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Antibodies anti-Shiga toxin 2 B subunit from chicken egg yolk: Isolation, purification and neutralization efficacy

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ABSTRACT

Shiga toxins (Stx1 and Stx2) are the main virulence factors of enterohemorrhagic *Escherichia coli* (EHEC), a foodborne pathogen associated with diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. The aim of this study was to evaluate the antibodies against Stx2 obtained from egg yolks of laying hens immunized with a recombinant Stx2B subunit. A high specific response in serum was observed 25 days after the first immunization and IgY antibodies were extracted from day 47th and purified from egg yolk. A concentration of 0.84 mg of total IgY/ml of egg yolk was obtained, of which 8% were antigen specific. The ability of anti-Stx2B IgY to recognize Stx2B and Stx2 either in solid-phase or in solution were evaluated and compared with anti-Stx2B rabbit antibodies by Western blotting and ELISA. The protective efficacy of IgY against Stx2 was determined by *in vitro* and *in vivo* experiments. The results show that IgY was able to recognize Stx2B and Stx2 in denatured conditions, attached to a solid-phase and free in solution. The anti-Stx2B IgY could effectively block the biological activity of Stx2 on Vero cells and protect mice from Stx2 challenge.

The data suggest that immunization of hens with Stx2B could be a strategy to obtain at low cost a relatively high concentration of anti-Stx2 egg yolk IgY, able to neutralize Stx2 lethal activity. IgY technology could be an useful tool for research, diagnosis and therapy of EHEC infection.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), a subset of Shiga toxin producing *E. coli* (STEC), are important human foodborne pathogens (Kaper et al., 2004). Transmission is most frequently associated with the consumption of contaminated food or unpasteurized dairy products and infections can also be acquired through person-to person contact (Griffin, 1995). The clinical manifestations of EHEC infections range from watery diarrhea, or hemorrhagic colitis (HC), to the most severe outcome, the life threatening hemolytic uremic syndrome (HUS) (Nataro and

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Kaper, 1998). HUS occurs in about 5–10% of individuals, primarily very young and elderly subjects (Rivas et al., 2010). Although EHEC infections are a serious problem in developed countries, Argentina has the highest incidence of HUS in the world, with approximately 500 new cases observed each year in children under 5 years of age (Rivas et al., 2010).

STEC also referred to as verotoxin producing *E. coli* (VTEC), produce cytotoxins structurally related to those produced by *Shigella dysenteriae type 1.* Shiga toxins (Stx1 and Stx2) are the main virulence factors involved in the pathogenesis of HUS and belong to the class known as AB5 toxins composed of one A subunit and five identical B subunits. The A subunit (32 kDa), possesses enzymatic RNA N-glycosidase activity that hydrolyzes the N-glycoside bond of adenosine of the 28 S rRNA of 60 S ribosomes, and hence inhibits protein synthesis; B subunits (7 kDa) are mainly involved in receptor binding (Jeong et al., 2010). STEC strains produce Stx1, Stx2 (or their variants), or both of these toxins. Although the mechanisms of action of Stxs are thought to be similar, cytotoxicity of Stx2 may be stronger than that of Stx1; the 50% lethal dose in mice of purified Stx2 is 1 ng, whereas Stx1 has a 50% lethal dose of 400 ng (Tesh et al., 1993). Additionally, epidemiological data indicate that Stx2 producing strains are more frequently associated to severe illnesses such as HUS than Stx1 producing strains (Karmali et al., 1985). Due to their extreme toxicity, Shiga toxins are classified in category B of bioterrorism agents by the Center for Disease Control and Prevention (CDC, 2009).

Immunoglobulins are widely used for a variety of purposes, such as in diagnostic tests, purification columns, and passive immunotherapy (Kim et al., 2010). Therefore, research and diagnostic community constantly demand new alternatives and procedures to produce cost-effective antibodies. The use of laying hens to produce polyclonal antibodies is an alternative to the use of mammals, such as rabbits and, since more than two decades, egg yolk antibodies (IgY) are a low cost and ethical alternative (Schade et al., 1996; Schade and Chacana, 2007). Compared with the stressful bleeding of mammals to obtain serum, IgY can be easily obtained non-invasively from the egg yolk. From the economical point of view, the amount of antibodies produced by a single hen is similar to that of a large mammal such as a sheep or goats, whereas maintenance costs are much lower (Schade et al., 2005; Zhang, 2003). IgY from serum is actively transferred into the yolk by a receptor –mediated process (Schade et al., 2005) and the amount of the immunoglobulin varies between 100 and 250 mg per egg (Schade et al., 1996). Thus, a substantial amount of antibody can be produced from just one hen (up to 40 g of total IgY per chicken per year), of which 1–10% is expected to be specific to the antigen of interest (Mine and Kovacs-Nolan, 2002). In contrast to mammalian IgG, IgY antibodies do not activate mammalian complement, do not cross-react with Fc receptors, mammalian rheumatoid factor, or human anti-mouse antibodies, thus eliminating false-positive results in serological assays (Schade et al., 2005). Also, chickens are able to develop a better response against mammalian antigens, due to the phylogenetic distance between mammals and birds (Schade et al., 2005).

