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Generation and characterization of chicken egg yolk rotavirus antivac IgY antibodies for its prospective use in oral passive therapy

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Abstract

Among diarrheal diseases in developing countries, Rotavirus gastroenteritis plays the major reason for infant mortality and hospitalizations in India under 5years of age. In India, nearly 80,000 to 1lakh children die and 9lakh hospitalizations, 32.7 lakh hospital visits happen yearly due to this infection. Since the incidence rate is very high and only oral rehydration therapy is available, there is an urgent need for an alternative therapy to treat the infection. Antibody therapy proves to be an effective alternate oral therapy to treat the rotavirus infection. Chicken Egg yolk antibodies against the vaccine strain of rotavirus were generated by immunizing white leghorn chicken intramuscularly. Antivac IgY antibodies were purified by PEG precipitation method followed by Dialysis. Total protein content of the purified antibodies was found to be 40.96 mg/ml and the total IgY antibodies were found to be 14.91 mg/ml. Also, the purified Antivac IgY antibodies correspond to 180kDa protein as visualized in SDS PAGE Analysis. The antibody titer was around 1:50,000 as determined by Indirect ELISA method. The antibodies were stable up to the temperature of 40°C and the activity started diminishing above 60°C.The antibody activity was affected by acidic pH of 3 and below and the activity was diminished to an extent in the alkaline conditions of pH 9 and above. The activity was unaffected between pH 4 to pH 8.0. Hence, these Antivac IgY antibodies can be used in passive oral immunotherapy for treating a rotaviral infection.

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Introduction

According to WHO report on child mortality, nearly 9 million child deaths happen worldwide and 70% of them are preventable reasons. One of the major reason for infant mortality and hospitalization was due to diarrheal disease next to the primary reason of Pneumonia. Among the major developing countries, India ranks number one in the highest mortality rate of infants because of moderate to severe diarrhea as found out from the Global Enteric Multicenter Study (GEMS Study). *Rotavirus* was the major contributor to the mortality followed by *Cryptosporidium* and Enterotoxigenic *E. coli* strain for causing moderate to severe diarrhea in children below 5 years of age group.

The children in India and other African countries are being exposed to the virus at least once before they attain the age of 5 even after the introduction of vaccines too. Oral rehydration therapy with zinc supplementation is the possible treatment option, but still, the hospitalization rates are much higher up to 9 million cases yearly. Hence an alternative therapeutic method is of a greater concern to treat the children apart from the conventional rehydration therapy to recover quickly from the infection.

In recent decades, passive therapy approaches are gaining attention due to its specificity towards the pathogen responsible for the infection and minimal risk factors involved in treating the patients with immune products with antibodies. Hence antibody treatment through oral passive therapy can be implemented for treating rotavirus infection in children community as an oral feed supplement or other oral products too which shows proven results of neutralizing the virus (Vega *et al.*,2012).

Chicken egg yolk IgY antibodies have become a potential alternative to conventional mammalian antibodies with the characteristics of ease of its production from the yolks of hyperimmunized chicken eliminating the need to bleed the animals to obtain maximum quantity thereby sticking on to the ethics of animals. Also, IgY antibodies do not activate the complement system and do not interfere with the Rheumatoid factor for its possible use in diagnostics to replace the mammalian antibodies in detection systems too (Reschova *et al.,* 2000).

In the recent decades, studies showed that these IgY antibodies have the potential to neutralize or eliminate most of the enteric pathogens causing diarrhea and other gastrointestinal problems in humans as well as animals too. Bacterial enteric pathogens including *Helicobacter pylori* (Abdou *et al.,* 2014), *E. coli* (Carlander *et al.,*2000),*Vibrio cholera*(Hirai *et al.,*2010) has been studied in recent years. Hence IgY antibodies can be successfully used to treat enteric pathogens.

