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Research Article

CHARACTERIZATION AND IN VITRO NEUTRALIZATION OF *STREPTOCOCCUS MUTANS EGG YOLK ANTIBODIES* (IGY)

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ABSTRACT

Objective: The objective of the present investigation was to develop Chicken Egg Yolk antibodies as an alternative and affordable approach for diagnosis and treatment against dental caries in humans.

Materials and Methods: Twenty four week old white leghorn chicken was immunized with formalin killed S*treptococcus mutans* (MTCC 890) to produce Egg Yolk antibodies (IgY). The amount of egg yolk antibody increased gradually as the booster injection antigen concentration increases. The antibodies were partially purified from immunized chicken egg yolk by poly ethylene glycol (PEG)/ammonium sulphate precipitation method and further purification was done by eluting the adsorbed antibodies from DEAE cellulose by linear gradient technique.

Result: In micro agglutination test the reaction was observed up to 1:1280 dilutions. Indirect antigen capture assay (IACA) showed that the antibodies were generated in chicken against *Streptococcus mutans* antigens with the high peak titre of 1:10000 antibody on 63rd day of observation. Antigen-antibody interaction was maximum around pH 6, Temperature around 30^{0}C, ionic strength from 10-50 mM and organic solvents [5% - 30%] like ethanol, methanol and isopropanol does not showed any decrease in antigen-antibody interaction.

Conclusion: The results indicated that chicken IgY could be used for diagnosing dental caries caused by *Streptococcus mutans* and as a therapeutic agent.

Keywords: *S.mutans*, Chicken antibodies (IgY), ELISA

INTRODUCTION

Dental caries is a disease caused by specific type of bacteria live in human mouth. These bacteria produce acid that destroys tooth enamel and results in cavities on its surface. Among all cariogenic streptococci, *Streptococcus mutans* predominates in dental cavities. *S. mutans* glucosyltransferase synthesize extracellular polysaccharides, mainly hydrophobic glycan from sucrose, colonize the tooth surface and initiate plaque formations [1]. Either inhibition of the above enzyme or elimination of *S. mutans* is essential for controlling dental plaque and prevention respectively. Several natural substances have been reported for bactericidal activity against *S. mutans.* However, it causes undesirable side effects. Antibiotics are often prescribed empirically and penicillin is still considered as the drug of choice for the treatment of such disease. *S.mutans* resists prolong usage of antibiotics. Recently, passive immunization using murine monoclonal antibodies, egg-yolk and bovine milk immunoglobulins generated against *S.mutans* has been used to control the dental caries in humans [2]. Various reports on Egg yolk immunoglobulins suggest that IgY act as a promising alternative candidate to prevent bacterial and viral infections [3]. Hens immunized with Streptococcus mutans glucan binding protein B (GBP-B) and its IgY showed protective effect against dental caries [4]. Egg yolk immunoglobulin generated against glucosyltransferases completely protected rats infected with *S. mutans* [5]. Chicken egg yolk antibodies against *S.mitis* may be considered as an alternative source for diagnosis and therapeutic purposes [6]. Therefore, the aim of the present study was to develop chicken egg yolk antibodies against predominant *Streptococcus mutans* and to evaluate the in vitro activity of egg yolk immunoglobulin (IgY).

MATERIALS AND METHODS

Experimental animals

Twenty four week old, single comb white leghorn chickens obtained from L.K.Poultry Farm, Coimbatore and were maintained in our animal house under hygienic conditions with proper food and water. These were used in the production of *Streptococcus mutans* antibodies (IgY).

