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Research Article

IgY: A Promising Alternative to IgG in Immunodiagnostics

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Abstract

IgY is a predominant antibody found in reptiles, birds and amphibians. IgY is equivalent to IgG of mammals. In the present study, IgY antibodies against *Shigella flexneri* was produced by immunizing chicken with 0.2 ml of 3.6x109 CFU ml^{-1} of formalin inactivated *Shigella flexneri* ATCC 9199 strain. Eggs were collected and IgY antibodies were harvested using PEG precipitation method. Yield of ~25 mg of IgY per egg yolk was recovered. Dot ELISA with immunized IgY showed reactivity with *Shigella flexneri* and also other *Shigella* species, while naïve IgY did not exhibit any reactivity. IgY cross-reactivity with other *Shigella* members indicate the prosect of using IgY in detection and neutralization of pathogenic *Shigella* strains.

Keywords: Immunoglobulin Y; Egg Yolk; *Shigella flexneri*; Dot ELISA; UV Spectrum

Abbreviations

ELISA: Enzyme Linked Immunosorbent Assay; PBS: Phosphate Buffered Saline; SDS-PAGE: Sodium Dodecylsulphate – Polyacrylamide Gel Electrophoresis; FCA: Freund's Complete Adjuvant; FIA: Freund's Incomplete Adjuvant.

Introduction

Immunoglobulins are the proteins belonging to the class of glycoproteins that can be secreted by plasma cells upon infection and plays a key role in humoral immunity. Immunoglobulins are majorily employed in diagnosis and research that aids the quantification of antigens in serum and other biological fluids using techniques such as ELISA and western blotting [1]. Traditional methods include mammalian immunoglobulins that can be extracted from blood by means of retro orbital bleeding or cardiac puncture that leaves the animal in pain and distress or even death. IgY from the immunized chicken will be an outstanding solution that can replace the existing mammalian antibodies [2].

IgY contains a characteristic "Y" shaped structure comprising two light and two heavy polypeptide chains. Comparative studies with respect to mammalian antibodies IgG have thrown light on the basic differences between two structures that involve the heavier molecular weight i.e, (~180 KDa) because of the presence of an additional constant domain (CH4) on it's heavy chain and it lacks the hinge region and also contains an unique oligosaccharide embedded in its structure [3]. Physiological conditions to maintain the stability of IgY include pH 4-9 and temperature up to 65°C. Three isotypes are reported in birds (IgA, IgY and IgM) where IgY is predominant i.e, (5 to 15 mg/ml). Chicken IgY is systemic in nature but not secretory, hence can also be found in duodenal contents, seminal plasma and tracheal washings in addition to the egg yolk [4]. Mammalian antibodies, despite their profuse use, come across few disadvantages while diagnosis and detection of pathogens. These hurdles include cross-reactivity with immunoglobulin binding proteins like Protein 'A' of *Staphylococcus aureus*, Protein 'M' of *Mycoplasma genitalium*, Protein 'G' of Streptococcal bacteria and Protein 'L' of *Peptostreptococcus magnus* [5]. Because of the structural discrepancies of IgY i.e, presence of an extra constant domain (CH4) on the heavy chain unlike mammalian IgG, the interaction with the immunoglobulin binding proteins remains incapable. Also, it is unable to activate the complement system. Major advantage lies in the non-invasive protocol of the antibody extraction. Higher yields i.e, 40-80 mg of total IgY per egg makes IgY more advantageous in comparison with mammalian IgG [6]. Additional constant domain and the heavier molecular weight (~180 Kda) makes the IgY least available to all the immunoglobulin binding proteins thereby decreasing the cross-reactivity to a considerable extent. Also higher yields will make the immunodiagnostics cost-effective. In addition to their application in immunodiagnostics, the edible nature of egg yolks makes the IgY also being used for therapeutic purposes. Orally administered IgY antibodies serves as the most promising approach for the treatment of gastrointestinal infections caused by enteric pathogens such as retrovirus, *Escherichia coli* etc [7].

