incubated for 8 h at 37°C.

After 0, 2, 4, 6, and 8 h, an aliquot of each culture was serially diluted in BHI and 100 µl were plated onto MacConkey Agar (Britania Argentina). After overnight incubation, the colonies were counted and their growth curves plotted.

#### Isolation of bovine intestinal cells

Intestinal fragments of proximal region of jejunum from adult animals were obtained from a local slaughterhouse. After several washes in warm (37°C) divalent ion free PBS, fragments were washed in warm PBS supplemented with 1% antibiotic-antimycotic solution (Gibco), 2.7 mg/ml D-glucose, and 4 mM L-glutamine and transported within 30 min to the laboratory.

For the isolation of bovine intestinal cells, the distended intestinal sac method described previously in pigs (Fan et al., 2001) with minor modifications was used. Briefly, 20 cm of proximal small intestinal segment was dissected and incubated with preincubation buffer (27 mM sodium citrate, 0.2 mM, phenylmethanesulfonyl fluoride (PMSF) and 0.5 mM dithiothreitol (DTT), pH 7.4) at 37°C for 15 min. After preincubation, the segment was incubated with isolation buffer (1.5 mM $Na_2$-EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 5 mM D-glucose, at 37°C for either 20 or 30 min). After incubation, the content of the segment, including buffer and isolated cells, were collected into a 15-ml conical centrifuge tubes. This procedure was repeated 6 times to yield 6 “cell fractions” (designated F1 through F6). Each of the first three cell fractions (F1 to F3) was collected after a separate 20 min incubation, whereas each of the last three fractions (F4 to F6) was collected after a separate 30 min incubation. The last cell fraction were washed twice as follows: was centrifuged at 400 x *g* for 4 min at 4°C, the supernatant discarded, and the cell pellet was dispersed with D-MEM supplemented with 1 ng/ml Epidermal Growth Factor, 1 µg/ml insulin, 1% Glutamax (Gibco®), 1% Antibiotic-Antimycotic solution and 10% FBS. Microphotographs of cells were taken using an inverted microscope (Nikon, Eclipse TS100) and a camera (Zeiss, Axiocam ERc5s).

#### Bacterial adhesion assay

Approximately 5 x $10^5$ bovine intestinal cells were seeded in 24-well plates, grown overnight in Williams E medium and infected statically overnight with 5 ml of bovine ETEC culture (1 x $10^8$ CFU/ml) (control 1 Group). Infected bovine intestinal cells were incubated for 4 h in 5% $CO_2$ at 37°C. For the treatment groups, ETEC was previously incubated for 2 h with anti-ETEC IgY (20 and 40 mg/ml, treatment 1 and 2 respectively). For the control 2 group, ETEC was previously incubated for 2 h with non-specific IgY (40 mg/ml). The supernatants were removed and the cells were washed with PBS. Then, 1% Triton X-100 was added for 20 mins for bacterial liberation. The bacterial suspension was serially diluted, and plated on MacConkey agar to count CFUs.

#### Statistical analyses

Statistical analyses were performed using Infostat software (Grupo Infostat/FCA, 1998). Data were represented as means ± standard deviation (SD). Differences were considered significant at the level of $p<0.05$. Student’s t-test was applied to detect differences in protein and IgY concentration between anti-ETEC IgY and non-specific IgY groups. In the bacterial growth assay, levels of significance were evaluated by one-way ANOVA followed by Tukey´s test and the least significant difference test for multiple comparisons. In the adhesion assay, the geometric mean number of CFU log in each group was compared with a non parametric Kruskal-Wallis test.

## RESULTS

### Electrophoresis and western blot analysis of WSF

The WD method was effective in isolating IgY from egg yolks as documented by SDS-PAGE and Western blotting (Figure 1 A and B). The IgY precipitation with sodium sulfate was useful to reduce impurity levels in preparations. The results show there are two main subunits, in lanes 1 and 2 (Figure 1B), that represent the heavy and light chains of IgY.

