Antibodies against dextransucrase from *Streptococcus mutans* display anti-biofilm and growth suppressing activities

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Abstract

Dextransucrase produced by *Streptococcus mutans* play an essential role in the formation of dental caries by synthesizing exopolysaccharides from sucrose, an important metabolite of the organism. In this study we report the location of dextransucrase in *Streptococcus mutans* cells and describe that antibodies raised against dextransucrase inhibited biofilm formation and reduced the adherence and hydrophobic properties of *Streptococcus mutans.* Western blot analysis and immunoelectron microscopy revealed that dextransucrase is located abundantly in the membrane fraction in *S. mutans* cells. Scanning electron microscopy and fluorescence microscopy revealed reduced cell density, impaired bioflim (plaque) formation in presence of dextransucrase antibodies. Genes associated with bioflim formation in *S. mutans* such as GtfB, GtfC, BrpA, relA, Smu630, vicK were down regulated (50−97%) in presence of the enzyme antibody. Presence of enzyme antibodies reduced adherence of *S. mutans* cells to glass surfaces by 58% and hydrophobicity by 55.2%. However dextransucrase antibodies did not affect acid production by *S. mutans*, under the experimental conditions. Immunohistochemistry studies with certain human samples displayed no cross reactivity with dextransucrase antibody. These findings suggest that antibodies against dextransucrase exhibit a profound inhibitory effect on the vital cariogenic factors of *S. mutans* and have no cross reactivity with human tissues tested, thus implying that dextransucrase could be a promising antigen to study its anticariogenic potential.

Introduction

Dextransucrase (EC 2.4.1.5) produced by *Streptococcus mutans* is primarily an extracellular enzyme but its exact location in *S. mutans* cells is unknown. It synthesizes mainly α-(1−3) rich and α-(1−6) dextran polysaccharides from its exclusive substrate sucrose[1] which help in the attachment of microbes to the tooth surface in the form of plaque and biofilm formation, causing the infection[2]. The attachment of microbes onto the tooth surface is the result of hydrophobic interactions between complementary molecules on the microbe and the tooth surface[3]. Various factors associated with the establishment of caries include adhesion, biofilm formation, acidogenicity and aciduricity. *S. mutans* has a remarkable ability to tolerate acidic environment[4] and the acids produced by the metabolism of dietary carbohydrates lead to the dissolution of tooth enamel and subsequent decalcification, cavity formation and hence degradation of calcified dental tissue[5]. The adaptation to high acidic environment is followed by changes in the expression of different genes and proteins associated with protein transport, nuclear acid metabolism, signal transduction, energy metabolism which includes overexpression of 169 genes and repression of 108 genes[6]. The enzymes and proteins whose expression gets changed at acidic pH mostly belongs to stress proteins, amino acid synthesis, acid production, glycolysis pathways and membrane proteins. The adhesion is also facilitated by adhering to the pre-adhered bacteria via lectin links which helps in bioflim synthesis leading to the plaque formation[7]. Adhesion of bacteria on the oral surfaces is also promoted by various surface proteins which includes antigen B, antigen I / II, IF, SpaP, P1, and SR. Gtf genes like GtfB, GtfC, and Gtf D also appears to be essential for the cariogenesis. Gtf B forms water-insoluble α-1,3 binding glucans, GtfC produces α-1,3 and α-1,6 *binding* glucans and GtfD produces

water soluble α-1,6 binding glucans or dextran, which are required for the biofilm mediated plaque formation[8],[9]. Glucan binding proteins synthesized by *S. mutans* helps in the maintenance and architecture of biofilms formed by other oral organisms also[8]. The ability of *S. mutans* to form biofilms is the potential factor for the development of caries formation[10].

Several studies regarding immunotherapy for caries based on both *in vivo* and *in vitro* studies have been explored[11]. Antibody induction by active immunization with *S. mutans* antigens has been reported to be an effective method to provide protection against experimental dental caries. Other adhesion molecules of *S. mutans*, which have been used as antigens for vaccine development include AgI/II, GTFs, GBPs and Pst system (phosphate- binding-protein)[12]. The antibodies elicited by these antigenic molecules target the specific virulence factors of the pathogens and hence minimises the development of caries formation. The subunit vaccines containing AgI/II or GBP or GTFs from *S. mutans*[13], recombinant vaccines prepared from the genome targeting the expression of specific antigen of the pathogen[14] have also been reported. However antibodies against these molecules are reported to have cross reactivity against skeleton muscles and heart tissues[15], but antibodies against dextransucrase when tested for cross reactivity with human heart tissues and various mammalian tissues revealed negative results[16].