Organism used and Preparation of antigen

Lyophilized form of S*treptococcus mutans* was procured from IMTECH Chandigarh (MTCC 890) and used for our present study. The Isolates of *Streptococcus mutans* were cultured on Blood agar plates. *Streptococcus mutans* was transferred aseptically from blood agar plates into 250ml conical flasks containing 150ml of sterile Todd Hewitt broth and incubated at 37˚C for 24 hours. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes. The pellet was suspended in sterile saline and centrifuged again. These cells were resuspended in 5ml sterile saline containing 3% (v/v) formalin and kept at 40°C overnight. The cells were washed with PBS twice to remove formalin and dispersed in PBS. The inactivation was confirmed by culturing on Blood agar plates. The inactivated cells were adjusted to the final concentration of 1x10^8 cells/ml by Mcferlands opacity set**.**

Development of *Streptococcus mutans* antibodies in chicken

Streptococcus mutans (10^3 cells/ml) cells were dispersed separately in 0.9% phosphate buffered saline (PBS) and injected intramuscularly at the multiple sites of breast muscles in 24-week-old white leghorn chickens. Chickens received subsequent booster injections with increasing concentration of antigens (10^3 cells/ml – 10^6 cells/ml) at 7 days interval by the same route of administration. Test bleedings were made frequently to check the presence of *Streptococcus mutans* antibodies in the serum. Eggs were collected from day 0 till the end of the experiment and stored at 4°C.

Purification of anti-*Streptococcus mutans* from egg yolk

The antibodies were extracted from egg yolk by Polyethylene glycol and Ammonium sulphate precipitation method [7]. The partially purified chicken egg yolk antibodies were desalted by dialysis against 25mM Phosphate buffer pH 8.0 [10 KDa cut off; 1:1000}. The above partially purified IgY dialysate was further separated from unwanted proteins by DEAE cellulose ion exchange column (1.0 x 60 cm, Sigma, USA) chromatography. The column was packed with DEAE Cellulose and equilibrated with 25mM Phosphate buffer pH 8.0. Dialysate were loaded and

very weakly bound proteins were washed thoroughly with same buffer. The column was eluted by passing linearly increasing sodium chloride [0 - 2M]. Three milliliter fractions were collected at the flow rate of 0.6ml/min. Total protein was estimated by Lowry *et al.* (1951) [8]. The IgY fraction was then concentrated with Poly Vinyl Pyrolidone (PVP) at room temperature. The relative molecular weight of Chicken egg yolk antibodies and its purity was assessed by SDS-PAGE [9].

IgY determination

The specific IgY concentration in fraction was determined by adding 0.5 ml of the crude IgY solution and 1 ml of a 0.01% antigen solution in PBS in the same tube and incubated overnight at 37°C. The supernatant was separated by centrifugation, and the absorbance was measured at 280 nm [10].

Titration of antibodies by Indirect ELISA

The antibody titre of the antibodies generated against *Streptococcus mutans was* determined by indirect antigen capture ELISA [11]. Briefly, Nunc polysorp ELISA wells were coated with formalin killed, *Streptococcus mutans, antigens* using coating buffer (0.05M carbonate bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. The coated wells were washed thrice with PBST. The empty sites were blocked with 1% BSA (200 µl / well) and incubated at 37°C for 1 hour. Wells were subsequently washed and incubated with *Streptococcus mutans* antibodies (100 µl / well). PBST and preimmune sera served as controls. Wells were washed thrice with PBST and 100 µl of diluted (1:1000) rabbit antichicken immunoglobulin coupled to Horse Radish Peroxidase (Genei Pvt Ltd, Bangalore) was added to each well and incubated. Then the wells were washed and 100 µl of freshly prepared substrate solution (TMB/H_2O_2, Genei Pvt Ltd, bangalore) was added. These plates were allowed to stand at room temperature in dark for 20 minutes. The reaction was stopped by adding 50 µl of 4N sulphuric acid and read at 490 nm in an ELISA reader. All experiments were done in triplicates.

Micro agglutination test

Micro agglutination test was carried out in 'U' bottom Microtitre plates. Two rows were assigned for the test, into first well of each row added 80µl of sterile saline and the remaining wells of each rows contained 50µl of sterile saline. 20µl of *Streptococcus mutans* IgY was added into the first well of the first row and doubling dilution was done by transferring 50µl from the first well to second well, second to third and so on. The same procedure was repeated in the second row (control) except *Streptococcus mutans* IgY, 20µl of non specific antibody was present. Finally 50µl of bacterial cell suspension of desired optical density (0.42 at 620 nm) was added to all the wells of each rows and incubated overnight at 25°C. After incubation, plate was observed and the titre was defined as the highest dilution of *Streptococcus mutans* antibody showing agglutination when compared with controls.