### Concentrations of protein, total IgY and specific IgY in IgY powder

Protein and total IgY concentration of anti-ETEC IgY was found to be similar to those obtained in the sample of non-specific IgY. However, the specific IgY titre was

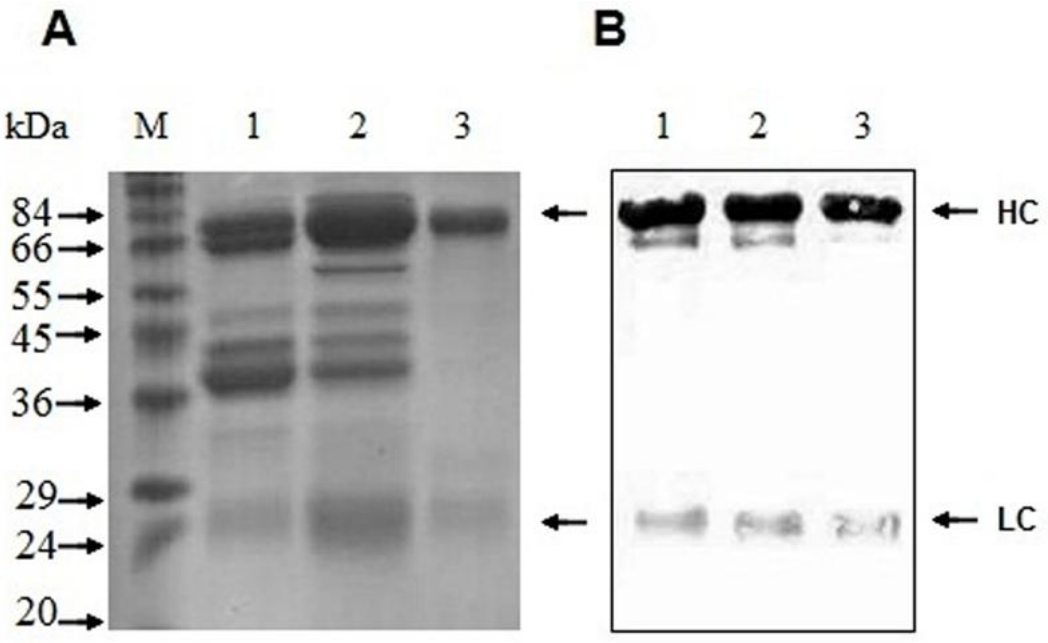


**Figure 1.** A- SDS-PAGE pattern of IgY purified by two steps. Reducing condition of SDS-15 % PAGE. Lanes: (M) wide molecular size marker; (1) fraction collected from $Na_2SO4$ precipitation; (2) fraction collected from WSF; (3) standard IgY (Sigma). B- Western blot analysis of IgY fractions. Lanes: (1) fraction collected from sodium sulfate precipitation; (2) fraction collected from WSF; (3) standard IgY. The arrows indicate the heavy chain (HC) and light chain (LC) of IgY.

**Table 1**. The concentrations of protein and total IgY, and specific IgY titre in IgY powder prepared from the WSF containing ETEC-specific or non-specific IgY.

| IgY powder | Protein (mg/g) | Total IgY (mg/g) | Specific IgY (titre) |
|---|---|---|---|
| Anti-ETEC | 686.2 ± 58.6 | 195.3 ± 1.7* | 1:100,000* |
| Non-specific | 556.1 ± 42.4 | 114.2 ± 3.4 | 1:1,000 |

The values in each column are the average of three different assays. Different *Indicate significant difference ($p<0.05$) between both groups.

1/1,000 and 1/100,000 for non-specific and anti-ETEC IgY. Both total concentration and specific titre of IgY were determined by an ELISA developed by our group (Table 1).

## Bacterial growth assay

As shown in Figure 2, treatment 2 (anti-ETEC IgY-20 mg/ml) showed significant ($p<0.05$) inhibition of ETEC growth after 2 h of incubation compared with the control 1 group. Cell counts of the treatment 2 group and control 1 group rose by 0.26 log CFU/ml and 1.14 log CFU/ml, respectively, during 2 to 6 h of incubation. The highest inhibition was found at 8 h with treatment 2 with a 1.24 log reduction of CFU/ml compared with the control 1 group.

## Isolation of bovine intestinal cells

The sequential isolation method allowed the efficient isolation of morphologically identifiable intestinal epithelial cells. In the last fraction, this method approximately yielded $1.2 \times 10^7$ cells/ml. These cells had mainly columnar and spherical morphology (Figure 3). At least 95% of cells were viable at the time of plating as determined by trypan blue exclusion method.