Antibodies against vaccine strain of rotavirus were generated in White Leghorn chicken by intramuscular immunization. From the egg yolks of hyperimmunized chicken, Antivac IgY antibodies were purified and characterized by analysis of its total Protein content, Total IgY, purity using SDS PAGE protein profile, and checking the antibody titer by Indirect ELISA. Also, the stability of the antibodies was checked by exposing them to different temperature and different pH ranges facilitating the use of them in passive therapy. These antibodies will be useful to treat the patients with rotavirus infection in a unvaccinated community of children or in countries where vaccination fails to safeguard the infants and these Antivac antibodies can play a significant role to eliminate the virus thereby protecting the children in early stages of infection (Diraviyam *et al.,* 2014).

Materials and methods

Antigen preparation and Immunization of experimental birds

Twenty-one weeks old White Leghorn chicken with good health and known history of vaccinations were used for raising antibodies against vaccine strain of rotavirus. The birds were maintained under hygienic conditions and vaccinated periodically throughout the period of study. Room temperature, relative humidity, and light/dark cycles were controlled.

The birds were split into two groups with three birds in each as a test group and an unimmunized control group.

Rotavac, commercially available Live attenuated rotavirus vaccine (manufactured by Bharat Biotech, India) was used as the antigen to immunize the chicken in the test group to raise Antivac IgY antibodies. 0.5ml of vaccine containing virus particles of not less than 10^5 FFU was mixed with 0.5ml of Freund's Complete Adjuvant was used for initial immunization followed by Freund's Incomplete Adjuvant for booster doses. The birds were immunized by intramuscular injection (*Musculus pectoralis*, left and right, injection volume 0.5 ml). The birds were given four booster doses at two weeks interval. The unimmunized chicken served as control group.

Collection of hyperimmune eggs and antibody purification

Following immunization, eggs laid by the chicken in both the test and control groups were collected daily and the egg laying performance of the chicken were monitored to study the effect of immunization on egg laying pattern of the birds. The eggs were stored at 4°C for further purification of antibodies in cold storage facility. The values were represented as a mean number of eggs with the standard deviation in the test and control group respectively as shown in Fig. 1.

Antivac IgY antibodies were purified by PEG Precipitation method as described previously by Polson *et al.*,1985. Briefly, the yolks were separated from the white albumin part of the egg. Separated yolks (10-15ml per yolk) was 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 gets completely dissolved. The sample was centrifuged at 10,000rpm for 20 mins at room temperature. The lipid fraction was trapped by filtration of the supernatant through a cotton wool plug. 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 rpm for 20 mins at room temperature. The supernatant was discarded and the pellet was dissolved 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 10,000rpm 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 buffered saline.

The PEG-purified antibodies were precipitated using 40% Ammonium sulphate at 4°C for overnight (Harlow *et al.,* 1988). Followed by, Dialysis was performed against saline overnight with two to three buffer changes. The purified Antivac IgY antibodies were stored at 4°C for characterization studies. Also, the antibodies were lyophilized into powder and stored at -20°C for long-term storage.

Titration of antibodies by indirect ELISA method

An indirect non-competitive enzyme-linked immunosorbent assay (ELISA) was used to measure the titer of IgY antibodies against vaccine strain of rotavirus by the protocol as described by Yuan e Yang *et al.,* 2014. Briefly, vaccine strain of virus was used to coat ELISA plates and incubated for overnight at 4°C. After washing the wells thrice with PBS, serial dilutions of Antivac IgY were added to the wells, and the plate was incubated at 37°C for 1 h. The plate was washed three times with PBS containing 0.05% Tween20 and blocked for 1 h at 37°C with PBS containing 1% bovine serum albumin. Rabbit anti-chicken IgY antibodies (Invitrogen, USA) in the dilution of 1: 5000 in PBST (100µl per well) was added and incubated at 37°C for 1h.The plates were washed three times with PBS containing 0.5% Tween-20 and 100µl of Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, USA) solution containing 0.1mmol/l citrate phosphate buffer and 1 µl/ml H_2O_2 was added to each well and incubated at 37°C for 20 min under dark condition. 75µl of 4N H2SO4 was added to each well for termination of the reaction. Colour changes were observed and the plates were

read directly on a microplate reader at 450 nm (Varioskan Flash by Thermo Scientific). PBS was used as a blank control, and IgY derived from non-immunizedhens was used as a negative control.