The direct relationship between dextransucrase activity and cariogenicity makes it a good candidate to study its anticariogenic potential. Thus in the present study we evaluated the localization of dextransucrase in *S. mutans* cells and the effect of anti-dextransucrase antibodies on the bioflim formation and other associated cariogenic factors. These results are reported herein.

Results

Dextransucrase Localization in *S. mutans* cells.

The site of location of dextransucrase in *S. mutans* was determined by measuring dextransucrase activity in different fractions of *S. mutans* cells which was further validated by immunoelectron microscopy and western blot analysis.

Cell pellet obtained after centrifugation of 24 hours. culture of *S. mutans* was lysed by sonication which was subjected to differential ultracentrifugation. The pellets obtained at 2000g, 8000g, 40000g and 100000g centrifugation contained undisrupted cells, cell wall fraction, cell membrane fraction and cytoplasmic fraction respectively[24]. The pellets were suspended in 20mM sodium maleate buffer pH 6.8 and each fraction was analysed for dextransuccrase activity. Dextransuccrase activity in 40000g fraction was high as compared to other cell fractions indicating that dextransucrase is largely associated with the membrane fraction of the *S. mutans* cells.

Immunoelectron microscopic location of dextransucrase in *S. mutans* cells

Using the antibody raised against dextransucrase as well as gold-labeled IgG, dextransucrase antigen was localised by detecting the immune complexes formed by dextransuccrase antibody and the dextransucrase present in the *S. mutans* cells. As shown in Fig. 1 the immuno-gold labelling was observed more on the surface of the cells as compared to the cytosol, which demonstrated that dextransucrase is largely associated with the membrane part of *S. mutans* cells. This was apparent even in the cells undergoing cell division. The immune-gold particles seen outside the cells presumably represent the excretory enzyme in the medium.

Reactivity of *S. mutans* fractions with dextransucrase antibody.

The immunoblot analysis (Fig. 2a) revealed that reactivity was more pronounced in membrane fractions of *S. mutans* as compared to the cytosolic fractions, this further corroborated that dextransuccrase is largely associated with the membrane fraction of the cells. Densitometric scan of the immunoblot carried out by ImageJ software is shown in Fig. 2b.

Dextransucrase antibody inhibited bioflim formation by *S. mutans in vitro.*

S. mutans were allowed to form biofilms for 20h at 37°C on glass cover slips that were deposited in 6-well culture plates in BHI media supplemented with 5% sucrose. *S.mutans* cells grown on cover slips were fixed with 2% glutaraldehyde and 4% formaldehyde and sputter coated with platinum in carbon. Images were taken from magnifications 500X, 1000X, 3000X and 6000X. As shown in Fig. 3 (a & b) there was a significant difference in the cell aggregation and depth of biofilms in the control and antibody treated samples. Cluster formation was seen in control samples where as in treated samples the cells are segregated which demonstrated considerable reduction of bioflim formation in presence of dextransucrase antibodies in test samples.

The effect of dextransucrase antibody on bioflim formation was also studied using propidium iodide (PI) staining by fluorescence microscopy. *S. mutans* cells grown on saliva coated glass coverslips in presence and absence of dextransucrase antibody were fixed and stained with propidium iodide solution (1µg/ml) as mentioned in materials and methods. These results illustrated that dextransucrase antibodies reduced the bioflim formation by *S. mutans.* The cells in the control samples showed clumping of cells in the polysaccharide matrix however the cells grown in presence of the antibody were well dispersed, suggesting inhibitory effect of antibody on bioflim formation by *S. mutans* (Fig. 4).

Dextransucrase antibodies down regulated the expression of biofilm forming genes in *S. mutans*

Transcriptional expression of biofilm forming genes GtfB, GtfC, BrpA, Smu630, RelA and VicK was determined by using Real-Time PCR system. The specific primers and reaction conditions are described in Table 1 of material and methods section.16sRNA was used as an invariant internal control. The data of quantitative analysis is represented as ΔCT (ΔCT = CT target − CT 16sRNA) and $2^{-\Delta\Delta CT}$ method was used for the determination of fold change in mRNA expression [25].

