Protection against Dental Carries by Passive Immunization with Hen Egg Yolk Antibody Using Cell Associated Glucosyltransferase of Streptococcus mutans

Sentila R¹, Karthika S¹, Michael A² and Gandhimathi A³

¹Department of Microbiology, PSG College of Arts and Science, Coimbatore, India
²Associate Professor and Head, Dept of Microbiology, PSG College of Arts and Science, Coimbatore, India
³PhD Scholar, Department of Microbiology, Karpagam University, Coimbatore, India

Abstract

**Background:** Dental caries is one of the most frequently encountered diseases. Of the oral bacteria, *Streptococcus mutans* is considered to be causative agent of dental caries in humans.

**Aim:** This study aims at formulating a dental composition using Chicken Egg yolk Antibodies (IgY).

**Methodology:** IgY was raised in 22 weeks old white leghorn chicken against CA-GTF antigen of *Streptococcus mutans*. The level of the antibody in serum was monitored and booster doses were given whenever necessary. The antibodies were purified from the egg yolk using PEG and Ammonium sulphate precipitation and DEAE cellulose ion exchange column chromatography.

**Results and discussion:** High titre of more than 1:10000 antibodies were detected by Indirect ELISA at 150th day of observation. The IgY concentration in egg yolk was increased during the immunization period and reached maximum of 3.9 mg/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 not distinct when 25 μg/ml of IgY was added to the culture. Similarly, GTF activity was quantified by phenol-sulfuric acid method.

**Conclusion:** Total glucan synthesis by cell-associated GTF preparation from *Streptococcus mutans* was significantly inhibited by the addition of antibodies to CA-GTF. This raises the possibility of conferring passive protection against *Streptococcus mutans*-induced dental caries by using IgY from the eggs of the hens hyperimmune to *Streptococcus mutans* CA-GTF 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.

Keywords: Dental caries; Glucosyl transferase; *Streptococcus mutans*; IgY; Prevention

Introduction

The term caries means decay of tissue. It has affected the teeth of all nations irrespective of geographic and cultural differences. The incidence of caries increased as civilization advanced, mainly because of dietary habits. In India the incidence has gone up from 40 to around 80 percent during the past four decades. Presumably rising standards of living have led to increasing sugar intake. According to the survey made by the WHO, Dental caries has been increasing both in prevalence and severity for the last five decades [1].

Dental caries is caused predominantly due to the common oral bacterium, *Streptococcus mutans* (*S. mutans*). These bacteria are present in virtually all individuals. Various selective media like MSFA, MS, TYSCB, and BHI etc. supports the growth of *S. mutans*. On Mitis Salivarius sucrose bacitracin agar *S. mutans* has a characteristic colonial morphology, which permits its differentiation from other oral *Streptococci*. However in many instances, identification by the morphological criteria requires considerable experience.

Cell associated glucosyltransferase of *Streptococcus mutans* plays an important role in the development of dental caries in humans. Sucrose is a natural source of energy for this enzyme, and GTF is the key enzyme that catalyzes the conversion of sucrose to long-chain polysaccharides [2]. This insoluble glucan serves as a bridge for the bacterium to bind tightly to the tooth surface. After binding of *S. mutans*, it releases large amount of acids by fermenting a variety of sugars from the diet, which decalcify enamel, thus causing tooth decay or dental caries [3]. The reason behind the interest of studying GTF enzymes of *S. mutans* (serotypes C), is due to their ability to elicit protective immunity against experimental dental caries caused by implanted mutants streptococcal species [4].

The costs for operative dental treatment are significant both for individuals and society. Therefore, a need exists to target preventive measures and active treatment for the dental caries. A toothbrush can be used to remove plaque on accessible surfaces, but not between teeth or inside pits and fissures on chewing surfaces. When used correctly, dental floss removes plaque from areas which could otherwise develop proximal caries. Mouthwashes claim to prevent or reduce plaque

*Corresponding author: Sentila R, Department of Microbiology, PSG College of Arts and Science, Coimbatore, India, E-mail: sentilarajan@yahoo.in

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and chlorhexidine was probably the most effective and commonly used antibiotic [5]. Although most of the pathogenic and benign microorganisms are killed some of the strains of the Streptococcus have been found to be resistant to this chemical due to the formation of biofilm on the tooth surface in vivo. Dental caries causes many people to experience a great deal of continuous discomfort through impaired function and aesthetics as well as inconvenient treatment.