The antibody titer in the egg yolks of immunized hen was checked after checking the antibody titer increase in serum of the hen and were compared (data not shown). The antibody titer in the egg yolks of immunized hens against unimmunized control was studied and represented in Fig. 2.

Characterization of AntivacIgY antibodies

Total protein estimation by Bradford assay

Total protein in the purified IgY sample was estimated by Bradford assay for Protein estimation (Bradford *et al.,* 1976). Briefly, 5µl of the sample was taken and 250µl of commercially available Bradford reagent (Sigma, US) was added to it, in a 96well microtiter plate. The plate was incubated in dark for 45mins to 55mins and OD was checked at 595nm. Standards were prepared using Bovine Serum Albumin and the standard graph was plotted for samples ranging from 1mg/ml to 100mg/ml. Reagent alone served as a blank solution. The OD value of the sample at 595nm was plotted against the standard to determine the total protein concentration of the purified IgY suspension.

Total IgY estimation

Total IgY concentration in the sample was calculated as described by Pauly *et al.,* 2011. Briefly, absorbance at 280nm was recorded for the antibodies diluted in PBS at 1:50 dilution. The observed OD value was calculated in the standard formula used below to calculate the total IgY concentration.

$$IgY\ concentration\ \left(\frac{mg}{ml}\right) = \frac{Absorbance\ 280nm * 10\ (dilution\ factor)}{1.33\ (extinction\ coefficient\ for\ IgY)}$$

The total protein and total IgY antibodies in the purified sample as determined by above methods were recorded as in Fig.3.

SDS PAGE analysis

The protein profile of purified Antivac IgY antibodies was checked by SDS PAGE Analysis.

Briefly, 10% non-reducing SDS gels were prepared and 20µl of IgY sample was loaded onto the gel along with molecular weight standard markers.

The gel was electrophoresed at 60V for 3 hours (King & Laemmli 1971). After Electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 to visualize the protein band of 180kDa which corresponds to the IgY antibody as seen in Fig.4.

Stability studies of AntivacIgY antibodies

Temperature stability

IgY antibodies were exposed to various temperatures for a different time period to study the thermal stability of the antibodies. Briefly, Antivac IgY antibodies were incubated at -20 °C, 4°C, 10°C, 20°C, 30°C,40°C, 50°C, 60°C, 70°C, 80°C for 30 minutes. Aliquots of samples were collected at 5minutes time interval during the incubation period, cooled to room temperature and the remaining antibody activity was measured by ELISA method (Shin *et al.,* 2002, Jaradat *et al.,*2000). The results were analysed statistically and represented as mean ±standard deviation asin Fig.5.

pH stability

The stability of Antivac IgY antibody to acid and alkali conditions has been studied at different pH conditions. The pH of IgY solution was adjusted to the desired pH range between 2 to 12 with appropriate buffers and was incubated at 37°C for 4 hours. After incubation, each IgY solutions were neutralized with appropriate pH buffer and the remaining antibody activity was measured by ELISA (Shin *et al.,* 2000, Lee *et al.,* 2002). Antibody activity was represented as a percentage of the untreated control as in Fig.6

Stability analysis on lyophilization

The effect of freeze-drying or lyophilization on these purified antibodies were studied by the method described by Fu *et al.,* 2006. Briefly, the purified antibody samples were pooled together, frozen at– 20°C Deep freezer for overnight and then were placed in an ALPHA 1.2LD Plus Freeze Dryer (Christ,

Germany) for about 8 hrs. The condenser temperature of the freeze dryer was set at −55°C. Upon completion of freeze drying, the samples were removed and stored at – 20°C until used.

The stability of antibody in the samples of liquid egg yolk and the freeze-dried samples were checked by ELISA method and represented graphically as in Fig. 7.

Results

Monitoring egg-laying performance of immunized chicken:

Monitoring the effect of immunization of chicken with the rotavirus vaccine antigen on their egg laying performance showed that there was no significant change or effect on the pattern as such (Fig. 1).

