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Generation of Eggyolk Antibodies in Chicken (IgY) against *Streptococcus mutans* and its in-vitro neutralization efficacy

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ABSTRACT

Chicken Eggyolk Antibodies (IgY) were raised in 22 weeks old white leghorn chicken against formalin killed whole cell antigen of Streptococcus mutans (MTCC No:497). The level of the antibody in serum was monitored and booster doses were given wherever necessary. The antibodies were purified from the eggyolk of immunized chicken using PEG and Ammonium sulphate precipitation method and further purified by DEAE cellulose ion exchange column chromatography. High titre of more than 1:10000 antibodies were detected by Indirect antigen capture ELISA at 150th day of observation. The IgY concentration in egg yolk was increased during the immunization period and reached maximum of 5.8mg/ml. The minimum agglutination concentration of IgY against whole cell antigen of S.mutans was found to be 1:625. Similarly, when pre-immune IgY (control) were used no agglutination were observed. There was a decrease in Streptococcus mutans growth with increasing concentration of antibodies (IgY). The growth was completely inhibited when 25µg/ml of IgY was added to the culture.

Key Words: *Streptococcus mutans*, Dental Caries, Chicken Antibodies (IgY).

INTRODUCTION

Oral health and general well-being are inextricably bound. Many conditions that plague the body are manifested in the mouth, thus enabling to view the onset, progression and management of numerous systemic diseases. Dental Caries and periodontal problems are almost universal and are found in many populations and age groups across the globe and all economies. India is no exception to these problems and they are widely prevalent in India too. The oral cavity is sterile at birth but rapidly becomes colonized from the environment. *Streptococcus salivarius* is dominant and may make up 98% of the total oral flora until the appearance of the teeth. The eruption of the teeth during the first year leads to colonization by *Streptococcus mutans* and

Streptococcus sanguis[1]. The microbiological study on human dental plaque dates back to 1924 when Clarke first observed oral *Streptococci* [2]. He discovered that certain Streptococci, later identified as *Streptococcus mutans*, were the main causative agent of dental caries [3,4]. *Streptococcus mutans* causes caries in three phases: initial interaction with the tooth surface mediated by adhesins; accumulation of the bacteria in a biofilm and the production of glucose and glucans by the bacterial enzyme glucosyl transferase and the formation of lactic acid.

The current methods of caries management include traditional prevention (prophylaxis and fluoride) and early surgical intervention; fluoride treatment, mouth rinses and sealants. The best available evidence indicates that the level of dental caries is low in countries where the consumption of free sugars is below 15-20 kg/person/year. While it is not possible to eliminate all bacteria from the mouth, by limiting sugar consumption, it is possible to prevent the attachment of bacteria onto the surface of teeth. To prevent the adhesion of mutans streptococci to teeth surfaces, antibodies can be targeted to block the antigenic adhesion site of the pathogen.
One of the major strategies is to combat *Streptococcus mutans* by immunological procedures such as active immunization or passive oral administration of preformed antibodies to *Streptococcus mutans*. It has been found that antibodies are actively transported to the egg yolks from serum in large quantities during gestation in immunized hens. Chicken egg yolk has been recognized as an inexpensive alternative antibody source, and passive immunization with egg yolk immunoglobulin (IgY) has shown therapeutic value. This raises the possibility of conferring passive protection against *Streptococcus mutans*-induced dental caries by using antibody prepared from the eggs of the hens hyperimmune to *Streptococcus mutans* antigen. IgY specific to *Streptococcus mutans* can prevent the tooth decay by immobilizing bacteria and disabling bacteria's ability to convert sugar into acid, thereby preventing dental caries from developing.

MATERIALS AND METHODS

Experimental animal-Chickens

White Leghorn Laying chickens, 21weeks old, were bought from Suguna Poultry Farm, Namakkal. The hens were kept in isolated cages at the animal house and provided regular food and water. They were used in the study for the production of anti-*Streptococcus mutans* antibodies (IgY).