Shigellosis is an acute intestinal infection caused by *Shigella*. The four major species of them include *S. dysenteriae, S. flexneri, S. boydii, S. sonnei.* The incidence of shigellosis is creating a havoc i.e, around 8 million cases per year causing 1 million deaths annually. Shigella members are gram negative rods belonging to the enterobacteriaceae family. Because of the lower susceptibility towards stomach acids only a few CFU can cause disease [8]. Bacterial multiplication takes place in the small intestine followed by colonic

transmission i.e, entry into the colonic cells. Spread of *Shigella* is possible from person to person contact and from food and water contamination to a minor extent. Human will serve as natural reservoirs for the spread of the disease [9]. Onset of disease symptoms can be seen post 1-2 days after the bacterial exposure and later lasting up to 5-7 days [10].

Apart from antimicrobial therapy, treatment of *Shigella* infections has no alternatives. No vaccine is available yet for human application till date. However early detection eases timely treatment by using targeted drugs [11]. Due to increase in antimicrobial resistant *Shigella* strains, alternative therapies are needed along with fast and low cost diagnostics. In the present study, we explored the possibility of IgY application for diagnosis of *Shigella* members by dot ELISA.

Material and Methods

Formalin Inactivation of *Shigella* cells

Shigella flexneri ATCC 9199 was inoculated into 5ml of Brain Heart Infusion broth followed by incubation for overnight at 37°C in a shaking incubator. After incubation, the cells from the overnight culture were harvested by centrifugation at 10000 rpm for 8-10 minutes. Supernatant was discarded and the pellet was washed two times with acetone followed by two times with diethylether. Later the cells were resuspended with 1% formalin saline. Tween – 80 was added to the suspension to a final concentration of 3 – 4%. This procedure was repeated three times with saline solution and the inactivated bacterial cells were made up to a final cell concentration of ~3 x 10^8 cells/ ml [12].

Production of IgY from chickens

Production of IgY was carried out by immunizing chicken with 0.2 ml of 3x10^8 CFU/ml of formalin inactivated *Shigella flexneri* cells along with Freund's complete adjuvant during their egg laying period at an interval of 10 days followed by three booster doses with same number of CFU along with Freund's incomplete adjuvant. Immunization was done through intramuscular route in the breast muscle. Laid eggs were collected and separated for IgY extraction after 7th day of the last immunization. Eggs were collected up to a continuous stretch of 3-4 days followed by storing at 4°C for further extraction.

Primed chicken were housed separately under sterile conditions. White leghorn breed of layers were employed in this study. Chickens were fed with the feed that has all the required nutrients and minerals from a commercial vendor.

Isolation and purification of IgY

Laid eggs were collected from immunized hen. Egg yolk is filtered using filter paper, thereby removing the egg white part completely and transferred into a 50ml tube. Twice the volume of PBS was added to the yolk fraction, followed by the addition of 3.5% pulverized Polyethylene glycol (PEG), vortexed and rolled on a rolling mixer. Two phases got separated with yolk solids (fatty substances) from that of aqueous phase having IgY with other proteins. Aqueous phase was separated by centrifugation at 10000 rpm for 20 min at 4°C through a folded filter. PEG 6000 at 8.5% was added, vortexed and rolled in a rolling mixer followed by centrifugation at 10000 rpm for 20 min at 4°C. 1 ml of PBS was used to initially dissolve the pellet and final volume was made up to 10 ml followed by centrifugation at 10000 rpm for 20 min at 4°C, 800µl of PBS was used to dissolve the pellet carefully without any bubbles. The pellet containing IgY extract was dialyzed against PBS under 4°C and maintained at -20°C [13]. Spectrophotometric measurement of total protein (IgY) content was done at 280nm and applied Beer-Lambert law having an extinction Co-efficient of 1.33. Quality of the final preparation was analyzed by SDS-PAGE.