The mRNA expression of biofilm forming genes GtfB, GtfC, BrpA, Smu630, RelA and VicK was analysed in treated (in presence of serum IgG) and untreated (in absence of IgG) *S. mutans* cells.The data showed reduced expression of GtfB 0.5 fold (p<0.0162), GtfC 0.13 fold (p<0.014), BrpA 0.16 fold (p<0.014), RelA 0.030 fold (p<0.0001), Smu630 0.064 fold (p<0.0008), and VicK 0.148 fold (p<0.005) compared to control (Fig. 5).

Effect of dextransucrase antibodies on adherence of *S. mutans* to glass surfaces

Adhesion of bacterial cells on the tooth surface is the prerequisite for the bioflim formation which is initiated by attachment of cells to the surfaces followed by assembly of cells leading to plaque formation. The effect of dextransucrase antibody on the adherence of *S. mutans* cells on the glass surfaces was studied by allowing the growth of bacteria in presence (Test) and absence (Control) of dextransucrase antibody in glass tubes kept at 30° containing BHI medium with 5% (w/v) sucrose at 37°C for 24 h as mentioned in methods section. The effects of dextransucrase antibodies (30μg) on the adherence of *S.mutans* is shown in Fig. 6(a). Results with dextransucrase antibody exhibited 58.3% reduction in the adherence of *S. mutans* suggesting considerable inhibitory effect on the adherence of the organism.

Dextransucrase antibodies decreased cell surface hydrophobicity of *S. mutans*

The effect of dextransucrase antibody on the hydrophobicity of *S. mutans* grown in presence of 30 μg of antibody in BHI medium supplemented with 5% sucrose for 20 hours at 37°C was carried out. The samples without antibody were taken as the controls. These results are shown in Fig. 6(b). A considerable reduction in the hydrophobicity of *S. mutans* was observed under these conditions. The hydrophobicity index was 55.2% which was less than the standard value of hydrophobicity index.

Effect of antibodies on acid production by *S. mutans*

Acid production by *S. mutans* was analysed by culturing the bacteria in presence (Test) and absence (Control) of dextransucrase antibody at 37°C. Culture media without inoculum was taken as control. The pH of the culture was measured at 0 h and at 24 h. As shown in Fig. 6(c) there was essentially no effect of dextransucrase antibodies on the acid production by *S. mutans,* under the experimental conditions. The data indicated that dextransucrase antibodies did not affect the acid production by *S. mutans.*

Cross reactivity of dextransucrase antibody with human samples

The antibodies raised against dextransucrase were tested for the reactivity with different human tissues by performing immunohistochemistry. The positive control was prepared from the heart muscle tissue which was immersed in the antigenic protein and fixed before preparing the slides. As shown in Fig. 7 antibodies raised against dextransuccrase did not show any reactivity with any of the tissues tested, which further demonstrated that there was no cross reactivity with the human tissues tested.

Materials And Methods

Chemicals

Analytical grade chemicals were used in this study. Brain heart infusion broth (BHI), Agar, peptone, sucrose, exogenous dextrose, glucose, maleic acid, NaCl and ethanol were obtained from HiMedia Pvt. Ltd. (Mumbai, India). Electrophoresis reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glutaraldehyde, paraformaldehyde, Sephadex G-200, propidium iodide and all other chemicals used were obtained from E. Merck Pvt. Ltd. (Mumbai, India) or Sisco Research Lab Pvt. Ltd. (Mumbai, India).

Bacterial strain and growth conditions

Streptococcus mutans strain MTCC-890 was obtained from MTCC Institute of Microbial Technology (IMTECH), Chandigarh, India. *S. mutans* was grown in brain heart infusion (BHI) broth, supplemented with 1% peptone, 1% dextrose, 0.29% glucose, 0.25% sodium hydrogen phosphate and 0.05% NaCl (pH 7.4) to late-exponential phase at 37 °C.

Purification of dextransucrase from *S. mutans* and production of antibodies.

Dextransucrase was purified to homogeneity from the culture supernatant of *S. mutans* MTCC-890 grown at 37 °C for 36 h by 55% ammonium sulphate precipitation followed by gel filtration chromatography using Sephadex G-200 and treatment with PEG-400 (polyethylene glycol 400) as reported earlier[16].