Interaction of Chicken egg yolk antibodies at various pH and Temperature

Interaction of IgY at various pH

50µl [130 µg] of chicken egg yolk antibodies were incubated with 950 µl of 20mM various buffers (20mM sodium acetate pH 2.0 to pH 5.0, 20mM phosphate buffer pH 6.0 to pH 7.0) [E]. Sample pH was reversed to pH 7.1–7.5 with 150 µl of PBS and/or 200mM Na_2HPO_4 and incubated at 37°C for one hour. Indirect antigen capture assay was performed using 100 µl aliquots of incubated solutions. After washing the bound antibodies were allowed to interact with anti rabbit chicken immunoglobulin coupled to Horse Radish Peroxidase. The interaction was assayed with TMB and H_2O_2. The reaction was stopped by adding 50 µl of 4N sulphuric acid and wells were read at 490 nm in an ELISA reader. The value obtained at 20mM PBS, pH 7.2 at 37°C was considered as control [C] and 100% activity. Relative interaction [%] was calculated as ratio of difference between control and experiment values and control multiplied by 100.

$$\text{Relative interaction [\%]} = \frac{\text{Control [C]} - \text{Experiment [E]} \times 100}{\text{Control [C]}}$$

Interaction of IgY at various Temperature

50µl [130 µg] of chicken egg yolk antibodies were mixed with 950 µl of 20mM PBS pH 7.2 and incubated at different temperature (30°C, 40°C, 50°C, 60°C, 70°C, 80°C) for one hour [E]. Indirect antigen capture assay was performed by dispensing 100 µl aliquots of incubated solutions into the ELISA wells. After washing the bound antibodies were allowed to interact with anti rabbit chicken immunoglobulin coupled to Horse Radish Peroxidase. The interaction was assayed with TMB and H_2O_2. The reaction was stopped by adding 50 µl of 4N sulphuric acid and wells were read at 490 nm in an ELISA reader. The value obtained for 20mM PBS pH 7.2 & at 37°C was considered as control [C] and 100% activity. Relative interaction [%] was calculated as earlier.

Interaction of IgY at various molarity of buffer

50µl [130 µg] of chicken egg yolk antibodies were mixed with 950 µl of 20mM, 30mM, 50mM, 100mM, 200mM, 300mM & 400mM PBS pH 7.2 and incubated at 37°C for one hour [E]. Indirect antigen capture assay was performed by dispensing 100 µl aliquots of incubated solutions into the ELISA wells. After washing the bound antibodies were allowed to interact with anti rabbit chicken immunoglobulin coupled to Horse Radish Peroxidase. The interaction was assayed with TMB and H_2O_2. The reaction was stopped by adding 50 µl of 4N sulphuric acid and wells were read at 490 nm in an ELISA reader. The value obtained at 20mM, pH 7.2 & at 37°C was considered as control and 100% activity. Relative interaction [%] was calculated as discussed earlier.