At present, there are no established specific prophylaxis or therapy strategies for EHEC borne disease or the prevention of its complications (Orth et al., 2008; Tzipori et al., 2004), nor early and reliable predictors of the severity of the disease. But, the rapid diagnosis of STEC infection and early intervention before the onset of systemic diseases are desirable to prevent or ameliorate toxin-related complications, including HUS, as a proper supportive treatment can be determined. Besides, rapid laboratory diagnosis and subtyping of STEC isolates leads to prompt detection of outbreaks and implementation of control measures (CDC, 2009).

In this work, polyclonal antibodies were raised in chicken against the Stx2B subunit. The antibodies were extracted from egg yolk, purified and analyzed for their binding and neutralizing capabilities against Stx2 holotoxin. The comparative performance of anti-Stx2B IgY was evaluated by immunoblotting, ELISA and *in vitro* and *in vivo* neutralization against polyclonal antibodies obtained from immunized rabbits.

2. Materials and methods

2.1. Shiga toxin type 2 holotoxin and recombinant Stx2B subunit

The Stx2 holotoxin, corresponding to Stx2-EDL 933 variant (Krüger et al., 2011), was obtained from STEC 026:H11 strain T 59-2 isolated from cattle by Parma et al. (2000). Briefly, the bacteria were grown in Luria Bertani (LB) broth at 37 °C with shaking at 150 rpm until OD 600 nm reached 0,3. The culture was treated with mitomicin C (0.5 μg/ml) and incubated overnight at 37 °C with shaking. After centrifugation at 10,000 × g, 10 min at 4 °C, the supernatant was filter-sterilized and aliquots were stored at −20 °C. A negative control for the sandwich ELISA assay was prepared performing the same protocol with *E. coli* DH5α strain.

Stx2B subunit was produced by a cloning method based in site-specific recombination reactions that mediate the integration and excision of phage lambda into and from the *E. coli* chromosome (Gateway Cloning Technology, Invitrogen). A DNA fragment encoding Stx2B was obtained by PCR amplification using DNA extracted from the reference strain *E. coli* EDL 933 and the following pairs of synthetic primers that include recognition sites (attB) for the recombination:

5′GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTAGAA GAAGGAGATATACATATGAAGAAGATGTTTATGGCGGTT 3′(**attB1-forward**, sequence corresponding to attB1 is underlined).

5′GGGGACCACTTTGTACAAGAAAGCTGGGTGGATCCTTA TCA*GTGATGGTGATGGTGATG*CCGACCTTCGATGTCATTATTAA ACTGCACTT 3′ (**attB2-reverse**, sequence corresponding to attB2 is underlined and His-tag codons are in italics). The accuracy of the final DNA construction was confirmed by DNA sequencing of different clones. The plasmid obtained was called pDESTStx2B. For expression of recombinant Stx2B subunit, BL21-AI™ *E. coli* cells were transformed by pDESTStx2B. Isolated colonies were then grown with shaking at 200 rpm at 37 °C to mid exponential phase (OD 600 nm: 0,6) in 200 mL of Luria Bertani (LB) broth

supplemented with 100 μg/mL ampicillin (Sigma Aldrich). The cultures were induced with L (+) arabinose (Sigma Aldrich) at a final concentration of 0.2% and incubated with shaking at 37 °C overnight. The Stx2B was purified by affinity chromatography under native conditions using a Ni-NTA column (Qiagen). The eluted fractions were exchanged with PBS (pH 7.4) by repeated dilution and concentration by using centrifugal ultrafiltration with Vivaspin 20 (3000 MWCO, Sartorius). Protein yield was determined by the Bradford assay using BSA as the standard protein, purity and presence of the recombinant protein in the elution fractions were determined by SDS-PAGE and Western blot, respectively.

One gel was stained with Coomassie Blue and the other was electrotransferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). Membranes were blocked overnight with 5% skimmed milk in PBS-Tween 20 (PBS-T) 0.1% at 37 °C and then washed three times with PBS-T. This was followed by an incubation with 1:3000 dilution of anti- His-tag antibody (Invitrogen) in PBS-T and then with a 1:1000 dilution of the anti-mouse ECL secondary antibody (Sigma Aldrich). After a washing step, the membrane was developed with the ECL Western blotting detection kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The blot was exposed for 1 min to an x-ray film (Super RX; Fuji Photo).

2.2. Production of polyclonal antibodies

Two 16-week-old Lohmann Brown Layer-line hens were immunized intramuscularly in the pectoral muscles with 150 μg of purified recombinant Stx2B emulsified with Freund's complete adjuvant (FCA) (Difco). Booster immunizations were done at days 15 and 38 with 150 μg of antigen and at days 103 and 137 with 200 μg of antigen emulsified with Freund's incomplete adjuvant (FIA) (Difco). Serum samples were collected from the wing vein 7 days after each immunization. Eggs were collected daily starting one week after the third immunization and stored at 4 °C until further processing. Egg yolks were pooled and frozen for purification of IgY antibodies. Hens were housed in individual cages before the beginning of laying, maintained on 12/12 h light/dark cycle and received food and water *ad libitum*.

Two New Zealand White male rabbits (3 kg) were immunized subcutaneously with 150 μg of purified recombinant Stx2B emulsified with FCA at multiple sites in the dorsal region. Subsequent booster immunizations were done with an equivalent dose of Stx2B emulsified with FIA at days 20, 40, 60 and 80. Blood was collected from the marginal ear vein after the second booster. Final bleeding of the anaesthetized animals was done by cardiocenthesis. Animals were housed individually with *ad libitum* water access and commercial rabbit food was supplemented with hay and carrots once a week.