## Bacterial adhesion assay

The bacterial adhesion assay shows that treatment group significantly reduced the CFU number of ETEC adhered to the intestinal bovine cells compared with Control 1 group (5.48 ± 0.15 vs. 6.84 ± 0.09, $p<0.05$). It should be

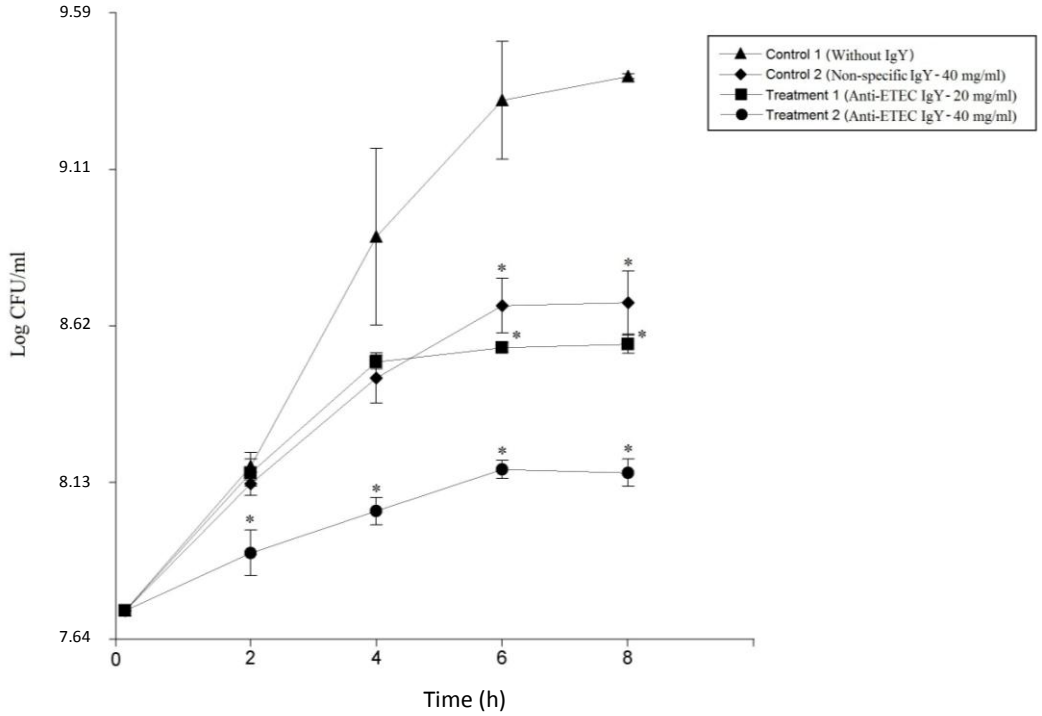


**Figure 2.** Effect of IgY on the growth of ETEC in BHI broth. CFUs were measured in triplicate by the spread plate method on Mac Conkey Agar. Values are the mean ± SD (n=3). *p<0.05, as compared with corresponding treatment in the control 1 group.

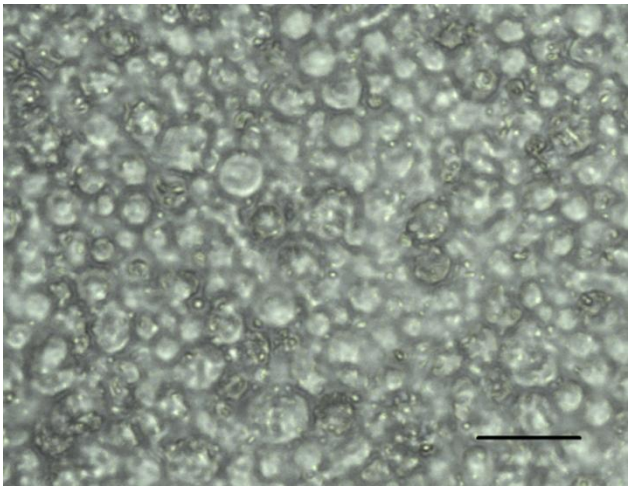

**Figure 3.** Micrograph of confluent monolayer of bovine intestinal epithelial cells after 2 days in culture. Barr = 100 µm.

noted that control 2 group also significantly reduced the adherence of ETEC, however, the rate of inhibition was lower than that of the treatment group (Figure 4).

## DISCUSSION

Although an effective protection against enteric pathogens has been obtained with the use of egg yolk antibodies in mice, pigs and calves, the *in vitro* evaluation of IgY effects on intestinal primary culture, as performed in this study, has not received much attention so far.