There are many reports suggesting the possibility of preventing dental caries by vaccination (active immunization) using antigens of mutants streptococci whole cell or one of its cariogenic factors CA-GTF. Chicken egg yolk has been recognized as an inexpensive alternative antibody source. Passive immunization with egg yolk immunoglobulin (IgY) against cell wall associated glucosyltransferase can prevent the tooth decay by immobilizing bacteria [6].

Hence, the present investigation is focused to generate chicken egg yolk antibodies against cell associated glucosyltransferase which effectively blocks the CA-GTase from binding to the tooth surfaces, thus inhibiting its growth and preventing the development of dental caries. Similar work was carried out previously with reliable records. Anti-CA-GTase antibody also inhibits the synthesis of insoluble glucan by the enzyme, thus assisting the elimination of the bacterium from the oral cavity [7].

Materials and Methods

Experimental animal

21 week old White leghorn chickens were purchased from LK poultry farm, Iyampalayam (Near Paladam), Coimbatore. Chickens were maintained in the Layer Shed and were given ad libitum access to water and food.

Confirmation of the standard strain

Streptococcus mutans serotype c (MTCC No: 497) standard strain obtained from Depository at MTCC, Chandigarh, India, in a lyophilized form and after recovery confirmed by gram reaction, cultural characteristics, biochemical tests, biofilm formation and glass adherence test.

Preparation of cell-associated glucosyltransferase antigen

Streptococcus mutans serotype c standard MTCC (497) strain was cultivated on Brain Heart Infusion Broth supplemented with sucrose and incubated at 37°C for 24 hours aerobically. Further, the culture was preserved in 15% glycerol at -20°C for further procedures.

The Streptococcus mutans c strain was cultivated onto a brain heart infusion broth with 5% sucrose and it was incubated for 18hours at 37°C. The culture was centrifuged at 10000 rpm for 15 minutes and the pellet was washed three times with saline. Appropriate quantities of 8 M urea solution was mixed with suspended washed pellet and the mixture was allowed to stand for one hour at room temperature in a metabolic shaker. This was subjected to centrifugation and the partially purified supernatant was again purified by Dialysis method. The purified form of mixture was centrifuged at 10000 rpm for 15 minutes and the supernatant was precipitated 60% saturated ammonium sulfate and the precipitate was dissolved in 5 ml of 50 M phosphate buffer (pH 7.5). This solution was then subjected to dialysis. The fully purified mixture was again centrifuged 10000 rpm for 15 minutes and the precipitate was removed. The resultant supernatant was used as an antigen [8].

Immunization of Hens

The White Leghorn hens were immunized intramuscularly at two different sites with the antigen solution. The first immunization was made by intramuscular injection with 1ml of antigen suspension prepared by emulsifying 0.5 ml streptococcus mutans glucosyl transferase with an equal volume of Freund's Complete Adjuvant (FCA) as described by Sunwoo et al. [9]. The second dose of antigen was given after three weeks of the first inoculation with the same route and volume as the first immunization but the FCA was replaced by Freund's Incomplete Adjuvant (FIA). Booster inoculations were given at four-week intervals without adjuvant. Antibody titre in the serum was monitored to confirm the raise in the level of antibody after the booster doses.

Collection and storage of eggs

The eggs were collected daily and labelled with an identification number, and date. The collected eggs were refrigerated at 4°C until the isolation of the immunoglobulins. Yolk antibodies are stable in situ in the refrigerator for at least 6 months.

