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DEVELOPMENT OF ELISPOT FOR RAPID DETECTION OF ROTAVIRUS INFECTION USING AVAIN IGY ANTIBODIES

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ABSTRACT

Immunoassays are widely being used globally for the detection of rotavirus infection. Keeping pace with the severety of infections due to the development of various strains, detection assays are also developed to identify the infection rate rapidly. A strip based ELISA assay, ELISPOT was developed for rapid detection of rotavirus infection and to determine the specificity and sensitivity of anti-recRVP6 IgY antibodies using western blotting assay. The limit of detection was 1µl/ml and the quantification was 1µg/ml of concentration was sufficient for the strip based ELISA detection. This asssay is quite handy and easily adoptable having higher sensitivity than currently available tests for rotavirus detection.

KEYWORDS: ELISPOT, IgY antibodies, rapid detection, western blotting, sensitivity.

INTRODUCTION

Many epidemiologic studies has reported that group A rotaviruses (RVA) are major cause of severe gastroenteritis in young children and animals (Estes and Kapikian, 2007; Dhama *et al.*, 2009; Martella *et al.*, 2010). The severity of diarrheal disease is enhanced by several environmental, nutritional and immunological factors (Saif and Smith, 1985; Bendali *et al.*, 1999b). Rotavirus infection occurs in young children below 5 years old and usually occurs in young claves of 2 to 8 weeks old. In both the cases the severity decreases as age increases. The incubation period is very short approximately 12 to 24 hours the infection develops and this diarrhea self limits within 5 to 7 days by itself unless there are no secondary infections occuring in the host system (Svennerholm and Steele, 2004; Dhama *et al.*, 2009). Good

health management and hygienic practices will pave a way to reduce the infection rates while antibiotics can be taken to control secondary infections. Fluid and rehydration therapy can help to rstore the fluids reserves which minimizes the mortality rates (Holland, 1990; Svennerholm and Steele, 2004). Rotavirus is a gastrointestinal pathogen which stimulates the responses of the local gut-associated lymphoid tissues (GALT) and thereby it becomes dfficult for the virus clearance and prevention of rotavirus infection. Hence, it is efficient to develop a passive maternal antibodies (Ab) like Chicken egg yolk antibodies (IgY) that are recently gaining more attention as an alternative source of antibodies against antibiotics, as IgY antibodies are naturally present in egg yolks in large quantities, making chickens as an ideal source of polyclonal antibodies effectively (Touchette *et al.,* 2003; Kovacs-Nolan and Mine, 2004). Several reports have been published with chicken egg yolk antibodies (IgY) proving that these antibodies are effective in controlling and protecting rotavirus infection (Ikemori *et al.,*1997; Kuroki *et al.,* 1994). 22 weeks old white leghorn chickens were immunized with recRVP6 protein for four immunizations intramuscularly to develop anti-recRVP6 IgY antibodies. The aim of this study was to generate, purify and *invitro* characterize the anti-recRVP6 IgY antibodies raised against the recRVP6 protein and also to develop a rapid ELISPOT assay for the detection of rotavirus infection using anti-recRVP6 IgY antibodies effectively.

1. METHODOLOGY AND RESULTS

1.1. Generation of anti-VP6 IgY Antibodies in Chickens

The recRVP6 protein purified by Ni- NTA Sepharose column under native condition was dialyzed against PBS and was used as antigen to immunize 22 weeks old white leghorn chickens for raising anti-recRVP6 IgY antibodies.

1.2. Antigen Preparation & Immunization of Birds

22 weeks old White leghorn chickens were obtained from Chandran poultry, Coimbatore. The birds were procured and maintained to our animal house environment for a week and their egg laying performances were monitored at regularly. The birds were divided into two groups as test (n=2) and control (n=1). Purified recRVP6 protein (100µg) in 0.5ml of 0.01M PBS pH7.4 was mixed with equal volume (0.5ml) of Freund's Complete Adjuvant (FCA) (Sigma, USA) were used as immunogen for the first dose. The chickens were intramuscular immunized in the breast muscle at two different sites. The second and third booster doses were given with 100µg of purified protein in 0.5ml of 0.01M PBS pH7.4 with 0.5ml of

Freund's Incomplete Adjuvant (FIA) at two weeks of intervals. The eggs from immunized and unimmunized chickens (controls) were collected daily, labelled and stored at 4°C for purification of antibodies (Table-1).