Polyclonal antisera against dextransucrase purified from *S. mutans* was raised in New Zealand White rabbits immunised subcutaneously with purified dextransucrase (500µg) mixed with complete Freund's Complete Adjuvant (F5881, Sigma, USA). Serum was collected after two weeks of last booster and analysed for dextransucrase specific antibody by Dot blot assay, confocal microscopy, ELISA and western blot analysis as described previously[16]. Serum IgG was purified by affinity chromatography using Protein A Sepharose Column (Bio Vision, USA).

Cell fractionation of *S. mutans*

Cell fractionation was carried out by separating the cells from culture media by centrifugation. The cell pellet was treated with lysozyme 0.25mg/ml in 50mM sodium maleate buffer pH 6.8 for half an hour in ice and then kept in a water bath at 37°C for 5 min. The cells were lysed by sonication using cell sonicator (Sonics- Vibra VCX-750 USA) for 10 min and the pulse rate was 10 sec on and 10 sec off. After cell disruption the sample preparation was subjected to differential centrifugation using ultracentrifuge Beckman coulter (Optima XPN-100). The sample preparation was centrifuged at 2000g to separate unbroken cells followed by 8000g for 10 min, 40000g for 30 min and 150000g for one hour to collect different fractions of *S. mutans* cells. All the procedures were carried out at 4°C. The pellets obtained at each step were suspended in 20mM sodium maleate buffer pH 6.8 and stored at -20°C and analysed for dextransucrase activity and affinity for dextransucrase antibody.

Immunoelectron Microscopy

Immunoelectron microscopy was carried out for detecting intracellular and surface location of dextransucrase utilizing antigen- antibody reactions[17]. The cells were fixed in 0.2% glutaraldehyde and 4% paraformaldehyde in PBS for 2-hours at room temperature. The fixed cells were dehydrated in ascending grade of ethanol (20%, 50%, 70%, 90%, and 100%) and embedded in LR white resin (Hard grade) (TAAB, UK) at 55°C. Thin sections of 80-90 nm thickness were cut using ultra microtome and mounted onto 300 mesh nickel grids. The grids were first incubated in 1% cold-water fish gelatin made in 0.01M phosphate buffer saline (PBS) for 30 minutes to block nonspecific sites followed by incubation in primary antibody (Dextransuccrase antibody) diluted in the ratio of 1:200 overnight at 4°C. The grids were washed three times one minute each in diluent buffer and incubated in secondary antibody (goat anti-rabbit) conjugated with 10 nm colloidal gold (dilution: 1: 50; TAAB, UK) for 2 hours at room temperature followed by washing three times with buffer and water one minute each. The grids were stained for five minutes with uranyl acetate followed by brief washing with distilled water. The grids were blotted dry and observed at a magnification 29000X at 200kV under transmission electron microscope Tecnai G20 (FEI Company, The Netherlands).

Scanning Electron microscopy (SEM)

Scanning electron microscopy was performed following the method described by Ansari et al.[18]. Cultures of *S.mutans* with OD 0.01were grown on glass coverslips immersed at the bottom of the 6-well culture plates to allow the formation of biofilms. Purified IgG was added to the wells and wells without addition of IgG served as the control. After 36 hours of incubation at 37 °C the glass coverslips were carefully removed from the wells and washed three to four times with PBS. The cells were fixed by 4% formaldehyde and 2% glutaraldehyde in PBS pH 7.4 for 2 hours followed by 3-4 times PBS washings. After fixation the glass coverslips were dehydrated by suspending in different percentages of ethanol (10-100%) for 15min each and dried using critical point dryer CPD (EMS 850 JEOL Japan). Sample specimens were sputter coated with platinum in argon gas using sputter coater (JEC-3000FC JEOL Japan) and observed under SEM (JSM-IT300 JEOL Japan). The images of treated cells and control were compared.