RESULTS

Freeze dried culture of S*treptococcus mutans* was obtained from IMTEC Chandigarh (MTCC 890) and cultured on Blood agar plates. The organism was transferred into 150ml of Todd Hewitt broth and incubated at 37°C for 24 hours. The cells were harvested by centrifugation and inactivated by formalin. The formalin killed S*treptococcus mutans* cells were injected intramuscularly at multiple sites of 24-week-old white leghorn chicken breast muscles. Anti-*Streptococcus mutans* was checked in chicken serum and in egg yolk. Antibodies against Streptococcus mutans was detected by Indirect antigen Capture ELISA. The egg yolk antibodies was partially purified by polyethylene glycol and ammonium sulphate precipitation method and anion exchange chromatographic technique was followed to get rid of unwanted proteins (Fig 1). Its relative molecular weight was checked by SDS PAGE and found to be 180 KDa (Plate 1). The total amount of protein in each fraction was estimated. The protein concentration varied in the range of 0.60 – 6.7mg/ml of yolk throughout the immunization period. The specific IgY was found to be 24.1%. In microagglutination test the agglutination was observed up to 1:1280 dilutions (Plate 2). The titre of antibodies in the immunized chicken egg yolk was carried out by ELISA. Indirect antigen capture assay (IACA) showed that the antibodies were generated in egg yolk against inactivated, *Streptococcus mutans*, antigens. High peak titre of 1:10000 antibody were observed during 63rd day of observation (Fig 2 & Plate 3). Characterization studies like effect of pH, temperature, ionic strength of buffer and various organic solvents on antigen-antibody interactions were carried out. IgY showed good interaction from pH 4 to pH 8 after incubating O/N at respective pH. IgY was inactivated at pH below 4 and above 9. These results suggested that the chicken egg yolk antibodies interacted strongly with bound antigens when pH was from 4 to 8 (Fig. 3). IgY showed good interaction with antigen when temperature was 30°C, 37°C and 50°C. IgY was inactivated at temperature above 60°C. These results suggested that the chicken egg yolk antibodies interactions are strong when the temperature was from 30°C to 60°C (Fig. 4). Antigen-antibody interaction was maximum at ionic strength of 10-50 mM and organic solvents [5% - 30%] like ethanol, methanol and isopropanol does not showed any adverse effect on antigen-antibody interaction.

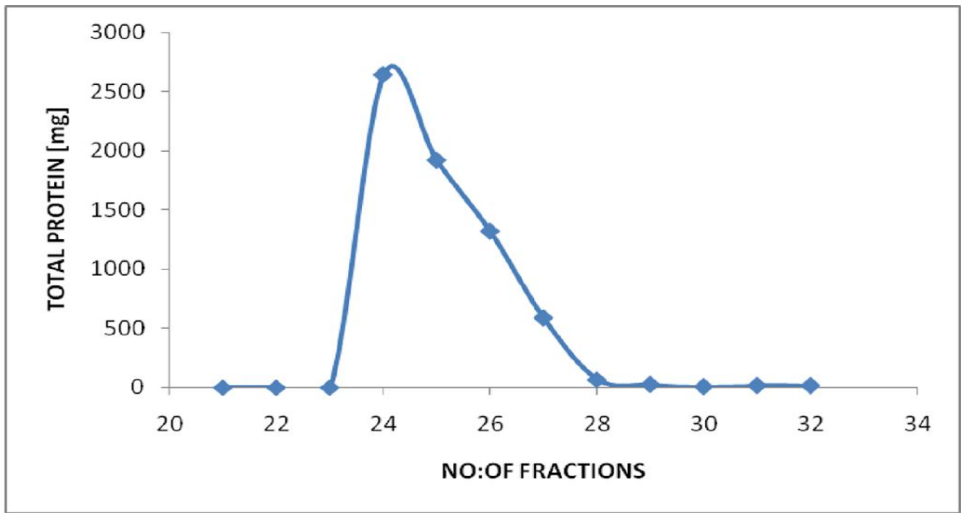

Fig. 1: Column Chromatography using DEAE Cellulose

Purification of IgY by DEAE Cellulose Ion Exchange Column Chromatography. The IgY was eluted by passing linearly increasing sodium chloride [0 - 2M]. Most of the IgY was present in 23rd to 27th fractions.