Organism Used

Streptococcus mutans c standard strain was obtained from Depository at MTCC, Chandigarh, India, in a lyophilized form (MTCC No: 497). The strain was cultivated on Brain Heart Infusion Broth supplemented with 5% sucrose and incubated at 37^0C for 24hours anaerobically. Further, the culture was maintained on BHI Agar medium aseptically, after confirmation by Grams reaction, Cultural and Biochemical tests.

Preparation of Antigen

The *Streptococcus mutans* c strain was transferred aseptically from BHI agar plates to 150ml conical fl asks containing sterile Brain Heart Infusion broth supplemented with 5% sucrose and incubated at 37˚C for 24 hours under anaerobic condition. The culture was treated with 0.5% formalin for 24 hours and then bacterial cells collected by centrifugation (10000g, 15 min). The pellets were washed thrice with sterile saline containing 0.5% formalin and resuspended in sterile

saline using a Vortex mixer. This formalin killed whole cell antigen samples were kept frozen at -20 °C until required after the concentration adjustment to 2 x10^9 CFU/ml and its protein profile detected using SDS-PAGE.

Generation of antibodies against *S. mutans* in chicken

Twenty-two week old white leghorn chicken was immunized intramuscularly with 1ml of the prepared antigen containing 1x10^4 CFU/ml(cells dissolved separately in PBS) of formalin treated whole cell *S.mutans* to generate antibodies against the same. Chickens received subsequent booster injections with increasing concentration of antigens (10^4cells/ml – 10^9cells/ml) at 15 days interval by the same route of administration. The pre-immune sera and hyperimmune sera were collected at specified time intervals during and after the immunization schedule to monitor the presence of anti-*Streptococcus mutans* antibodies. Likewise, the eggs were also collected collected from day 0 until the end of the experiment and stored at 4°C until testing by the indirect ELISA.

Purification and Characterization of anti-*Streptococcus mutans* antibodies from egg yolk

The antibodies were extracted from egg yolk by using Polyethylene Glycol [5] and Ammonium sulphate precipitation method. Briefly, the egg yolk was separated from white, washed with distilled water to remove as much albumin as possible and rolled on a paper towel to remove adhering egg white. The membrane was punctured and the yolk without the membrane was allowed to flow into a graduated cylinder. An equal amount of bufferS (10mMphosphate, 100mM NaCl, pH 7.5, containing 0.01% sodium azide) was added to the yolk and stirred. To this mixture 10.5% PEG 8000 in buffer S was added to a final concentration of 3.5%. The mixture was stirred for 30 minutes at room temperature and centrifuged at 11000 rpm for 20 minutes. The supernatant was filtered through double-layered cheesecloth. The 42% PEG in buffer S was added to make final concentration of 12% PEG. The mixture was stirred thoroughly and centrifuged at 11000 rpm for 20 minutes. The pellet was redissolved in buffer S to the original yolk volume and an equal volume of 4M Ammonium sulphate (pH7) was added and the precipitate was centrifuged at 11,000 rpm for 20 minutes, the pellet was redissolved in 1ml of buffer S (without NaCl) over night. Then the content was desalted by dialysis process. The crude fraction of IgY thus obtained was further purifi ed by DEAE cellulose ion exchange column chromatography. The IgY fraction was then concentrated with Poly Vinyl Pyrolidone (PVP) at room temperature. The protein content of the purified IgY fraction was determined by the method described by Lowry [6]. The purity of Chicken egg yolk antibodies was checked by SDS-PAGE.

Determination of antibody titer by Indirect ELISA

The immunological specificity of IgY elaborated against whole cells of *S. mutans* MTCC497 antigen was examined by enzyme-linked immunosorbent assay (ELISA) as described by Hamada [7]. In brief, wells of Microtiter plates were coated with 100 µI of antigen solution (A660nm=1.0) appropriately diluted with 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed, and 200µ1 of PBS (pH 7.4) containing bovine serum albumin (1% in PBS) was added to the wells in order to block the uncoated surface. After being blocked each well was washed three times with 200 µL of PBS (0.85% NaCl-0.01 M phosphate buffer, pH 7.2)-Tween (containing 0.05% Tween 20), and IgY from immunized hens at different time intervals was applied to the well in triplicate for reaction with the antigen for 2 hours at