Table 1: Immunization schedule.

Immunization Days	Doses	Antigen concentration of recRVP6 protein	Adjuvant used	Volume of adjuvants	Route of immunization
0th Day	First	100µg/ml	FCA	0.5ml	Intramuscular
14th day	Second	100µg/ml	FIA	0.5ml	Intramuscular
28th day	Third	100µg/ml	FIA	0.5ml	Intramuscular

*FCA – Freund's Complete Adjuvant *FIA – Freund's Incomplete Adjuvant

2.3. Purification of anti-recRVP6 IgY antibodies using (PEG-6000) Polson *et al.*, 1980 method

The stored eggs collected from both immunized (test) and unimmunized chickens (control) were taken for purification of immunoglobulins from the egg yolk. The separated yolks from the eggs were mixed with an equal volume of 100mM phosphate buffer, pH 7.6 and mixed thoroughly with a glass rod. 3.5% (w/v) polyethylene glycol (PEG 6000) was added and mixed until the PEG completely dissolved. The sample was centrifuged at 10,000 x g for 20 mins at room temperature. A cotton wool plug was firmly placed at the base of a funnel, and the supernatant was filtered through it. The lipid fraction is trapped by the cotton wool. The filtrate volume was recorded and the PEG concentration was increased by 8.5%. The PEG was dissolved completely by mixing. The suspension was centrifuged at 10,000 x g for 20 mins at room temperature. The supernatant was discarded and the pellet being dissolve in phosphate buffer equal to the egg yolk volume. PEG was again added to a final concentration of 12% (w/v), mixed thoroughly, and centrifuged at 10,000 x g for 20 mins at room temperature. The supernatant was discarded and the final pellet containing the antibodies was resuspended in 1⁄6 of the original egg yolk volume in phosphate buffer.

2.4. Preparation of dialysis tubing

Dialysis membrane were taken and cut into pieces as per the required length needed for the salting out process. The bags were activated by boiling it for 10 minutes in distilled water and washed with cold distilled water. Then it is placed in a large volume of sodium bicarbonate buffer, rinsed thoroughly in cold distilled water and then boiled in 1mM EDTA (pH 8.0). The tubing was cooled down and stored at 4°C in 50% ethanol.

2.5. Desalting IgY fraction

The PEG Purified pooled IgY fraction was transferred to an active dialysis bag. The bags containing pooled contents were dialyzed against 1L of phosphate buffer pH-7.2. The setup was continuously stirred by means of a magnetic stirrer and the temperature was maintained at 4ºC. It was carried out over 16 hours with over two changes of buffer. The contents were dispensed into Eppendorf tubes after dialysis and centrifuged in order to remove the remaining impurities.

2.6. Concentration of antibodies

The pooled IgY fraction was transferred to a dialysis bag and tied after including some air and twisting the open end of the dialysis bag to develop reasonable pressure. It was placed in a Petri dish and covered with finely powdered polyvinyl pyrolidone (PVP) at room temperature. The Petri dish was tilted to keep the bag on higher side and to allow the fluid to come out of bag, to facilitate the flow of water and other low molecular weight material to ooze out of the bag and to form a viscous solution of PVP. To maintain the initial rapid rate of flow of fluid, the dialysis bag was squeezed by twisting from one end and tied from time to time.

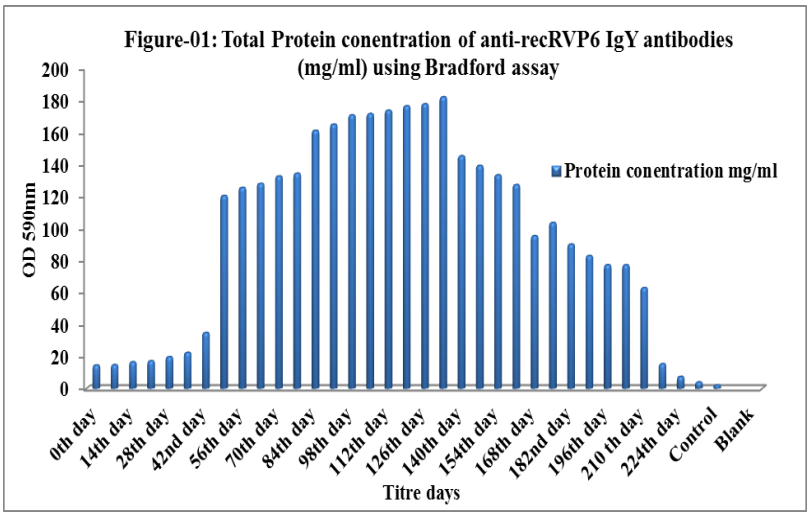
2.7. Characterization of the purified IgY