Prior to the first immunization, serum samples from hens and rabbits and egg yolks samples were taken and used as pre-immune negative controls. Presence of specific antibodies in chicken and rabbit sera was tested in a Dot blot assay, using recombinant Stx2B subunit as antigen.

2.3. Isolation of total IgY and IgG

Egg yolk antibodies were purified by the water dilution method (WD) (Akita and Nakai, 1992). Briefly, egg yolk content was mixed with water in a 1:5 dilution and kept at −20 °C for at least 72 h and thereafter thawed at 4 °C. The disrupted emulsion was centrifuged at 8000 × g, 15 min at 4 °C. The liquid phase containing the IgY was filtered through a gauze tissue and ammonium sulfate was added (0.24 g/ml supernatant).

After centrifugation at 10,000 × g, 15 min at 4 °C, the pellet was resuspended with ammonium sulfate 2 M and centrifuged again. Finally, the pellet was resuspended and dialyzed against PBS (pH 7.4) at 4 °C.

For rabbit sera antibodies, blood samples were kept overnight at room temperature, sera were separated by centrifugation and thereafter stored at −20 °C until use. IgG was precipitated with ammonium sulfate (45% saturation) and stirred overnight at 4 °C (Spira et al., 1987). After centrifugation at 10,000 × g for 3 min at 4 °C, supernatants were discarded and pellets were dissolved and dialyzed against PBS (pH 7.4).

Pre-immune serum antibodies from non-immunized chickens and rabbits were also processed. Purity of IgY and IgG was checked by SDS-PAGE and Western Blot and protein concentration was determined by the Bradford assay, using BSA as the standard protein.

2.4. Affinity chromatographic purification of anti-Stx2B IgY and IgG

Specific antibodies were purified using HiTrap NHS-activated HP 1 ml columns (GE). A coupling procedure was done using recombinant Stx2B (1.24 mg in coupling buffer) according to the manufacturer's protocol. About 4 ml of either IgY or IgG antibodies isolated as described in Section 2.3, were applied onto the column. Unbound material was eluted with 10 column volumes of binding buffer (75 mM Tris–HCl pH 8.0). Bound antibodies were eluted with 10 column volumes of elution buffer (100 mM glycine, 0.5 M NaCl pH 2.7) in 0.5 ml fractions into collection tubes containing 100 μl 1 M Tris–HCl pH 9.0. Protein concentration in the eluted fractions was determined by the Bradford assay.

2.5. Immunoasssays

2.5.1. Western immunoblotting

Recombinant Stx2B and Stx2 holotoxin were separated by 12.5% SDS-PAGE (under reducing conditions) and transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). The membrane was blocked overnight at 4 °C with 5% skimmed milk in PBS-T 0.1%, and incubated with a 1:5000 dilution of either IgY or rabbit serum in PBS-T for 1 h at 37 °C. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-chicken IgY (1:6000) or goat anti-rabbit IgG (1:5000) for 1 h at 37 °C. Finally, membranes were revealed using DAB/H2O2 system (Pierce).

2.5.2. Indirect ELISA

Microplates (Nunc Maxisorp) were coated overnight at 4 °C with 100 ng/well of recombinant Stx2B or Stx2 holotoxin in carbonate/bicarbonate buffer pH 9.6 and blocked at 37 °C for 1 h with 5% skimmed milk in PBS-T 0.05%. After a washing step, IgG and IgY (or pre-immune samples) were 2-fold serially diluted in PBS-T and incubated at 37 °C for 1 h. Previously, both antibodies were properly diluted to give the same starting protein concentration determined by the Bradford assay (initial concentration: 1.4 mg/ml). Plates were washed and incubated with horseradish peroxidase-conjugated goat anti-chicken IgY (1:6000) or goat anti-rabbit IgG (1:5000) for 1 h at 37 °C. The plates were washed with PBS-T and developed with ABTS/H2O2 system (Pierce) and absorbance was read at 405 nm.

Antibody titers were defined as the reciprocal of the dilution of IgY and IgG antibodies corresponding to a OD 405 nm of 0,5. For chicken sera, 4-fold serial dilutions were made and antibody titers were defined as the reciprocal of the highest dilution of anti-Stx2B generating a signal about 2-fold higher than the pre-immune serum.

2.5.3. Sandwich ELISA

Microplates (Nunc, Maxisorp) were coated overnight at 4 °C with 100 μl of IgY or IgG at 500 ng/well in carbonate/bicarbonate buffer pH 9.6. After washing with PBS-T 0.05%, plates were blocked with 5% skimmed milk in PBS-T for 1 h at 37 °C. Wild type Stx2 holotoxin 3-fold serially diluted in PBS-T was added and incubated at 37 °C for 1 h.

As a negative control, DH5α supernatant was used in the same dilutions as Stx2. Plates were washed and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated anti-Stx2B IgG antibodies, coupled in the lab by using EZ-Link Plus activated peroxidase (Thermo Scientific). Plates were incubated 1 h at 37 °C and then developed with OPD (Sigma) and absorbance was read at 490 nm. Minimun concentration of Stx2 detected by IgY and IgG was defined as the reciprocal of the highest dilution generating a signal about 2-fold higher than that of DH5α supernatant.