Purification of IgY antibodies from egg yolk

The egg yolk was separated from white and was washed with water to remove albumins. Approximately 10-15 ml of yolk is obtained from an average sized egg. An equal amount of buffer S (10 Mm phosphate buffer, 100 mM 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 so that lipids got separated. The stirred mixture was centrifuged at 11000 rpm for 20 minutes. The supernatant was filtered through double layered cheese cloth so that proteins and impurities were separated other than antibodies. The 42% PEG in buffer S was added to make a 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 the equal volume of 4 M ammonium sulfate (pH 7) was added and incubated at 0°C for 30 minutes so that antibodies got saturated. The precipitate was centrifuged at 11000 rpm for 20 minutes and the pellet was redissolved in buffer S without sodium chloride for overnight. This suspension was then subjected to dialysis using the same buffer. IgY fraction obtained by dialysis was subjected to [10] further purification using DEAE cellulose ion exchange column chromatography.

Determination of protein content in IgY fraction

The total protein content was estimated by the method described by Lowry et al. [11]. The optical density (OD) was measured at 660 nm. The OD value of IgY was compared with standard graph.

SDS-PAGE

The pooled IgY fractions (antibody samples) from other proteins were separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) [12] with a 4% stacking and 10% separating gel. The samples were loaded into sample wells along with a known molecular weight markers (Low and Mid range markers, Genei Pvt. Ltd, Bangalore) and the electrophoresis was performed at 100V current in a Tris glycine buffer system. When Bromo phenol blue dye reached at the bottom of the gel, the electrophoresis was stopped. The gel was removed and was stained with coomassie brilliant blue R-250 for 30 minutes. The gel was rinsed with distilled water and destained. The molecular weight of the proteins resolved estimated in comparison to the molecular weight markers.
Titration of antibodies-ELISA

The immunological specificity of IgY elaborated against Streptococcus mutans antigen was examined by enzyme-linked immunosorbent assay (ELISA) [13]. In brief, wells of Microtiter plates were coated with antigen solution appropriately diluted with 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed, and 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 thrice with PBS, and IgY from immunized hens at different time intervals was applied to the well in duplicate for reaction with the antigen for 2 hours at 37°C. After each well was washed again with PBSTween, HRP-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 and the reaction was stopped after 20 min with 4N H₂SO₄. The intensity of color developed was measured at 490 nm with a microplate reader.

In vitro Assays

Assay of GTF activity

About 3 ml of sodium acetate buffer (100 mM; pH 6.2), 5% sucrose was added with 1.5 ml of crude bacterial enzyme, again 0.5 ml of potassium phosphate buffer was reacted with above mixture. The crude mixture was incubated at 37°C for 2 hours. The water insoluble glucan was separated by centrifugation at 10,000 g for 5 minutes [14] and it was quantified by phenol sulphuric acid method.

CA-GTF inhibition assay

To evaluate total glucan synthesis inhibition by antibody a reaction mixture containing 10 g/L sucrose and GTF (2.5 µg of protein) and various concentrations of antibody in a total volume of 50 µL of KBP (pH 6.0) was incubated at 37°C for 3 h. After incubation, 75 µL of 100% ethanol was added to the mixture, and synthesized glucan was precipitated at -30°C for 30 min. The precipitated glucan was centrifuged at 16,000 x g for 15 min and washed three times with 60% ethanol. The precipitates were suspended in 50 µL of KPB (pH 6.0), and the total amount of glucan was determined by the phenol-sulfuric acid method with glucose as a standard [15].

Results

21 week old white leghorn Hens were immunized intramuscularly with prepared bacterial antigens to generate anti-GTF antibodies. Booster doses were given in four weeks intervals and chickens were maintained with regular feed and water. The pre-immune sera and hyper immune sera were collected at specified time intervals during and after the various immunization schedules. After immunization the eggs were collected and stored at 4°C with proper marking of the sample name and date. Then the stored eggs were used for the purification of antibodies from yolks. The method used for purification of chicken egg yolk antibodies was PEG and ammonium sulphate precipitation described by [16] method. The suspension was precipitated at -30°C for 30 min. The precipitated glucan was separated by centrifugation at 16,000 x g for 15 min and washed three times with 60% ethanol. The precipitates were suspended in 50 µL of KPB (pH 6.0), and the total amount of glucan was determined by the phenol-sulfuric acid method with glucose as a standard [15].