SDS-PAGE

SDS-PAGE electrophoresis was used for fractionation of IgY. Bio-Rad vertical electrophoresis unit was used for this experiment. 12% concentration of acrylamide gels were used for separation and compositions were mentioned in the table below. Briefly, both thick and thin plates were attached and placed into the casting frame and clamped to gel stand and 12% of resolving gel was added in between both of the plates and allowed it for solidification for a period of 10-15 min. To the already solidified resolved gel 5 ml of stacking gel was poured and the comb was placed and left for a period of 15-20 min to ensure complete polymerization. After polymerization, gel plates were carefully attached to the tetra electrode assembly and kept in the vertical gel tank that is filled with Tris-glycine buffer. IgY samples along with prestained protein ladder (Takara) was loaded to the wells and made it to run at 100 V until the tracking dye disappears and leaves the gel. Once the electrophoresis was completed the gel was carefully removed using gel releaser and allowed it to stain for overnight in Coomassie brilliant blue stain on a gel rocker. After sufficient staining of the gel destaining was done using destaining solution (Water: Methanol: Glacial Acetic Acid in the ratio 5: 4: 1 until the bands were clearly seen [14].

List of buffers used were mentioned below.

S.no	Components	Concentration
2X Laemmli buffer	Tris-Cl	0.125 M
	Glycerol	4%
	Bromophenol blue	20%
	SDS	0.004%
Tris-Glycine buffer or Running buffer	Tris	25 mM
	Glycine	250 mM
	SDS	1%
Coommassie staining solution	Commassaie brilliant blue	0.25 %
Destaining solution	Water: Methanol: Glacial acetic acid	50:40:10

Table a

Reactivity of IgY

Dot ELISA

Grids on nitrocellulose membrane were marked with 1x1 cm dimension. Each grid was coated with 10µl of overnight grown *Shigella* strains followed by the incubation at 40°C for 30 min. Mem-

brane was blocked with 2% BSA solution followed by incubation at room temperature for 1 hr. The blocking solution was decanted and washing was done using Phosphate buffer saline tween-20 (PBST) for 4-5 times. Membrane was flooded with the primary antibody (Immunized IgY) diluted to 1:1000 dilutions with Phosphate buffer saline and incubated for 1 h. The primary antibody solution was decanted and the membrane was washed with PBST for 3 times. Secondary antibody (Goat Anti-Chicken Antibody) was also diluted to 1:2000 dilution with Phosphate buffer saline, added to the membrane and incubated at room temperature for 30 minutes. 0.1 mg/ml of diaminobenzidine tetrachloride (Sigma) and 1 µl/ml of 30% H_2O_2 were used for the development of membrane after incubating for 5-10 min with gentle rocking. Finally the membrane was rinsed with distilled water and the result was analyzed by visual inspection.

Results and Discussion

Immunization schedule

Chicken were first immunized with the said antigen using Freund's complete adjuvant (FCA) then followed by Freund's incomplete adjuvant (FIA) within a ten day interval. Chicken were administered with 0.2 ml of 3.0 x 10^8 CFU/ml of formalin inactivated *Shigella flexneri* cells. Immunization schedule followed as described below.

No of days	Activity
Day: 0	First Immunization
Day: 10	Second Immunization
Day: 20	Third Immunization
Day:30	Fourth Immunization
Day: 35	Eggs were collected (having antibodies)

Table 1: The immunization schedule of Chicken.

IgY purification

IgY purification from the immunized chicken egg yolks was done using PEG 6000 precipitation followed by extensive dialysis and reconstitution in PBS. The quality of final preparation was analyzed by SDS-PAGE and UV absorbance at 280 nm. IgY concentration was estimated to be approximately 20 mg/ml. However, the yield of IgY was measured by Folin - Lowry assay and yield from each egg is ~25 mg. Dialysis was done in 1X PBS for the eluents to remove the traces of other interfering proteins that might come from egg white.