2.6. Cytotoxicity test

African green monkey kidney (Vero) cells were plated at 10^4/well on 96-well plates in DMEM medium containing 10% fetal bovine serum and incubated overnight at 37 °C under 5% CO_2. Cytotoxic dose 50% (CD_{50}) was calculated from dose–response curves geometrically as the reciprocal of the toxin dilution causing 50% reduction in cellular viability. IgY and IgG antibodies (initial concentration: 1.4 mg/ml) (or pre-immune samples) were serially diluted 2-fold in Dulbecco's Modified Eagle Medium (DMEM) and pre-incubated for 1 h at 37 °C with an equal volume of Stx2 holotoxin (8 CD_{50}) (final Stx2 concentration: 4 CD_{50}). For the toxin control, serially 10-fold dilutions of Stx2 in DMEM without antibody were prepared. The Stx2-IgY and Stx2-IgG mixtures and Stx2 alone were added to the Vero cell monolayer (100 μl/well) and incubated for 48 h at 37 °C under 5% CO_2. The viability of the Vero cells was determined by crystal violet staining (Gentry and Dalrymple, 1980) and absorbance was read at 490 nm.Percentage of neutralization was calculated by using the following formula: OD (toxin + antibody) – OD (toxin only)/OD (no toxin) – OD (toxin only) × 100. Results were expressed as percent of neutralization compared with total IgG or IgY concentration in μg/ml. All data represent the average of triplicate assays.

2.7. Mouse neutralization assay

NIH mice (ANLIS-Malbrán) of 10–14 g, were allocated randomly over the groups. During the test period mice were housed in wire-topped plastic cages with a layer of sawdust as bedding. Cages were located in a room with controlled lighting (14 h/day), constant temperature (18–20 °C) and constant relative humidity (55–65%). Mice toxicity of holotoxin preparation was determined. Serially doubling dilutions were made in saline solution and the 5 successive dilutions, expected to cover the 0-to-100% mortality range, were tested in separate groups of 4 mice each. Median lethal dose (LD_{50}) was calculated by the method of Reed and Muench (1938). Two-fold dilutions of IgY and IgG diluted in PBS (initial concentration: 1.4 mg/ml) were incubated with 5 LD_{50} of wild type Stx2 holotoxin for 1 h at 37 °C. Groups of four mice were injected intravenously (IV) with 0.5 ml of each immunoglobulin dilution-Stx2 mixture. The mice were monitored over the next 4 days and symptoms of disease and death were recorded. Control mice injected with only Stx2 holotoxin were included in each assay and the results were used to confirm the L + /5 test dose of the toxin and to correct the antitoxin values obtained. Control mice (non-injected or injected with saline solution) were also included. Antibody titers were calculated by the Spearman-Karber method (Markus et al., 1995). All procedures involving animals were reviewed by the Animal Care and Use Committee at The National Institute of Agriculture Technology.

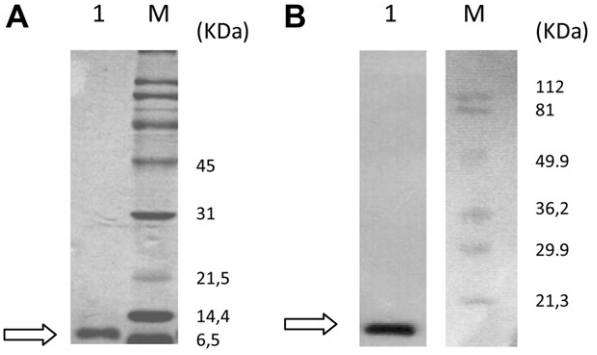

Fig. 1. Recombinant Stx2B protein purified by affinity chromatography. **A.** SDS-PAGE (12.5% acrylamide, under reducing conditions). Lane 1. Purified recombinant Stx2B protein. Lane M. Protein standard. Gel was stained with Coomassie Blue. **B.** Western immunoblot. Lane 1. Purified recombinant Stx2B protein. Lane M. Prestained protein standard. Membrane was incubated with anti-His antibody and anti-mouse ECL. *Arrow*: Stx2B subunit position.