37°C. After each well was washed again with 200µL of PBSTween, 100µL of horseradish peroxidase-conjugated rabbit antichicken IgG (Sigma Chemical Co.) diluted (1:1000) with PBSTween was added to each well, and the plate was incubated at 37°C for 2 hours. Each well was washed again with 200 µL of PBS-Tween, and then 100 µL of TMB solution with H_2O_2 (Genei Pvt. Ltd., Bangalore). The reaction was stopped after 20 min with 4N H_2SO_4 (50µl per well), and the intensity of color developed was measured at 490 nm with a microplate reader. The ELISA titer was defined as the dilution giving an A490 of 0.2. Crude IgY from nonimmunized hens were used as control.

Growth Inhibition Assay

The ability of the anti- *Streptococcus mutans* IgY to inhibit *Streptococcus mutans* growth in liquid medium is detected by this test. *Streptococcus mutans* was inoculated into 5ml Brain Heart Infusion Broth (BHI) supplemented with 5%sucrose, and into another 6 similar sets of 5 ml of same medium containing increasing concentrations of anti-*Streptococcus mutans* chicken egg yolk antibodies (5µg/ml of IgY - 30µg/ml of IgY) and incubated overnight at 37°C, anaerobically. PBS alone served as negative control and 50-70 µg/ml of Amoxycillin was the positive control. After incubation the contents were subcultured onto BHI agar and incubated overnight at 37°C, anaerobically. The OD of the tubes were checked using turbidity method to check the growth inhibition of *Streptococcus mutans* by the anti-*Streptococcus mutans* chicken egg yolk antibodies (IgY).

Agglutination of *S. mutans* cells by anti-*Streptococcus mutans* IgY

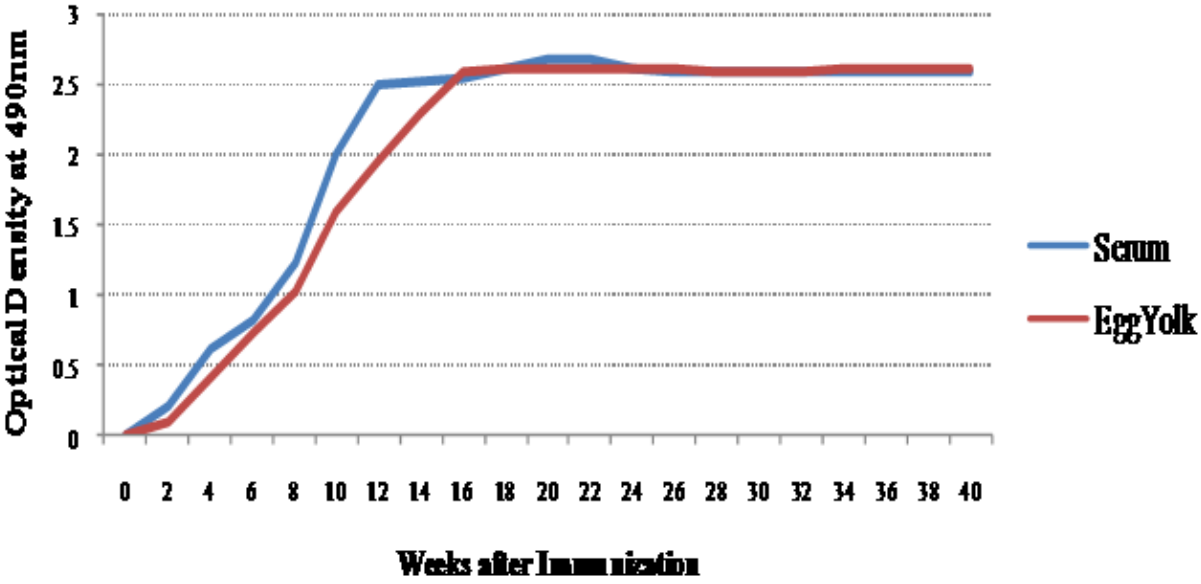
Antibacterial effect of IgY against *Streptococcus mutans was* tested and identified by Slide Agglutination Test. Formalin treated whole cells of *Streptococcus mutans* were suspended in saline. The cell suspension was mixed with an equal volume of twofold-diluted anti-*Streptococcus mutans* IgY and incubated for 2 hours at 37°C and then overnight at 4°C, and agglutination was determined visually. The antibody titer was expressed as the minimum concentration of IgY in the reaction mixture that gave positive agglutination.