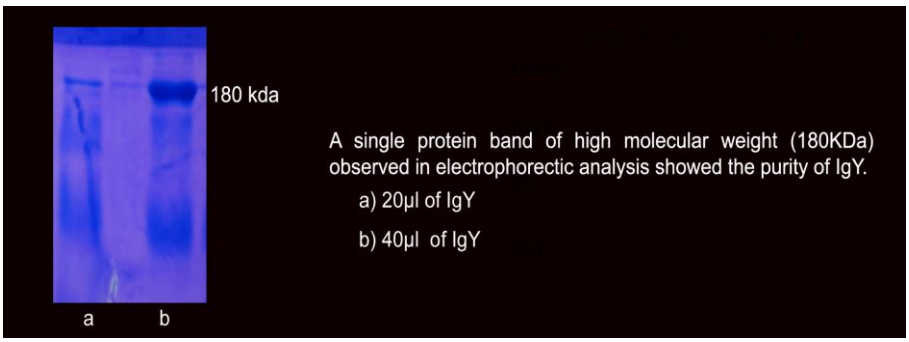

Plate 1: Protein profile of IgY by SDS-PAGE

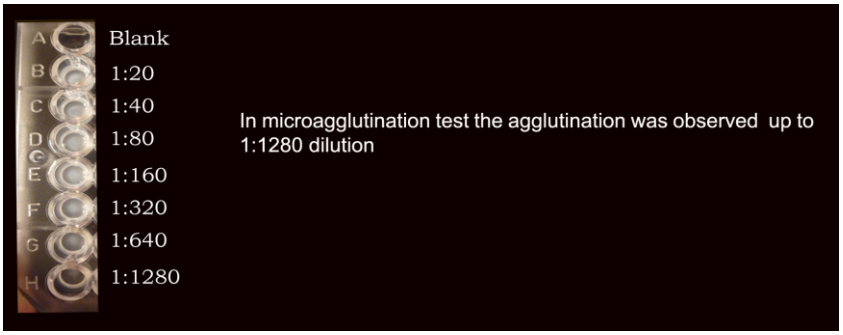

Plate 2: In micro agglutination test the agglutination was observed up to 1:1280 dilution

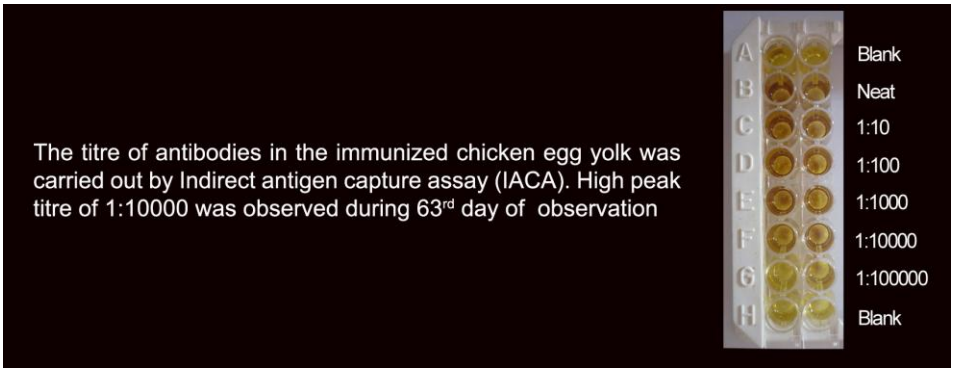

Plate 3: Titration of antibody by ELISA

The positive reaction shows up to 1:10,000 dilution indicates high amount of antibodies are produced in immunized chicken egg yolk