The WD method was showed to be simple, rapid and inexpensive and it does not require the use of chemicals, thus we suggest its use in future purifications of specific IgY. Akita and Nakai (1993) compared different methods

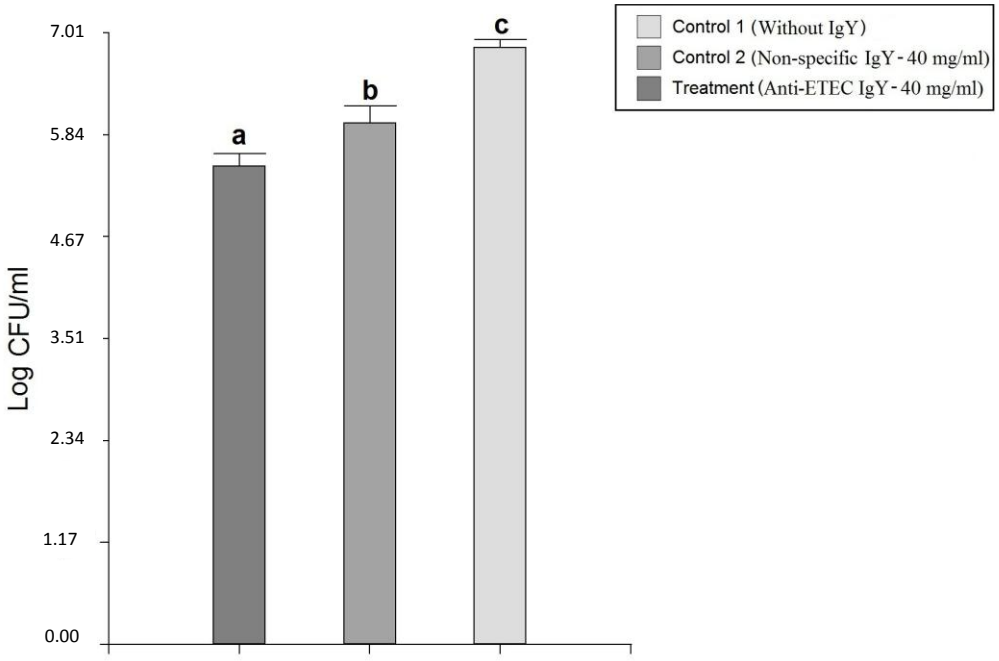


**Figure 4.** Effect of IgY on the adherence of ETEC to bovine intestinal cells. CFUs were measured in triplicate by the spread plate method on MacConkey agar. Values are the mean ± SD (n=3). Different letters indicate significant difference ($p<0.05$).

for IgY purification and WD was found superior in terms of ease of use and large scale production of IgY. Isolation of IgY with Polyethylene glycol has been described recently as another effective method to obtain chicken egg yolk antibodies (Meenatchisundaram and Michael, 2011; Pauly et al., 2011). Previous studies evaluated the efficacy of immune yolk and semi purified IgY to protect the infectious bursal disease in infected birds and showed that concentrated egg immune yolk can give full protection. However, when it is purified by chemical methods, it does not give full protection (Farooq et al., 2012). Addition of salts, such as ammonium or sodium sulfate, causes precipitation of mammals IgG. Ammonium sulfate has been used in previous studies to precipitate IgY (Trott et al., 2009; Zhen et al., 2008). In this study, the IgY was extracted from egg yolk with WD method and further precipitated with sodium sulfate. SDS-PAGE shows a reduce in the levels of impurities at the IgY preparations. Western blot revealed no alterations in its biological integrity. The freeze drying process seems to be a suitable method to conserve IgY and to preserve its biological stability. Also, Fu et al. (2006) reports no significant reduction in reactive activity and good thermal stability after freeze drying process.

Protein and total IgY concentrations of non-specific IgY and anti-ETEC IgY powders were similar. In contrast, Mahdavi et al. (2010), found that total IgY concentration and IgY purity of specific IgY powder were approximately 30% higher than the non-specific IgY powder ($p<0.05$). Specific IgY titre in the non-specific IgY powder was significantly lower than that in specific IgY powder, as expected. The ELISA performed in this study appears to be a sensitive technique to quantify the amount of specific and non-specific antibodies.