Reactivity of immunized IgY

The IgY antibodies raised against *Shigella flexneri* was tested for their reactivity by simple Dot-ELISA assay. Immunized IgY antibodies showed reactivity with both formalin inactivated *Shigella flexneri* cells and also live overnight *Shigella flexneri* cells. In addition, immunized IgY also demonstrated reactivity to other strains of Shigella flexneri and other species of *Shigella* namely *Shigella sonnei* and *Shigella boydii* (figure 3). On the other hand, native IgY did not show any reactivity to any *Shigella* strains.

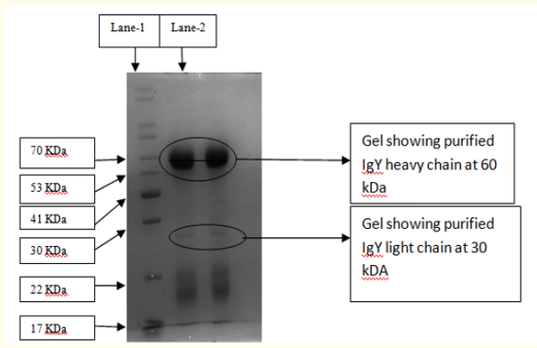

Figure 1: 12% resolving SDS-PAGE gel showing the purity of IgY from primed egg yolk after final preparation using 8.5% PEG. Lane 1: Takara Prestained Protien ladder, Lane 2: IgY after 8.5% PEG precipitation. The IgY HC~60 kDa, and the LC~ 30 kDa.

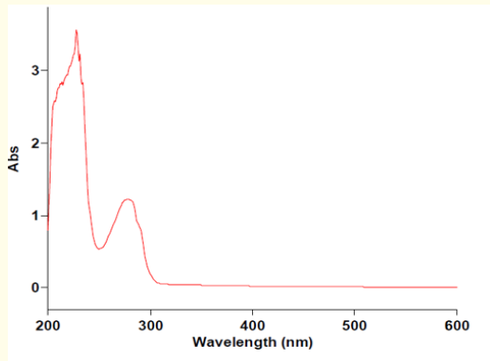

Figure 2: UV Spectrum showing the purity of IgY final preparation from egg yolk using 8.5% PEG precipitation. Peak at 220 nm indicates the presence of aromatic amino acids and peak at 280 nm indicates the presence of IgY.

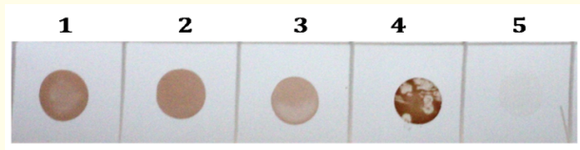

Figure 3: Dot-ELISA showing reactivity of IgY against *Shigella* species. 1 - Formalin treated *S. flexneri* ATCC 9199 cells; 2 – Overnight *S. flexneri* ATCC 9199 cells 3 - Overnight *S. sonnei* ATCC 25931; 4 - Overnight *S.boydii* ATCC 9207; 5 - DH5α strain.

Conclusion

IgY antibodies were packed with countless advantages such as lower assay background, stronger avidity and accuracy. A few of the other advantages include distinct Fc region having no reactivity towards immunoglobulin binding proteins and have a minimal role in complement activation. Finally, IgY can be an outstanding alternative that can reduce or replace invasive procedures that include antibody extraction from blood that imparts pain and distress or even death of the experimental animals. Moreover, IgY is cost effec-

tive and larger quantities can be produced with minimal costs. This article proves the reactivity of IgY with different strains of *Shigella* thereby showing the efficacy of IgY for the detection of *Shigella*. Future work will include the use of IgY as therapeutic antibodies for oral administration offering effective treatment for Shigellosis.

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Conflict of Interest

Authors declare no conflict of interest.

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