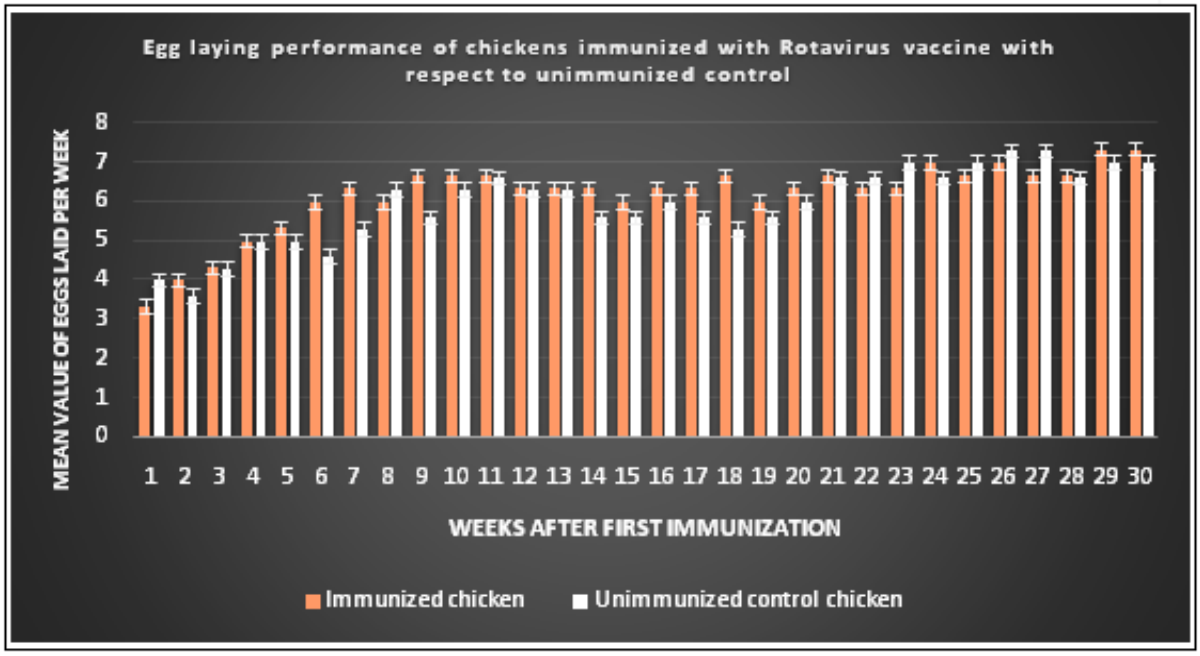

Fig. 1. Monitoring the egg-laying performance of chickens after immunization.