It must be noted that, the comparison of the IgY concentration obtained in this work with that obtained by other authors may be different due to the influence of factors, such as, the variety and concentrations of antigens used, as well as the inoculation routes and, type of adjuvant. Other factors that may influence the yield of IgY in the yolk are the egg yolk weight and the percentage hen-day production, which are considered to be important factors for the efficient production of IgY (Li et al., 1998). Pauly et al. (2009) monitored IgY levels in hens over a two-year period and found that although the laying capacity in the second year was lower than in the first year, the total IgY content was greater and therefore compensated for the reduced egg number.

The *in vitro* bacterial growth inhibition assay is an important investigative tool for evaluating the biological activity of alternative anti-microbial agents such as IgY. Previous results suggested a potential neutralizing effect of IgY preparations in growth inhibition assays (Amaral et al., 2008). The present study showed that IgY anti-bovine ETEC was able to inhibit the ETEC growth; when the concentrations of IgY was high, the time to inhibit the bacterial growth was more efficient.

Similar inhibition values were reported by Mahdavi et al. (2010) with chicken egg yolk antibodies against *E. coli* O78:K80. However, in contrast to these authors, with

40 mg/ml of non-specific IgY, a significant inhibitory effect on bacterial growth after 6 h of incubation was achieved. This growth inhibition effect is due to the fact that we found anti-ETEC IgY in purified fractions from non-immunized chickens. This may have been as a result of natural exposure of hens to antigenic components of non-pathogenic *E. coli* in the hatchery. It may be appropriate to use purified fimbrial antigens for the immunization of animals to obtain polyclonal antibodies. Several studies have reported conclusive evidence from trials using biochemically pure fimbrial antigens for immunization (Cook et al., 2007; Liou et al., 2011). However, most of the studies used whole bacterial cells for the immunization of hens (Sunwoo et al., 2002; Amaral et al., 2008; Zhen et al., 2008).

The sequential isolation of enterocytes appears to be an appropriate method to obtain a primary culture with low contamination with other types of cells. With the addition of epidermal growth factor, these culture conditions favor the proliferation of epithelial cells. The cell showed a spherical morphology which is typical of mid villus and deep crypts (Kaeffer, 2002). Therefore, we considered that primary bovine enterocyte culture may be used as a tool for the study of intestinal diseases and was employed to assess the effects of anti-ETEC IgY on ETEC adherence.

In this study, a low rate of bacterial growth inhibition was found. However, anti-ETEC IgY and non-specific IgY demonstrated significant reduction of the adherence of ETEC $F17^+$ to bovine intestinal epithelial cells *in vitro*. Cook et al. (2007) demonstrated that the inhibition of adherence was due to the influence of the antibodies on specific pathogenic processes and not a result of direct inhibition of bacterial growth. Recently, Ma and Zhang (2011) found that IgY and Fab' fragment of egg yolk immunoglobulin raised against LPS may be used to inhibit and treat tissue injury caused by LPS. ETEC develop their pathogenicity by cell attachment, which is a prerequisite for intestinal colonization. Such colonization is promoted by bacterial adherence to receptor structures present on host epithelial surfaces. The effect of non-specific IgY may be mediated by the innate levels of anti-*E. coli* IgY present in non-immunized egg yolks used in this study. These antibodies could be due to the natural exposure of hens to *E. coli* and may be sufficient to inhibit adherence.

The results of our study agree with those of Jin et al. (1998), who reported that anti-$K88^+$ IgY when added to $K88^+$ *E. coli* prevented their binding to receptors in the mucus isolated from piglet intestine. Same results were obtained by Girard et al. (2006) who demonstrated that anti-intimin IgY effectively prevented intimate attachment of EPEC and EHEC to porcine ileal *in vitro* organ culture. In concordance, Cook et al. (2007) found that both anti-EspA and anti-intimin IgY reduced bacterial adhesion and largely prevented host cell actin rearrangements. Previous studies (Sunwoo et al., 2002; Zhen et al., 2008) reported that specific IgY could adhere to bacterial surface resulting in structural modifications. These alterations could influence bacterial growth and biological functionality, such as adherence to cell.

## Conclusions

Our findings suggest that anti-ETEC IgY can be purified with high purity with an economic, simple and rapid method such us WD method accompanied with salt precipitation. Bovine intestinal cell culture has proved to be an excellent tool to study bovine intestinal pathogens such as ETEC. The inhibitory effect of specific IgY on growth and adherence of ETEC suggests that IgY can be considered as a possible treatment for farm animal welfare, particularly for protection against gastrointestinal infections.

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