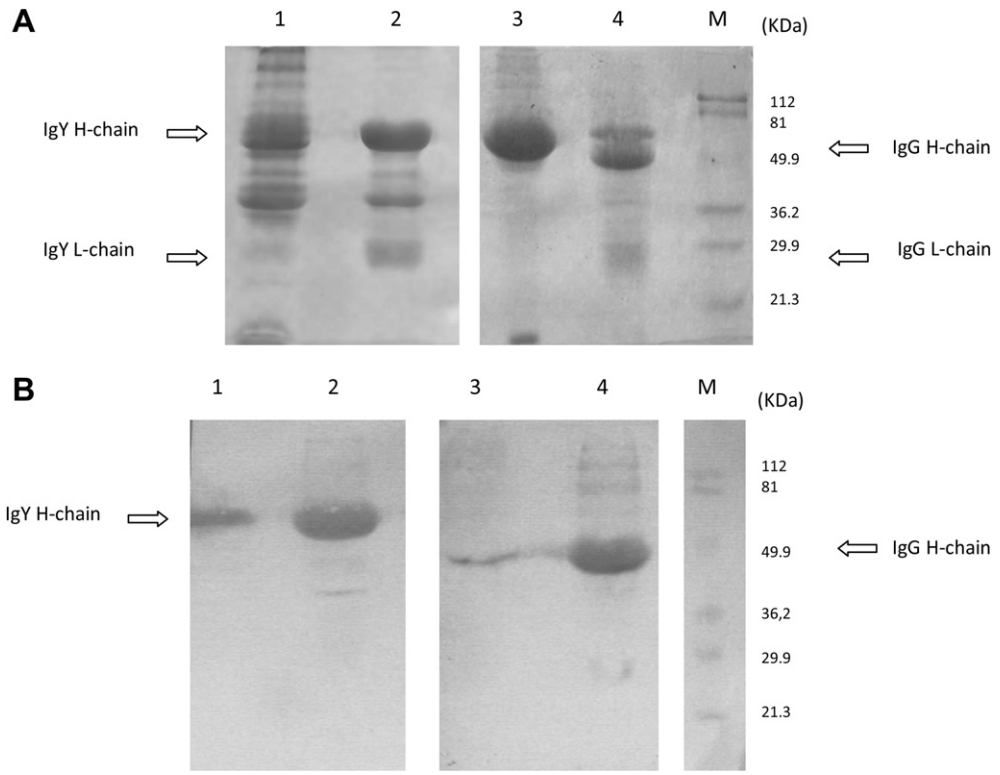

Fig. 2. Analysis of purified IgY from chicken yolk and IgG from rabbit sera by SDS-PAGE and Western blot. A. SDS-PAGE (12.5% acrylamide, under reducing conditions). Lane 1. Water dilution (1:5) of egg yolk (7 μl). Lane 2. Purified IgY by water dilution and ammonium sulfate precipitation (3 μg). Lane 3. Rabbit sera (3 μl). Lane 4. Purified IgG by ammonium sulfate precipitation (3 μg). Lane M. Prestained protein standard. Gel was stained with Coomassie Blue. **B.** Western immunoblot. Lane 1. 1:5 water dilution egg yolk (7 μl). Lane 2. Purified IgY by water dilution and ammonium sulfate precipitation (3 μg). Lane 3. Rabbit sera (3 μl). Lane 4. Purified IgG by ammonium sulfate precipitation (3 μg). Lane M. Prestained protein standard. Membrane was incubated with anti-IgY POD (lane 1 and 2) or with anti IgG POD (lane 3 and 4). DAB/H_2O_2 system was used to reveal.

3. Results

3.1. Expression and purification of recombinant Stx2B subunit

The average yield of purified recombinant Stx2B protein from 6 independent 200 ml culture preparations was 1.3 mg (6.5 mg/L). SDS-PAGE analysis of the purified Stx2B is shown in Fig. 1A. One band between 7 and 14 kDa was observed in the eluted fractions. This band likely corresponds to the recombinant Stx2B monomeric conformation of the protein (theoretical MW: 11 kDa) (Fig. 1B). Densitometry analysis of the protein bands on the SDS-PAGE gels revealed >98% purity in the purified protein preparations.

3.2. Characterization of polyclonal anti-Stx2B antibodies from chicken and rabbit

Hens had a different profile of antibody production, although in both birds the highest specific titer was found after the second immunization (day 25th, titer: 64,000). In hen n°1, specific titer diminished after the third (titer: 16,000) and fourth booster (titer: 4000) but it was increased with the last booster to approximately the same level of specific antibody than after the second immunization. In hen n°2, specific antibodies remained constant after 2nd and 3rd immunization (titer: 64,000) but diminished significantly after the final boosters (titer: 4000).

Highly purified total antibodies preparations were obtained as most of the other proteins present in sera and egg yolk could be removed (Fig. 2A and B). The final

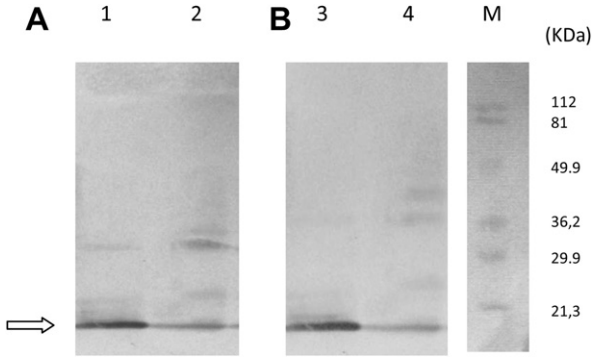

Fig. 3. Recognition of Stx2B and Stx2 holotoxin by IgY and IgG antibodies using Western immunoblotting. Lanes 1 and 3. Purified recombinant Stx2B protein. Lanes 2 and 4. Stx2 holotoxin. Lane M. prestained protein standard. Membrane was incubated with purified anti-Stx2B IgY and anti-IgY POD **(A)** or rabbit sera and anti-IgG POD **(B).** *Arrow*: Stx2B subunit position.