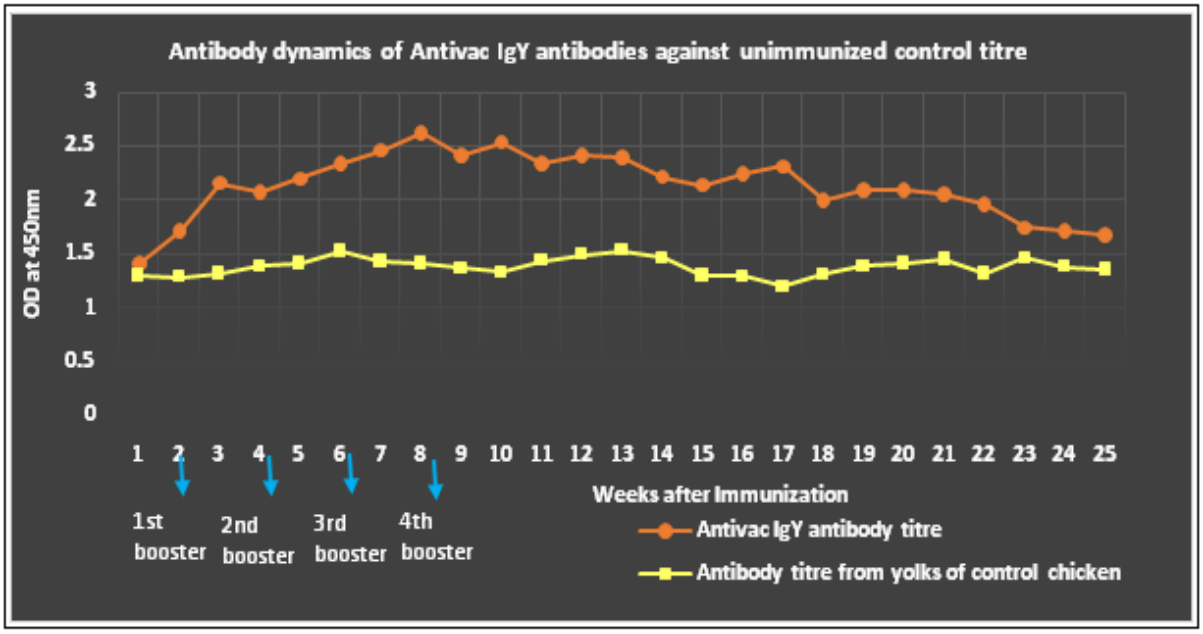

Fig. 2. Egg yolk Antivac IgY Antibody titer determination by Indirect ELISA.

The number of eggs collected per week was represented as the mean value of triplicates with standard deviation. It was found that the weekly mean of the number of eggs laid by the chicken in each group increased with the increase in the age of the chicken. Also, the weight of eggs laid increased with respect to the age of the experimental chicken to a certain period of time (Data not shown).

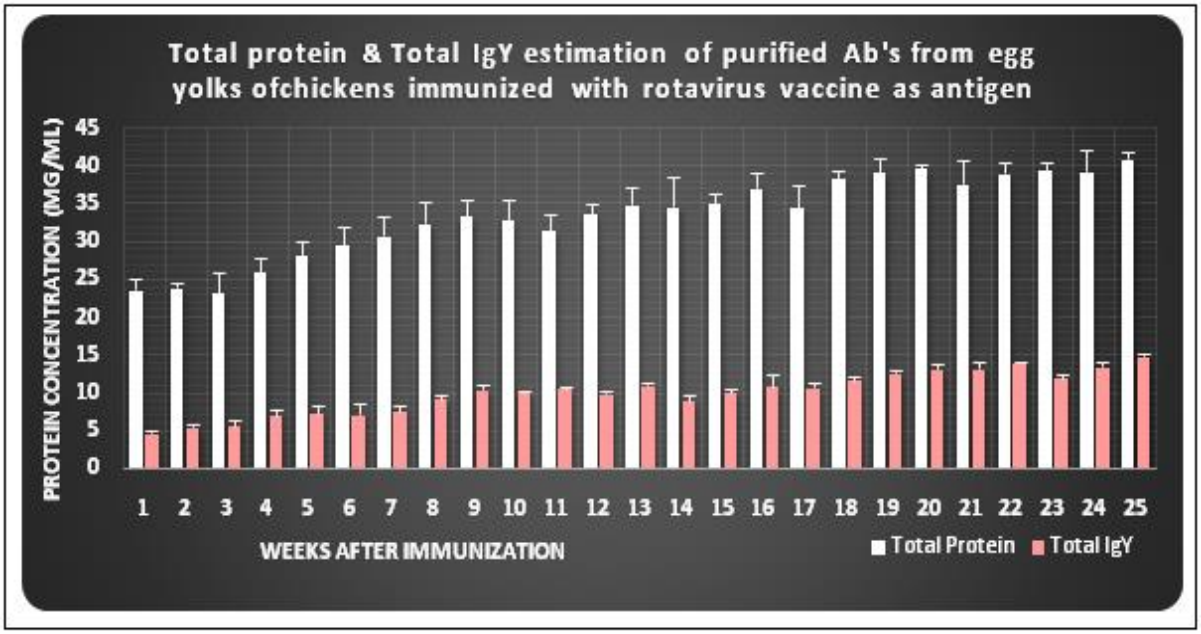

Fig. 3. Estimation of Total protein and Total IgY from the purified Antibody sample.

Antibody titer determination by Indirect ELISA

The titer of the purified Antivac IgY antibodies was determined by Indirect ELISA method which showed a significant titer of up to 1:50000 during the 56th day of the experiment which was found to be maintained stable until the 150th day of the experiment with subsequent booster doses. The titer of antibodies from the egg yolks of unimmunized control chicken against the rotavirus antigen showed a much lower titer which is been compared in Fig.2 below.