Fluorescence microscopy

Cultures of *S.mutans* were grown on glass cover slips in 6-well culture plates with OD 0.01 to allow the formation of biofilms on the glass coverslips. Purified IgG fraction of dextransucrase antibody was added to the wells in triplicates labelled as treated and wells without addition of IgG served as control. After 24 hours of incubation at 37°C the glass coverslips were carefully removed from the wells and washed three to four times with PBS. The cells were fixed in fixation buffer containing 4% formaldehyde and 2% glutaraldehyde in PBS pH 7.4 for 2 hours followed by 3-4 times washings with PBS. The samples were stained with propidium iodide (1µg/ml) for one hour followed by washing with PBS and analysed under

fluorescence microscope (EVOS M7000 Imaging System Thermos Fisher Scientific) as described by Wen et al.[19].

Cell adherence

The effect of the dextransucrase antibodies on the adhesion of *S. mutans* to smooth glass surface was studied by the method of Hamada and Torii[20]. The organism was grown in a glass tube at an angle of 30° containing 10 ml of BHI medium with 5% (w/v) sucrose at 37°C for 24 h. The appropriate controls of BHI were run simultaneously. The cell adherence was quantified spectrophotometrically by measuring O.D. at 600 nm using untreated culture as the control. All the determinations were done in triplicates.

Percentage adherence = (O.D. of adhered cells/O.D. of total cells) × 100.

Cell Surface hydrophobicity.

Cell surface hydrophobicity was determined by following the modified method of Martin et al.[21]. *S. mutans* MTCC 890 were inoculated to fresh BHI media and incubated for 12 hours in presence and absence of antibody (IgG). The bacterial cells were pelleted out and washed twice with sterile saline (0.85%) and optical density of the suspended cells in normal saline was adjusted to 0.3 at 600 nm. Thereafter 0.50 mL of toluene was added to 3.0 mL of the bacterial cell suspension in glass tubes which were agitated uniformly on a vortex mixer for 2 minutes and allowed to equilibrate for 10 minutes at room temperature. The absorbance at 600 nm of the aqueous phase separated from hydrocarbon phase was estimated using spectrophotometer. The hydrophobicity index, expressed as a percentage, was calculated as: [(OD initial - OD final) / OD initial] × 100.

Acid production

The method of Ciardi et al.[22] was used to determine acid production by *S. mutans*. 5% (w/v) of sucrose in 5 ml of BHI broth containing serum antibody was added with 100 µl of 18 h cultures of *S. mutans*. The pH of the bacterial medium was assessed at 0 h and after 24 h of incubation. Except otherwise stated all determinations were done in triplicates and suitable controls were run simultaneously.

RNA extraction and cDNA Synthesis

Total RNA was extracted from the bacterial cells using TRizol (Sigma Aldrich USA) reagent according to manufacturer's instructions. RNA quantification and purity was determined by the Nano Drop ND-1000 spectrophotometer. RNA extraction was immediately proceeded for cDNA synthesis after quantitative assessment.

cDNA was synthesized from 1µg of RNA isolated from *S. mutans* cells using random hexamer primers and Revert Aid First Strand cDNA synthesis Kit (Thermo Scientific) as per the user instructions in total reaction mixture of 20µl. The reaction mixture was incubated at 65°C for 5 minutes and then mixed with 5X reaction mix 4µl, RiboLock RNase inhibitor (20 Units/µl) 1µl 10mM dNTP mix 2µl, RevertAid M-MuLV

RT (200 U/μl) 1μl. The reaction was incubated at 42°C for 60 minutes followed by 70°C for 10 minutes. The reaction was terminated and cDNA was stored at -20°C till used for qRT-PCR analysis.

qRT-PCR

Real time Polymerase Chain Reaction (RT-PCR) (relative quantification) was done using StepOne Plus Real-Time PCR system (Applied Biosystems, Life Technologies) by SYBER Green method. The relative expression of GtfB, GtfC, relA, brpA, smu630 and vicK was carried out using specific primers as given in the Table 1. 16sRNA was used as invariant internal control. The data of quantitative analysis was determined by $2^{-\Delta\Delta CT}$ method where ΔCT = CT (Target gene) - CT (Reference gene). ΔΔCT = ΔCT (Target sample) - ΔCT (Reference sample). CT value (cycle threshold) is inversely proportional to gene expression and is represented as the number of amplification cycles required for the fluorescent signal to cross the threshold. PCR conditions for the amplification of 16sRNA and BrpA were 94°C for 5-minutes (Initial denaturation) followed by 40 cycles of 94°C 40 sec, 60°C 40 sec and 72°C 45 sec. GtfC, vicK and Smu630 were 94°C 5-minutes (Initial denaturation) 40 cycles of 94°C 40 sec 61°C 40 sec 72°C 45 sec and for GtfB, relA were 94°C 5minutes (Initial denaturation) 40 cycles of 94°C 40 sec, 62°C 40 sec and 72°C 45 sec.