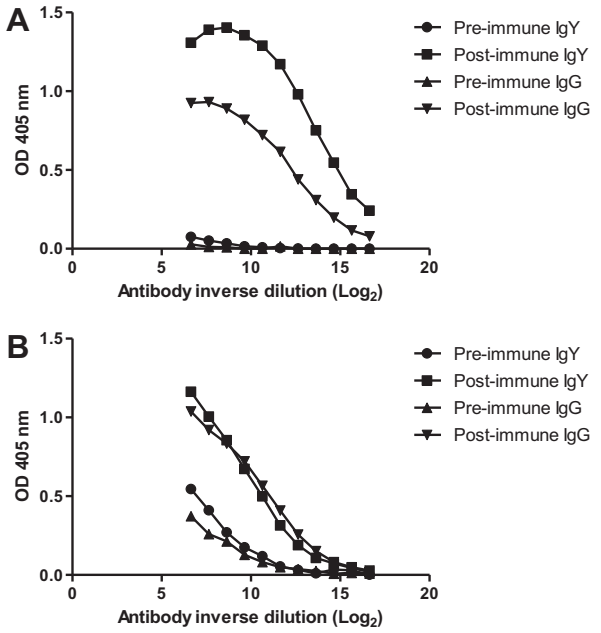

Fig. 4. Antibody recognition of recombinant Stx2B and native Stx2 holotoxin in solid phase. ELISA microplates coated with recombinant Stx2B **(A)** or Stx2 holotoxin **(B)** and incubated with 2-fold serial dilutions of purified IgY and IgG antibodies (at the same protein concentration). Preimmune IgY, and preimmune IgG correspond, respectively, to purified antibodies from eggs and sera collected before immunization.

concentration of total IgY was 0.84 mg/ml of egg yolk (equivalent to 8.4 mg of total IgY per egg).

Affinity purification of specific antibodies revealed that approximately 8% of total IgY and 6% of total IgG were antigen specific. IgY and IgG antibodies isolated as described in Section 2.3 were used at the same protein concentration for subsequent ELISA and *in vitro* and *in vivo* neutralization assays.

Western immunoblots (Fig. 3, lanes 1 and 3) and indirect ELISA (Fig. 4A) indicate that IgY and IgG were able to recognize both denatured and native forms of recombinant Stx2B protein, respectively. ELISA titers were higher for IgY (30,189) than for IgG (5219).

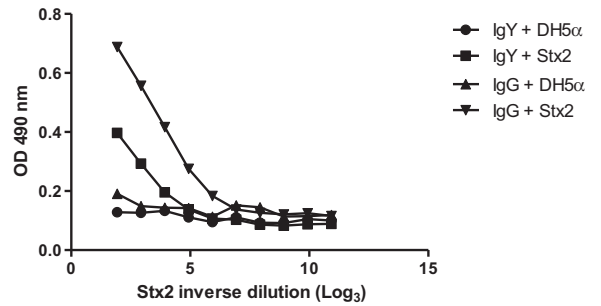

Fig. 5. Detection of antibody binding to Stx2 holotoxin in solution by a sandwich ELISA. Plates were coated with purified IgY or IgG antibodies, followed by incubation with 3-fold serial dilutions of Stx2 supernatant. Anti-Stx2B IgG POD was used as the second antibody. DH5α supernatant was used as negative control.

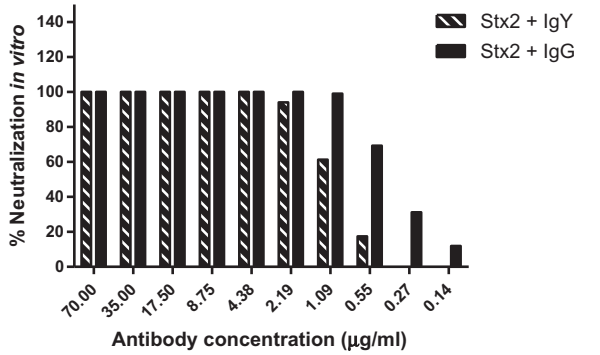

Fig. 6. *In vitro* Stx2-neutralization with IgY and IgG antibodies. Two-fold serial dilutions of IgY and IgG antibodies (starting from the same protein concentration) were incubated with 4 CD_{50} of Stx2 holotoxin. Vero cells viability was determined by crystal violet staining and absorbance was read at 490 nm.

3.3. Polyclonal anti-Stx2B antibodies from chicken and rabbit recognized both denatured and native form of Stx2 holotoxin

Specific anti-Stx2B polyclonal antibodies obtained from chicken egg yolk and rabbit sera recognized not only the denatured and native form of B subunit, used for immunization but the antibodies were also able to recognize the denatured and native wild type Stx2 holotoxin in western blot (Fig. 3), indirect ELISA (Fig. 4) and sandwich ELISA (Fig. 5). Unspecific signal was observed with pre-immune IgY and IgG antibodies, probably due to the recognition of