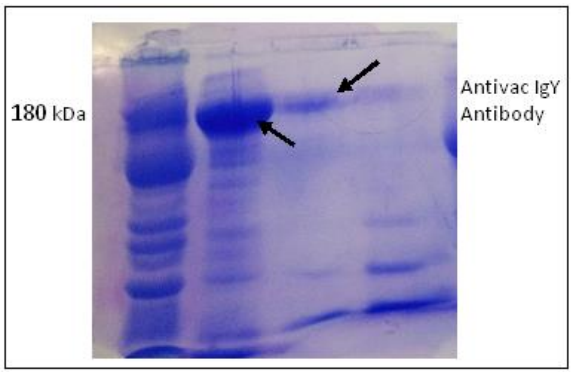

Fig. 4. SDS PAGE Analysis of purified Antivac IgY antibodies. Lane1: Diluted yolk sample Lane 2: PEG Purified antibodies Lane 3: Column purified antibodies Lane 4: Molecular weight marker.

Estimation of total protein and Total IgY

Total Protein as determined by Bradford assay from the yolk of chickens immunized with rotavirus vaccine was found to be 40.96 mg/ml during 175th day of the experiment and the total IgY as calculated from the formula mentioned previously was found to be 14.91 mg/ml during the 25th week of the experiment (Fig. 3).

SDS PAGE Analysis of Purified Antivac IgY antibodies

Electrophoresis of purified egg yolk antibodies as visualized in 10% non-reducing gel showed a clear 180kDa band which corresponds to the molecular weight of IgY antibodies as seen in Fig.4

Effects of temperature pH and freeze-drying on Antivac IgY antibodies

Temperature stability: The antibodies, when exposed to different temperatures, showed that the binding activity of IgY with antigen decreased with increasing temperature and heating time. Antivac IgY antibody was found to be stable at a temperature ranging between -20°C and 40°C

The activity of IgY started decreasing by heating for 25 min at 50°C or higher and IgY denatured seriously when thermally treated at temperatures higher than 80°C as seen in Fig.5 below.

pH Stability: Further experiment with Antivac IgY antibodies showed that around 62% to 97% of activity was retained between pH4 and pH8 whereas only 33% was retained at pH3. Under alkaline conditions of pH10 and above the activity was almost depleted and 51.1% was retained at pH9 (Fig.6).

The activities were expressed relative to the activity of purified IgY, which was considered as 100% using Indirect ELISA.