RESULTS

The antibody level in the serum and egg yolk of immunized hens were monitored after every booster dose. An increase in antibodies against *Streptococcus mutans* was detected in the serum of immunized chicken 7days after the initial immunization by ELISA. This humoral immunity reached a peak at about 45 days. In the egg yolk, the antibody level increased 2weeks after the initial immunization and persisted increase was observed till the 60^{th} day after which the antibody level remained stable (Figure 1). The IgY concentration in egg yolk was increased during the immunization period and reached maximum of 5.8mg/ml after immunization. The purity of chicken egg yolk antibodies and its molecular weight were determined by SDS-PAGE, were a single protein band of molecular weight protein (180KDa) showed the presence of IgY (Figure 2). A standard protein marker was also run in parallel along with the antibody samples. Indirect antibody capture assay (IACA) showed that the antibodies are generated in chickens against injected *Streptococcus mutans* antigens. The optimum titre of column purified egg yolk antibodies was found to be 1:10000 against 100µl of *Streptococcus mutans* antigen (Figure 3). The specificity of the chicken egg yolk antibodies (IgY) against *Streptococcus mutans* were carried about by agglutination assay. The column purified egg yolk antibodies derived from

immunized hen were added to the agglutination assay plate, which were coated with *S.mutans* antigen.

Fig 1 The antibody level in the serum and egg yolk of immunized hens were monitored after every booster dose. The IgY concentration in egg yolk was increased during the immunization period and reached maximum of 5.8mg/ml after immunization at 150th



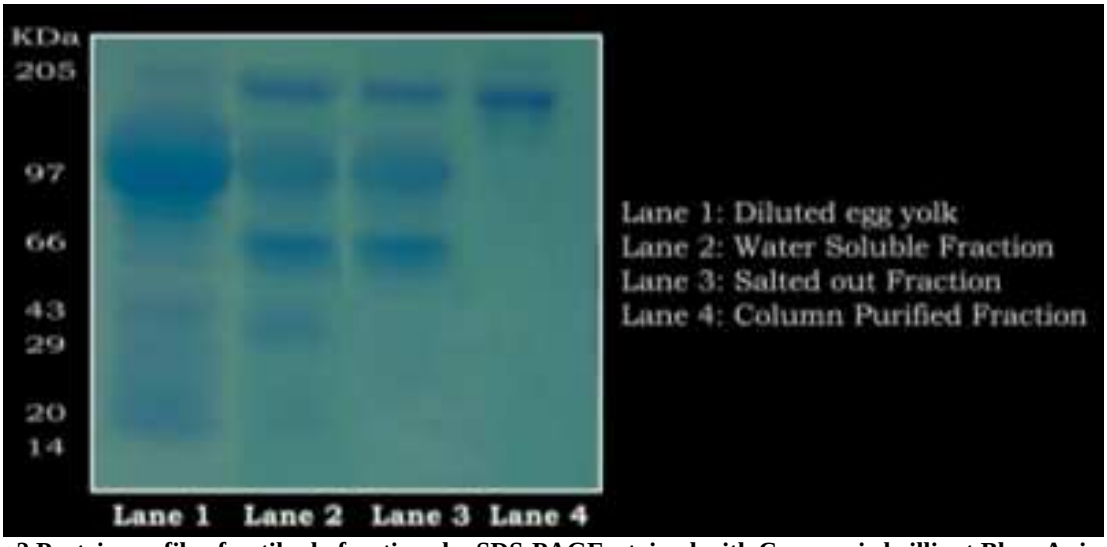


Fig 2 Protein profile of antibody fractions by SDS-PAGE, stained with Coomassie brilliant Blue. A single protein band of high molecular weight (180KDa) shows the purity of IgY in Lane 4