The concentrated protein was then checked for protein estimation by Lowry's and analysed by SDS-PAGE according to the method of Laemmli, 1970.

2.8. Estimation of Total Protein

Stock solution of Bovine Serum Albumin was prepared by adding 1mg/ml concentration of BSA and serial dilutions were performed. Bradford reagent was added to all the dilutions and incubated in dark for 60 minutes. The absorbance was read at 590 nm and the OD values plotted against the different concentrations of BSA to obtain the standard calibration graph. The purified IgY samples was checked for the total protein and it was found to be 181.73

mg/ml in 133rd day from the immunized chicken's egg yolk and 2.10 mg/ml in the yolk of unimmunized chicken's egg yolk (Figure-01).



2.9. SDS PAGE Analysis

The antibody samples were separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to Laemmli, 1970 with a 5% stacking and 12% separating gel. The samples were taken (20µl) and treated with an equal amount of sample loading buffer. The samples were loaded into sample wells and known protein molecular weight markers (Genei Pvt. Ltd, Bangalore). The electrophoresis was performed at 100V current using SDS electrophoresis buffer. The gel was stained with Coomassie brilliant blue R-250 for 30 minutes. The gel was rinsed with distilled water and destained. The IgY sample was viewed against 180kDa molecular weight marker (Figure-02).

2.10. Determination of antibody titre by Indirect ELISA

The immunological specificity of IgY elaborated against recRVP6 protein were analyzed using Indirect enzyme-linked immunosorbent assay (ELISA). In brief, wells of Microtiter plates were coated with 1µl/ml of antigen solution diluted with 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed with 100µ1 of PBST (pH 7.4) and blocked using 100 µ1 of 1% bovine serum albumin (BSA) were added to the wells. After being blocked, all the wells were washed three times with 100 µl of PBS-Tween-20 (PBST). After washing added 1 µl/ml of anti-recRVP6 IgY antibodies and incubated 37ºC for 1 hour. After each well was washed again with 100µl of PBST, 100µL of horseradish peroxidase-conjugated rabbit anti-chicken IgY diluted (1:1000) with PBST were added to each well and incubated at 37 °C for 1 hour. Each well was washed again with 100 µl of PBST. 100 µl of

TMB solution with H_2O_2 (Genei Pvt. Ltd., Bangalore) were added and incubated in dark for 20 minutes at room temperature. The reaction was stopped after 20 min with 4N H_2SO_4 (100µl per well), and the intensity of colour developed was measured at 490 nm with a micro-plate reader. IgY purified from unimmunized hens egg yolk was used as control. The highest titre of antibodies as 1:10000 were obtained during 19th week after initial immunizations (Figure-03).

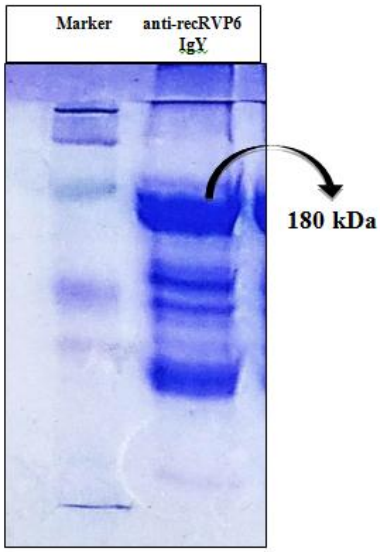


Figure 02: SDS-PAGE Analysis A

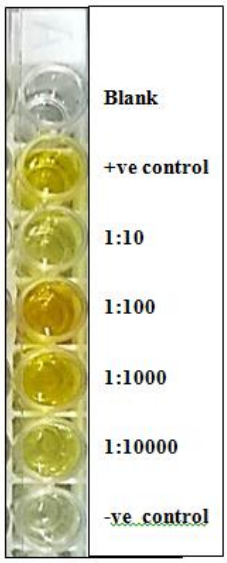


Figure-03: Indirect ELISA.