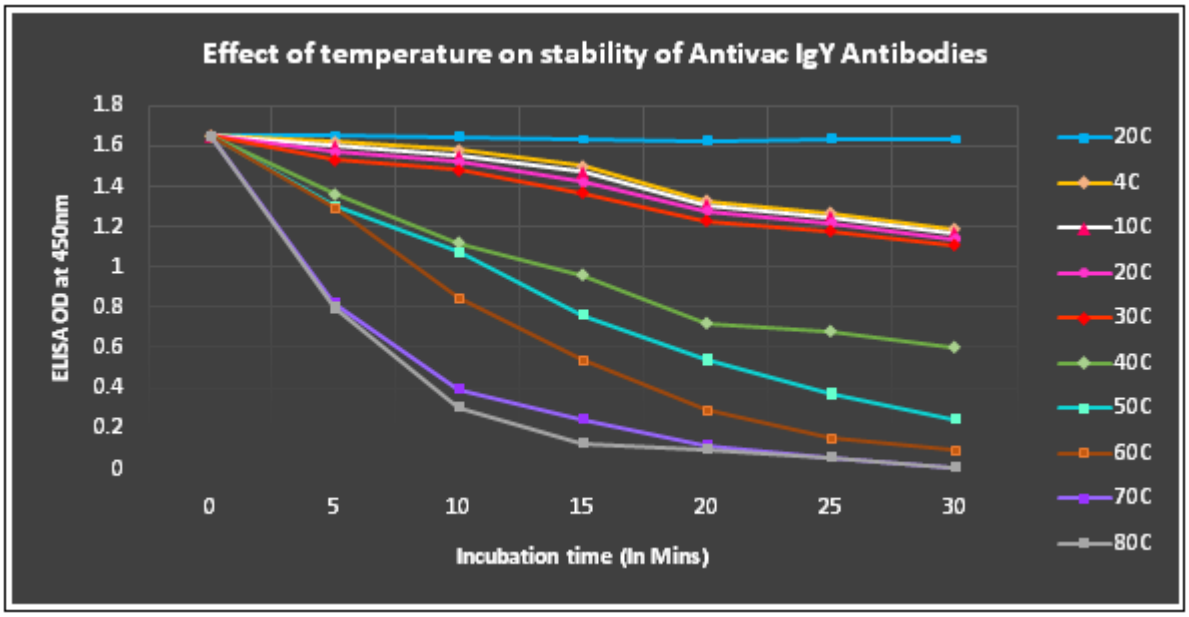

Fig. 5. Effect of temperature on stability of Antivac IgY antibodies.

Effect of Freeze drying or Lyophilization: It was observed that the process of freeze drying did not have an impact on the activity of IgY antibodies significantly and about 85% and 92% activity was observed in liquid yolk and lyophilized samples of purified antibody respectively (Fig. 7).

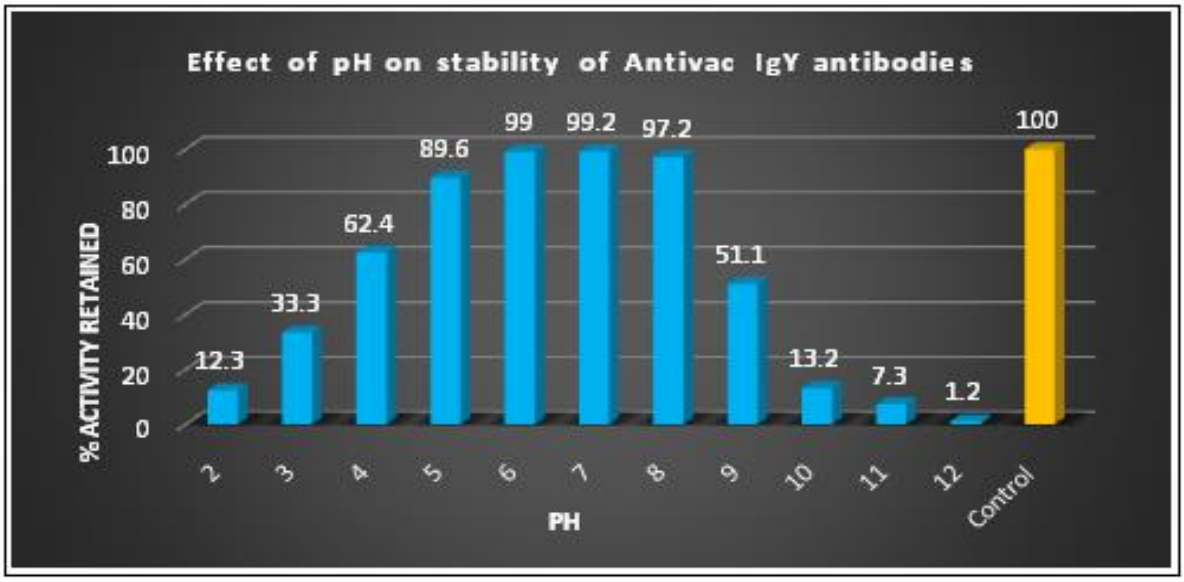

Fig. 6. Effect of pH on stability of Antivac IgY antibodies.

Discussion

In the present study, Chicken egg yolk IgY antibodies were raised against Vaccine strain of rotavirus (Rotavac, rotavirus vaccine) for checking its possible use in oral passive therapy for therapeutic measure against the rotaviral gastroenteritis in children. Preliminary experiments showed that the egg laying pattern of experimental chickens were not affected by the immunization procedures to develop antibodies. A constant routine pattern without any abnormalities were reported.