Table 1. Shows the sequence of primers used to amplify various genes.

Gene and primer sequence		Amplicon size (bp)
16sRNA		
Forward	GGTGCGCTACACCAATGA (Sense)	117
Reverse	CGCCTACTGGAACCCAAAC (AntiSense)	
GtfB		
Forward	GGTGCGCT ACACCAA TGA (Sense)	110
Reverse	CGCCT ACTGGAACCCAAAC (AntiSense)	
GtfC		
Forward	GGTGCGCT ACACCAA TGA (Sense)	110
Reverse	CGCCT ACTGGAACCCAAAC (AntiSense)	
relA		
Forward	CAAGAGCAGGGCT A TGTGG (Sense)	108
Reverse	ACCGACAGCCGCA T AAAG (AntiSense)	
Smu630		
Forward	GCAGTGCTAAGACTCCCGAA (Sense)	147
Reverse	GCGGAAGTGTGAGATTGGCA (AntiSense)	
BrpA		102
Forward	CTGCTGGAGGTGCT AAGA TG (Sense)	
Reverse	CCAACTGAACCAGCCCTT (AntiSense)	
vicK		
Forward	CTCATTACGCTTGCGCTTTG (Sense)	111
Reverse	CGGCGTTCACGTTCTTCT (AntiSense)	

Immunohistochemistry

Immunohistochemical analysis was carried out following method of Malhotra et al.[23] to check the cross reactivity of dextransuccrase antibody with the mammalian tissues. Reference slides were prepared from the tissue samples of the rabbit and pre-prepared slides of human tissues were obtained from Department of Histopathology, PGIMER Chandigarh (India).

Discussion

Dental caries is a preventable bacterial infectious disease with multifactorial etiology. To control this disease an antimicrobial strategy will be appropriate to minimize the aciduric and acidogenic bacteria to reduce the demineralisation process. A number of efforts have been made to identify the antimicrobial agents and products for the reduction and removal of bacteria from tooth surfaces[26, 27].

Dextransucrase is a secretory enzyme produced by *S. mutans*. It is present both in cell membrane and in supernatant fractions. The distribution of dextransucrase in the cell and supernatant fractions depends upon the presence of sucrose in the medium[28]. Cell associated dextransucrase is present either on the exterior surface of cell, which have an essential role in the cell adherence and is susceptible to proteolytic degradation[2]. In the present study localization of dextransucrase in the cells of *S. mutans* revealed that dextransucrase activity was present in all the pellet fractions of *S. mutans* cells obtained at 2000g, 8000g, 40000g and 100000g centrifugation, but was found mostly in the membrane fraction of the cells. Similar results were obtained by immunoelectron microscopic studies. The immunoblot analysis also showed more intense band in the membrane fraction as compared to the cytosolic fraction demonstrating that dextransucrase is largely associated with the membrane part than in the cytosolic region of *S.mutans*.

Biofilms formed by various pathogenic bacteria are involved in many diseases such as cystic fibrosis, endocarditis and many infections which arise from biofilms on artificial valves, catheters[29]. Biofilm formation proceeds in multiple steps which involve pellicle formation which is a combination of mucin, glycoproteins and other proteins, followed by bacterial adhesion to the pellicle and maturation of the biofilms[30]. Since biofilms develops tolerance towards various antimicrobial agents, thus a potent agent against caries must prevent the pathogen from establishing biofilm formation. In this study results from SEM demonstrated that biofilm was disrupted showing scattered cells in treated samples. The cell density was reduced in the treated samples depicting disruption in the 3D structure of the biofilms as compared to control samples where the cells were clumped forming aggregates in the exopolysaccharide matrix with higher cell density. Further the fluorescence microscopy data also corroborated these findings of reduction in the biofilm formation in presence of dextransucrase antibody. These observations are in line with the results of antibiofilm formation of secretary immunoglobulin A as reported by Huang et al.[30] and rPAc-specific IgG reduced significantly biofilm formation in *S. mutans* challenged rats described by Sun et al.[31]

Li et al.[32] have shown that adherence to tooth surface, biofilm formation and growth of *S. mutans* involves a range of genetic networks which have a significant role to interact and coordinate to various environmental signals like surface oxygen, cell density carbon source pH and nutrient availability[33].