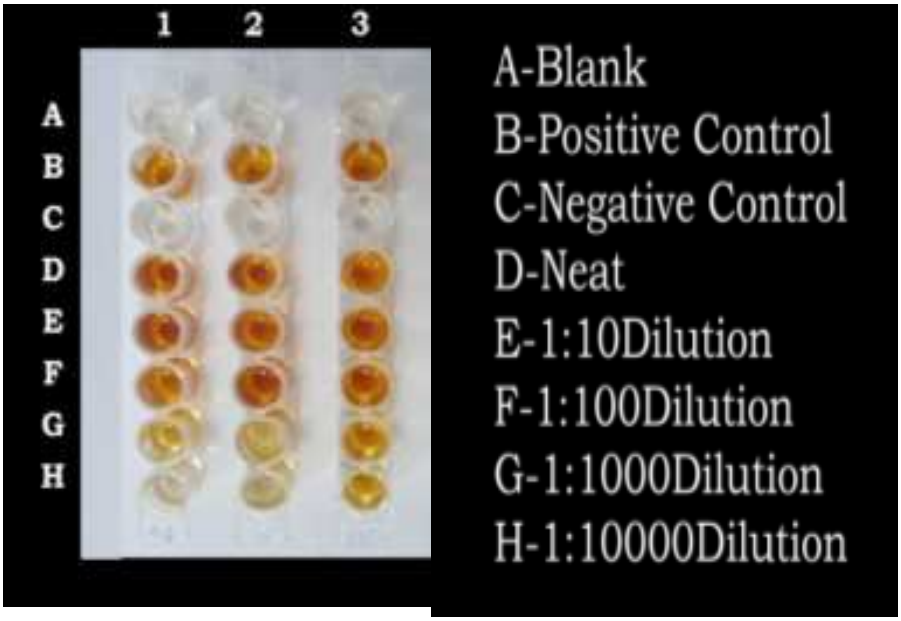


Fig 3 Titration of antibody by ELISA. The positive reaction shows up to 1:10,000 dilution indicates high amount of antibodies are produced in immunized chicken egg yolk

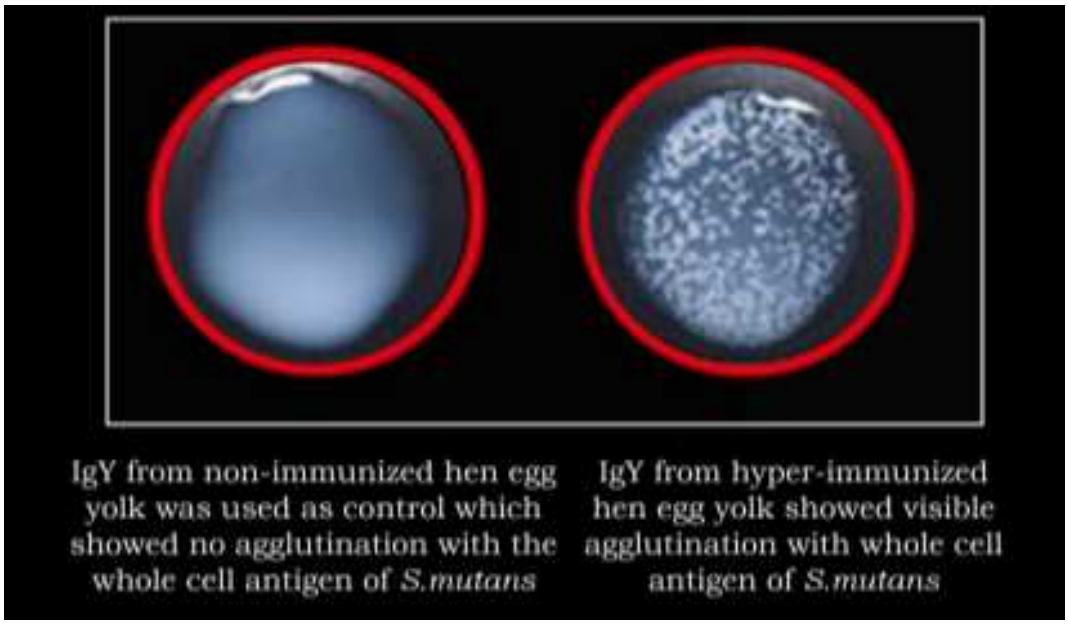


Fig 4 Demonstration of antibody specificity using Agglutination Assay. The binding pattern of IgY was visualized by agglutination