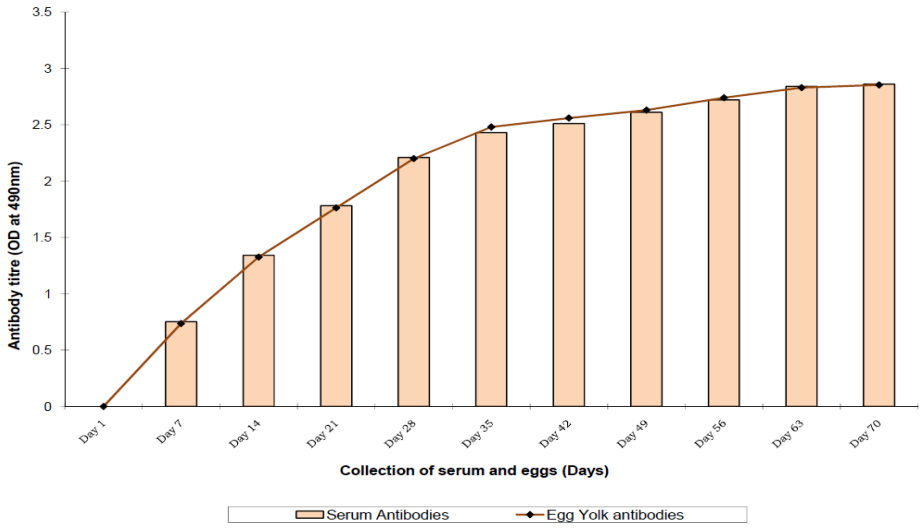

Fig. 2: Kinetics of antibody production in hens immunized with *Streptococcus mutans*.

The titre of antibody in immunized chicken egg yolk was estimated by ELISA. There was a gradual increase in antibody titre with subsequent booster dose administration

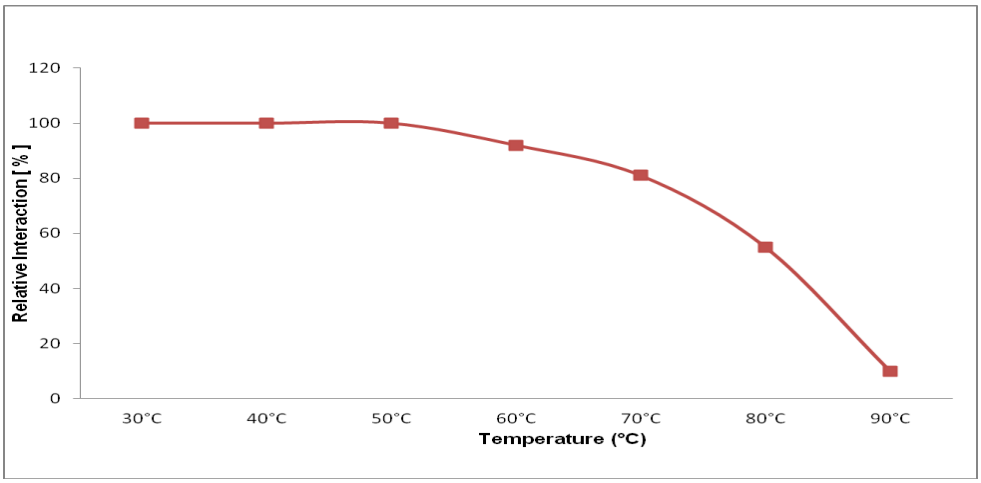

Fig. 3: Interaction of IgY at various temperature.

IgY showed good interaction at 30°C, 40°C and 50°C. IgY was inactivated at temperatue above 60°C. These results suggested that the chicken egg yolk antibodies are stable at temperature ranging from 30°C to 60°C