Discussion

Dental caries is a worldwide problem associated with plaque, microorganisms and the intake of refined carbohydrates. The mutants Streptococcus specifically S. mutans is strongly associated with dental caries. They produce acids by fermentation of dietary sugars, which in sufficient quantities demineralizes the dental enamel. An attempt had been also made to provide preliminary data on the use of chicken egg yolk immunoglobulin raised against cell associated Streptococcus mutans antigen. Chickens were immunized with CA-GTF antigen of Streptococcus mutans to raise antibodies.

Chicken eggs present an ideal alternative antibody source to mammals, as the IgY in the chickens’ blood is transported to the egg and accumulates in the egg yolk in large quantities. Hens usually lay about 280 eggs in a year. Egg yolk contains a considerable amount

![Figure 1: Characterization of IgY using SDS-PAGE.](Image)
the use of chicken for this purpose is of dual advantage. The primary
advantage is production of high levels of antibodies by chickens than
mammals, thus minimizing the number of animals needed. The
second advantage is the recognition that the egg yolk obtained from
immunized chicken as an excellent source of polyclonal antibodies
for over a decade. The amount of antibodies generated by this avian is
equal to or slightly greater than any other mammal used for obtaining
antibodies.

Antibodies were generated against the CA-GTF antigen of
Streptococcus mutans in chicken as an alternative source to mammalian
antibodies. The process involves the preparation of crude Streptococcus
mutans antigen. Immunization in chicken, characterization of
antibodies and finding out the antibody titre in serum and egg yolk
which was found to be 1:1280. Increase in the specific antibody
concentration of egg yolk from immunized hens over the course of
immunization period was monitored. The antibodies against CA-GTF
Streptococcus mutans first appeared in serum on 7th day after starting
the immunization schedule. Then the antibodies were detected in egg
yolk after a week. Protein content of freeze dried IgY antibodies were
estimated by the method using folin-ciocalteau reagent [11]. About 1 ml
of semi purified IgY antibody sample contains 3.9 mg of protein. The egg
yolk antibodies were further purified by DEAE cellulose ion exchange
column chromatography. The molecular weight of the purified IgY was
confirmed as 180 KDa through SDS PAGE [12]. The specificity of IgY
antibodies was determined by ELISA. These antibodies could be used for
developing a mouth rinse containing the specific monotype antibodies
along with stabilizing agents which when orally administered reduces
the number of streptococcus mutans in dental plaque and saliva by
forming antigen- antibody complexes thereby neutralizing the plaque.
The treatment of dental caries is otherwise done by chemicals and
antibiotics having harmful side effects or else by painful removal of the
teeth. The above approaches suggest that oral immunization could be
an efficient method of preventing human dental caries.

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Streptococcus mutans serotype F using monoclonal-antibody immunooaffinity
administration of a streptococcal antigen coupled to cholera toxin B subunit

Table 1: Estimation of Protein concentration in antibody fractions of chicken egg
yolk.

<table>
<thead>
<tr>
<th>Egg collection (Days)</th>
<th>Total Protein Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-immune egg yolk</td>
<td>0.3</td>
</tr>
<tr>
<td>Day 15</td>
<td>0.9</td>
</tr>
<tr>
<td>Day 30</td>
<td>1.1</td>
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<tr>
<td>Day 45</td>
<td>1.5</td>
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<tr>
<td>Day 60</td>
<td>2.3</td>
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<tr>
<td>Day 75</td>
<td>2.8</td>
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<td>Day 90</td>
<td>3.2</td>
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<tr>
<td>Day105</td>
<td>3.6</td>
</tr>
<tr>
<td>Day 120</td>
<td>3.9</td>
</tr>
</tbody>
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![Figure 2: Titration of IgY using ELISA.](image)

![Figure 3: S. mutans GTF in association with activity.](image)

![Figure 4: Inhibition of GTF activity.](image)