Fig 5 Growth Inhibition assay by Turbidity Method-There was a decrease in *S.mutans* growth when pre-incubated with increasing concentration of antibodies (IgY). The growth was completely inhibited when 25µg/ml of IgY was added to the culture

Table 1: Minimum Agglutination Assay.

Dilutions of Antibody	Visible Agglutination to *S.mutans* whole cell antigen
1:5	+++
1:25	++
1:125	++
1:625	+
1:3125	-
Control (IgY from Non-immunized Hen Egg yolk)	-
- *No Agglutination* + *Minimum Agglutination Titre* ++ *Presence of Agglutination* +++ *Strong Agglutination Reaction*	

The minimum agglutination concentration of IgY against whole cell antigen of *S.mutans* was found to be 1:625

DISCUSSION

The present study was conducted to determine the effectiveness of egg-yolk antibody (IgY) obtained from hens immunized against *Streptococcus mutans*. The increase in the specific antibody concentration of egg yolks from immunized hens over the course of immunization period was monitored. The antibodies against *Streptococcus mutans* first appeared in serum on 7^{th} day after starting the immunization schedule. Then the antibodies were detected in egg yolk after two weeks. The concentration of antibodies increased in the egg yolk with subsequent

booster doses with an average yield of 80mg per egg yolk at 150th day of immunization period. The activity of egg yolk antibodies was determined by ELISA, showed the presence of antibodies specific for *Streptococcus mutans*. Normal antibodies obtained from unimmunized chicken did not show any activity against *Streptococcus mutans* antigen. These results were comparable to the work already done which showed that antibodies started to appear in serum 10days after immunization began and reached high titre at 45th day and remained stable till 168th day observation [8]. A good correlation between ELISA and the potency of IgY raised against *S.mutans* was also as per results already observed [9]. In SDS-PAGE high molecular weight proteins (180KD) were detected by using Coomassie brilliant blue stain. It shows the purity of egg yolk antibodies. In ELISA a peak titre of 1.756 at 490nm was observed in immunized chicken egg yolk against *S. mutans* antigen during 150th day of observation. The minimum agglutination concentration of IgY against whole cell antigen of *S.mutans* was found to be 1:625 determined by the agglutination assay. Thus, considering their safety, it may be no problem to use eggs as a source of antibody for the prevention of dental caries by passive immunization. Passive immunization (orally administered yolk antibody against *Streptococcus mutans*) can provide a potential approach to the prevention of dental caries.

CONCLUSION

Dental caries is perhaps the most prevalent disease affecting humans today across the globe. India is no exception to these problems despite the advances in dental health. Streptococcus mutans serotype c is thought to be the principal causative bacterium of dental caries in humans. By far, no proper therapy has been involved in the treatment of caries other than personal hygiene and seal fissures. Several studies have been made regarding preventive measures for protecting the host from this disease. There are many reports suggesting the possibility of preventing dental caries by vaccination (active immunization) using as antigens mutans streptococci whole cells or one of its characteristic cariogenic factors. Many researchers have also reported the effectiveness of passive immunization against caries, in which specific antibodies against mutans streptococci are administered orally. Recently, passive immunization has gained much attention, as compared with active immunization, because of the possible side-effects caused by mutans streptococcal vaccine antigens. It is known that the hen transfers serum IgG to the egg yolk and that this antibody gives immunity to its offspring. The antibodies present in egg yolk have been termed IgY. Thus, it is possible to obtain pathogen specific IgY antibody from eggs laid by hens immunized against antigens. Eggs may be a suitable source of antibody for passive immunization, which requires large amounts of antibodies.

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