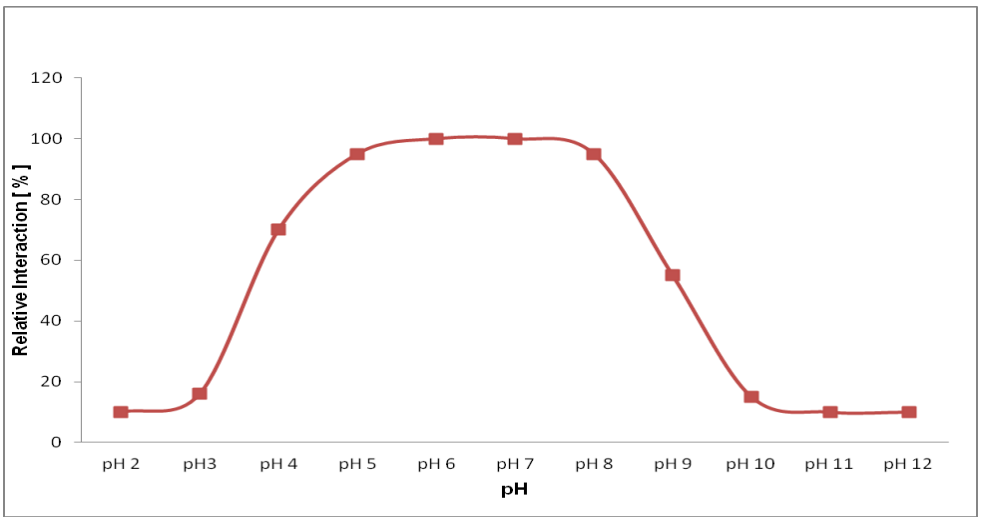

Fig. 4: Interaction of IgY at various pH.

IgY showed good interaction from pH 4 to 8 after incubating O/N at respective pH. IgY was inactivated at pH below 4 and above 9.

DISCUSSION

The oral ecosystem contains a wide variety of microbial species that causes tooth decay. Streptococcus sp being a major causative agent in dental caries and dominating in different parts of the oral cavity and gastrointestinal tract which leads to many clinical symptoms. Identification of such species is important for proper treatment. Streptococcus salivarius is found in saliva, on the tongue and buccal surfaces. Streptococcus mitis and Streptococcus mutans are found on the tooth surfaces [12, 13]. Among all streptococci, *Streptococcus mutans* play an important role in the development of dental caries in humans. These organisms produce acids that demineralize tooth enamel, damaging the hard tooth structure and resulting in cavities on its surface. *Streptococci* have become increasingly resistant to antibiotics including penicillin, cephalosporin, erythromycin, and tetracycline. Recent research showed that chicken egg yolk antibodies can be a promising alternative for mammalian antibodies. Recently, passive immunization has gained much attention, compared to active immunization, because of the possible side effects caused by mutans streptococcal vaccine antigens. Chicken egg yolk antibodies may serve as an alternative source to neutralize the *Streptococcus mitis* [6]. Chicken egg yolk antibodies are easy to produce in larger amounts, whereas other polyclonal and monoclonal antibodies are expensive and difficult to produce [14]. Hence, an attempt has been made to generate *Streptococcus mutans* antibodies in the yolk of the immunized chicken. In the present investigation antibodies are present in chicken serum and in egg yolk after 7 days of initial immunization period. The IgY concentration varied in the range of 0.60 – 6.7mg/ml of yolk throughout the immunization period. In micro agglutination test the reaction was observed up to 1:1280 dilutions. Indirect antigen capture assay (IACA) showed that the antibodies were generated in chicken against *Streptococcus mutans* antigens with the high peak titre of 1:10000 antibody on 63rd day of observation. Chicken egg yolk antibodies against enterotoxigenic *Escherichia coli* were detected in the serum after 8 days and in eggs after 10 days, with levels reaching peaks at 15 and 20 days after the first immunization [15]. Antibodies obtained from unimmunized chicken egg yolk did not show any activity against *Streptococcus mutans* antigen. A single protein band of high molecular weight (180KDa) observed in electrophorectic analysis on 10% gel showed the purity of IgY. Many reports suggested that high antibody titre of IgY will have greater purity and effectively neutralize various antigens in both in vitro and in vivo models [16, 17]. Antigen-antibody interaction was maximum around pH 6, Temperature around 30^{0}C, ionic strength from 10-50 mM and organic solvents [5% - 30%] like ethanol, methanol and isopropanol does not showed any decrease in antigen-antibody interaction. The above result implicates that highly purified chicken egg yolk antibodies could be used for therapy in *Streptococcus mutans* infections. Our future plan is to perform *in vivo* studies which could help us to generate tooth paste for effective treatment of dental caries.

CONCLUSION

The egg yolk derived immunoglobulin act as a promising alternative source for the diagnosis and treatment of *streptococcus mutans* dental caries. These alternative approach will reduce the side effects which are frequently caused when administration of broad spectrum antibiotics. Egg yolk immunoglobulins considered as a safe and efficient antibodies for the treatment of dental caries.

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