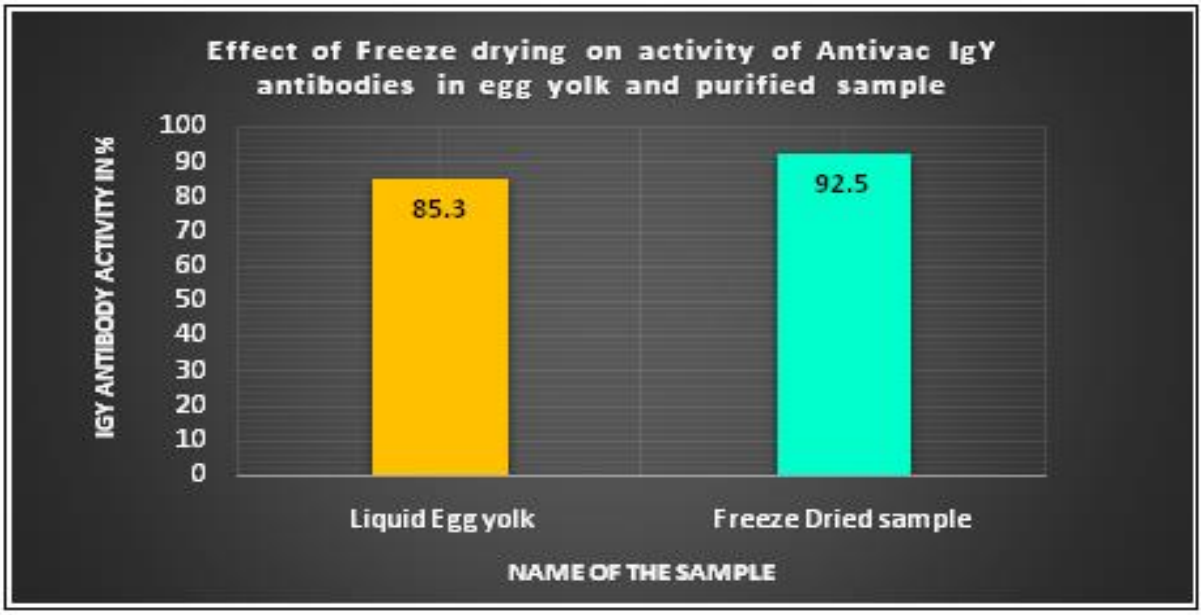

Fig. 7. Effect of freeze-drying on the stability of Antivac IgY antibodies.

The Antivac IgY antibodies purified by PEG precipitation method showed a antibody titer of about 1:50000 by Indirect ELISA during 56th day of the experiment and was stable up to 150th day with subsequent booster doses. The total protein content of purified Antivac IgY antibodies as estimated by Bradford Assay was found to be 40.96 mg/ml and the total IgY of about 14.91 mg/ml. In SDS PAGE analysis, 180 kDa protein that belongs to the IgY antibody in a non-reducing gel was visualized by Coomassie Brilliant blue staining protocol.

Studies conducted to check the stability of these antibodies to different temperature and pH showed that the antibodies were stable up to 50°C and maintained its activity in pH range of pH5.0 to pH8.0. Also, it was found that the process of freeze drying does not have any detrimental effects on the antibody activity after exposing to Lyophilization procedure which can be applied for long term storage of antibodies or yolk powder as such.

Similar studies were conducted by Vega *et al.,*(2012) to check the possible use of IgY antibodies against different strain of rotavirus in piglet models and showed that these antibodies protect the animals against rotavirus and its possible application in therapy. Also, study by Hirai *et al.,* (2010) supports the use of IgY antibodies to treat cholera infection. Shin *et al.,* (2002) have successfully reported these antibodies as an effective antibiotic alternative to treat *Helicobacter pylori* infection. Therefore, the use of IgY antibodies against enteric pathogens is evident from the previous studies which supports the current idea of using the Antivac IgY antibodies for oral passive therapy.

Hence, these egg yolk antibodies raised against vaccine strain of rotavirus can be efficiently employed for passive therapy to treat the infection in children in developing countries where the incidence is higher due to the main reason of children not getting vaccinated and easy transmission of the virus in the infant community.

The present work forms a basis for the development of an oral product such as infant formulae supplemented with anti-rotavirus IgY antibodies to treat the infection in the paediatric population.

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