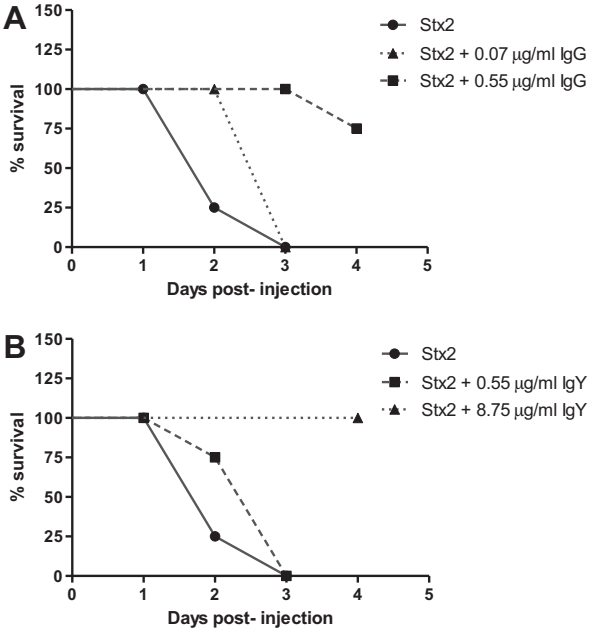

Fig. 7. *In vivo* Stx2-neutralization with IgY and IgG antibodies. Purified IgG **(A)** and IgY **(B)** antibodies were 2-fold serially diluted (starting from the same protein concentration) and incubated with 5 LD_{50} of Stx2 holotoxin. Mice were injected with this mixture and monitored over the next 4 days. A control was done with mice injected with native Stx2 without antibody (Stx2 curve). Percent of survival over time is shown for each antibody-toxin combination.

E. coli proteins from the supernatant of Stx2. Specific titer of chicken IgY and rabbit IgG against the holotoxin were of 1573 and 2149, respectively.

In the sandwich ELISA, soluble native Stx2 holotoxin was detected by IgY and IgG. The holotoxin could be detected in a 1/25 dilution when IgY was used as capturing antibody, whereas using IgG coupled to the ELISA plate it was possible to detect the holotoxin in a 1/75 dilution.

3.4. Polyclonal anti-Stx2B antibodies from chicken and rabbit neutralized Shiga toxin cytotoxicity in vitro and in vivo

The cytotoxicity of Stx2 holotoxin in Vero cells was 2000 CD_{50}/ml and the lethality in mice was 3165 LD_{50}/ml. The cytotoxicity assay based in Vero cells was used to test if anti-Stx2B antibodies from both species were able to prevent the toxin activity *in vitro*. Both IgY and IgG antibodies neutralized the cytotoxic effects on Vero cells (Fig. 6). The data obtained show that IgG antibodies were 4 times more efficient than IgY antibodies since a concentration of 1.09 μg/ml was able to neutralize 100% of the toxin cytotoxicity compared with IgY antibody that needed 4.38 μg/ml. Vero cells that were treated with the mixture Stx2-pre-immune antibodies or Stx2 alone subsequently died (data not shown).

In addition, neutralizing activity was evaluated *in vivo* during 4 days with native Stx2 holotoxin that had been previously incubated with IgY and IgG antibodies. All animals that received only native Stx2 (5 LD_{50}) died at the third day of the experiment, whereas mice that received 8.75 μg/ml of IgY antibody survived (Fig. 7B). In concordance to the results observed in the *in vitro* assay, neutralizing capability of IgG was higher than IgY antibodies. This can be easily visualized with 0.55 μg/ml of IgG (Fig. 7A) that prevented 3 of 4 mice from dying at the end of the experiment compared with mice that received IgY at the same concentration that all could not survive to the third day of the experiment (Fig. 7B).

Neutralizing activity was calculated by the Spearman-Karber method, as the antibody dilution that prevented mortality in 50% of the animals, resulting in 1:640 and 1:4305 dilution for IgY and IgG antibodies, respectively.

4. Discussion

In the present report, specific egg yolk IgY antibodies with binding and neutralizing capabilities against the wild type Stx2 toxin were obtained after immunization of laying hens. The antigen that was injected consisted in the recombinant B subunit Stx2B, obtained with a yield of 6.5 mg/L culture, a relatively elevated production compared with the 0.1 mg/L and 0.7 mg/L reached in the procedure described by Acheson et al. (1995) and Marcato et al. (2001), respectively.

In this work, significant levels of specific antibodies were measured by ELISA after 25 days of initiating immunization, showing that Stx2B (~11 kDa) is immunogenic for chickens. However, in some other mammal species as human (Ludwig et al., 2001) and mice (Imai et al., 2004), Stx2B was found to be a poor immunogen. Marcato et al. (2001) reported that high specific antibody titers against Stx2B in rabbits could only be achieved including endotoxin in the antigen preparation. So it can be hypothesized that a selective pressure to minimize a long-term immunity against the B subunit was favored in host species (Johannes, 2002). Although a high antibody response was achieved at the beginning of immunization, ELISA results from chicken sera showed that levels of specific antibodies were not constantly maintained in the immunized birds, in contrast to the results of Pauly et al. (2009). It is possible that conjugation of Stx2B with a carrier may be necessary (Marcato et al., 2005) although Wang et al. (2010) obtained a high titer of specific antibodies against B subunit from Shiga toxin type 1 without conjugation to a carrier and the response remained constant over one year. This suggests a possible negative effect of B subunit from Shiga toxin type 2 in laying hens. To the best of our knowledge, there is no information available regarding to this fact, though it has been reported that the Stx holotoxin and the B subunit have harmful effects, including lethality, in different mammal species (Huang et al., 2010). Early studies have established that chickens tend to be more resistant against toxins than other species (Bengtson, 1924).

The polyclonal chicken antibodies were tested in classical immunological assays, recognizing not only the denatured toxin, but also the native holotoxin and the B monomer either in solid-phase or in solution. The StxB subunit binding capability of egg yolk IgY was comparatively higher than rabbit IgG. However, recognition of the B subunit in the context of the whole assembled toxin was similar for IgY and IgG. In general, ideal antigenic B-cell epitopes are hydrophilic, surface orientated and flexible because in most natural environments, hydrophilic regions tend to reside on the surface of proteins, while hydrophobic regions are found hidden in the interior of the protein. Then, a possible justification for the difference in binding is that IgY antibodies recognized epitopes in the isolated B subunit that became inaccessible in the holotoxin.