2.11. Western blotting analysis

To confirm the sensitivity and specificity of antibodies, western blotting is always performed. The assay was initiated by running the sample (anti-recRVP6 IgY antibodies) in SDS-PAGE gel. After running the gel for 2 hours 30 minutes at 75V, the gel were placed on the blotting sheet holding the nitrocellulose membrane and packed like a stack. 1X TB (transfer buffer) was added enough for the transfer os samples to the nitrocellulose membrane using a Semi-dry western blot apparatus for approximately 1 hours 30 minutes. After transfer is complete, the membrane was washed with TBST and blocked with 10ml of 3% BSA for 2 hours at room temperature on a shaker. After incubation with BSA, membranes were washed three times with TBST for 15 mintues. Then added 10µl/ml of primary antibody (anti-recRVP6 IgY) and incubated overnight at 4ºC on a shaker. After overnight incubation, membrane was washed with TBST thrice for 15 mintues at room temperature and added 1 µl of secondary antibody (rabbit anti-chicken IgY) and incubated for 2 hours at room temperature on a shaker. Then the membrane waas washed again as the same and taken for developing of bands visibility using ECL solution (Biorad) and observed using GelDoc analyzer.

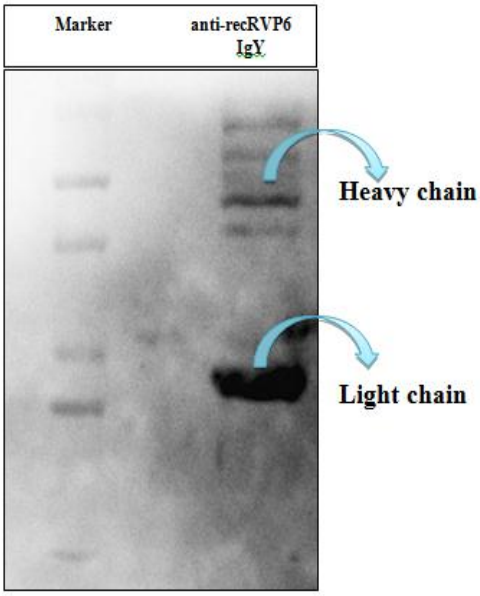


Figure-04: Western Blotting **Figure-05: ELISPOT assay**

2.12. Development of ELISPOT assay for detection

A sterile fresh strip was taken for the ELISPOT (strip based ELISA) assay development for detecting rotavirus infection effectively using anti-recRVP6 IgY antibodies. 2µl of antigen sample was applied onto the nitrocellulose membrane coated strip and allowed it to dry by plaing it in a 10cm petridish for 20 minutes at room temperature. After drying, the strip was soaked in 5% BSA in PBST for 30 minutes at room temperature. The strip was washed using PBST and allowed it to dry. Then added 1µl/ml of anti-recRVP6 IgY antibodies (primary) to the strip and incubated for 30 minutes at room temperature. Washed again with PBST and dried. Added 1µl/ml of secondary antibodies (rabbit anti-chicken IgY) and incubated for 30 minutes at room temperature. After incubation the strip was taken for washing with PBST and allowed to dry. 10µl of TMB substrate was added to the strip to develop a blue spot which indicates positive for the detection of antigen and antibody interaction. This rapid and less time consuming assay can be used for the detection of rotavirus infection effectively (Figure-05).

3. DISCUSSION AND CONCLUSION

Being one of the most sensitive immunoassays, ELISA offers a commercial value in laboratory scale research and diagnosis with quality control assurance from the infected samples. A simple, specific and sensitive polyclonal based strip ELISA (ELISPOT) assay has been developed, which detects rotavirus antigen at 1µl/ml of anti-recRVP6 IgY antibody. This assay can be easily used for large scale rapid screening effectively.

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