In addition to the ability for binding to the native toxin, the chicken antibodies were able to neutralize crude Stx preparation, both *in vitro* (cell culture cytotoxicity assay) and *in vivo* (mouse bioassay). Wang et al. (2010) also evaluated the neutralizing capability of anti-Stx IgY antibodies by *in vitro* and *in vivo* experiments, but they tested the protective efficacy against Stx1. In this work, we demonstrate that hens, and also rabbits, can produce neutralizing IgY antibodies against Stx2, which is associated to more severe course of illness (Karmali et al., 1985) and when administered systemically it is about 400 times more lethal to mice than Stx1 (Tesh et al., 1993). Also in our work, we completely neutralize the activity of Shiga toxin *in vitro* and *in vivo* with an IgY concentration of about 700 times lower than that used in Wang et al. (2010). Others authors have similarly generated neutralizing chicken antibodies against ricin toxin (Pauly et al., 2009), botulinum toxins (Gomez et al., 2006; Pauly et al., 2009), *Clostridium difficile* toxins, rabies and viper venom (Motoi et al., 2005).

In this work, rabbit anti-Stx2B IgG antibodies were about 4 times more effective in Stx2 neutralization than egg yolk antibodies (at a similar mass) *in vitro* and *in vivo*. However, to obtain 4 mg of specific anti-Stx2B antibody from one hen the collection of only 5 eggs was enough,

while to reach the same amount of specific IgG, the exsanguination of one rabbit was necessary. Total IgY yield per egg was 8.4 mg although Gassmann et al. (1990) described a yield of 64 mg IgY per egg. However, there was less difference in the amount of specific antibodies per egg: 0.67 mg from this work and 2 mg from Gassmann et al. (1990). Another group (Bizhanov and Vyshniauskis, 2000) reported a recovery rate of 2–10 mg of anti-Sendai virus IgY per ml of egg yolk.

Antibiotic therapy is not recommended for food poisoning caused by enterohemorrhagic *E. coli* infection, because increases the risk of serious complications, such as hemolytic uremic syndrome, due to the release of Shiga toxin from killed bacteria and by inducing expression of Stx through replication of phages that carry *stx* genes (Kozlov et al., 1988). Therefore, alternative therapeutic approaches, such as inhibiting Shiga toxin activity or absorption from the intestine, are required. Oral administration of IgY has proved successful for treatment of a variety of gastrointestinal infections, such as bovine and human rotaviruses, bovine coronavirus, *Yersinia ruckeri*, *C. difficile*, *Salmonella* spp., *Edwardsiella tarda*, *Staphylococcus* and *Pseudomonas*, (Mine and Kovacs-Nolan, 2002; Schade et al., 2005). Chicken egg yolk has previously been used as an inexpensive and effective source of IgY antibodies for the passive immunization or treatment of piglets suffering enterotoxigenic F4(K88)+ (Marquardt et al., 1999) and F18ab+ (Imberechts et al., 1997) *E. coli* infections, dental caries due to *Streptococcus mutans* in humans (Smith et al., 2001) and porcine epidemic diarrhea virus (PEDV) infection in piglets (Kweon et al., 2000). Therefore, anti-Stx IgY could be an economic alternative for prophylactic and therapeutic treatment of EHEC infections or Stx exposure, either alone or combined with other products. Furthermore, therapeutic use of IgY in primates was effectively studied by LeClaire et al. (2002) using neutralizing IgY antibodies against the highly toxic staphylococcal enterotoxin B (SEB).

Neutralization of Stx may also be useful to decrease cattle colonization by STEC (Hoffman et al., 2006), which is considered the main reservoir of the bacteria. Since Stx plays a role in both colonization and systemic disease, passive administration of anti-Stx antibodies to reduce or prevent EHEC infection in people has also been proposed (Krystle et al., 2010). This was demonstrated in mouse models with anti-Stx2 antibodies administered before infection (Donohue-Rolfe et al., 1999). In subsequent evaluations of the efficacy of such passive therapy, anti-Stx antibodies were shown to provide protection against STEC-mediated illness and death even when administered up to 4 days after bacterial challenge (Donohue-Rolfe et al., 1999; Yamagami et al., 2001). Besides, IgY is widely used in many applications ranging from immunofluorescence, immunohistochemistry, immuno-enzyme techniques (ELISA), Western blotting and immunoelectrophoresis (Lee et al., 2002; Tini et al., 2002). These antibodies are capable to detect multiple bacterial and parasitic organisms such as *Acanthamoeba* spp., *Helicobacter pylori* (Shin et al., 2002), microsporidia (Young et al., 2007), *E. coli* O157:H7 (Sunwoo et al., 2006).

In conclusion, although further studies should evaluate different adjuvants and immunization plans in chickens in order to maximize the anti-Stx2B IgY production, this work shows that IgY technology is a promising alternative to be applied in the detection of STEC and the prophylaxis or treatment of Hemolytic Uremic Syndrome.

Conflict of interest statement

There are no conflicts of interest related to this study.

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