The genes associated with biofilm formation have been reported by Bhagwat et al.[34]. In this study quantitative real time PCR analysis was used to quantify and compare the expression of GtfB, GtfC, BrpA, relA, Smu630, vicK genes. These genes help in bacterial adherence and biofilm formation [35, 36, 37]. The expression of various genes tested was down regulated when *S. mutans* were grown in presence of

dextransucrase antibody. There was a 0.5 fold and 0.13 fold reduction in the expression of GtfB and GtfC genes respectively which have an important role in glucan formation from carbohydrates[38]. The observed reduction in the expression of these genes may result in suppression of biofilm formation, cell adhesion and integrity of cell wall. Similarly BrpA which is involved with the oxidative stress, acid tolerance and bioflim formation has shown 0.16 fold reduction in its expression[39] and the stringent response gene relA involved in accumulation of the bacterial alarmones[40] showed a down regulation by 0.030 fold which may reduce the acid tolerance and defective bioflim formation. Bioflim associated hypothetical gene smu630 expression was downregulated by 0.064 fold. Also Vick which regulates the other essential virulence genes like GtfBCD, glucan binding protein B (gbpB) has a vital role in the biofilm formation [41] showed a fold decrease of 0.148 fold. The suppression of these genes may have a deleterious effect on the intracellular metabolism of the organism which may impair growth arrest. However examination of various other virulence genes involved may help in understanding therapeutic regime of caries prevention.

Adherence of bacteria to the saliva coated surfaces is essential for the genesis of dental caries [42]. Inhibition of adherence factors could be an effective measure to prevent progression of dental caries[43]. The present findings showed that dextransucrase antibody reduced the adherence of *S. mutans* by 58% as compared to control when grown in presence of 28µg of antibody for 20 hours. These results are similar to the findings of Huang et al.[31] who have reported that S-IgA antibodies reduced significantly the adherence of *S. mutans* in experimental rats. The adherence of *S. mutans* was also reduced by anti-caries pGJA-P/VAX antibodies as reported by Xu et al.[44]. The inhibition of *S. mutans* adherence may be due to the interactions of antibodies with the bacterial surface molecules which may alter their surface receptors. The reduction in cell adherence could minimize the bacterial colonization on tooth surfaces which may decrease plaque formation. Hydrophobic interactions play an essential role in the colonization of *S. mutans* on the tooth surfaces[45]. Hydrophobicity of the bacteria is due to the presence of lipoteichoic acids, hydrophobic proteins on the surfaces and external appendages[46]. Westergren and Olsson[47] reported that the changes in the cell surface hydrophobicity affects the adhesion of the bacteria to the surfaces. Therefore reduction in hydrophobicity may also influence the adherence of bacteria to the surfaces and hence caries formation[48]. Present findings showed that dextransucrase antibody reduced significantly the hydrophobicity of *S. mutans* cells when grown in presence of 28µg of antibody for 20 hours. The observed decrease in hydrophobicity may be due to binding of antibodies to the proteins associated with surface of *S. mutans* responsible for this phenomenon. *S.mutans* has the property of both acidogenicity and aciduricity and these physiological factors have an essential role in the pathogenesis of dental caries[49]. These virulence factors provide dominance of *S. mutans* over other oral commensals[4]. The acids produced by the fermentation of carbohydrates results in the destruction of tooth surface as a result of its demineralisation[50], leading to the progression of dental caries[51]. In the present study, there was essentially no effect of dextransucrase antibody on the acid production by *S. mutans* when the pH of the culture was measured at 0h and at 24h in the absence and presence of dextransucrase antibody. These experiments were carried out in presence of 28µg of antibody containing 5mL of media. It is likely that antibody concentration may be small in a large volume of the media than

that used in other experiments. Thus no effect on acid production by *S. mutans* could be observed under the experimental conditions. Also it is likely that under acidic conditions, the enzyme antibody become inactive to exhibit any detectable effect on pH of the culture medium. Thus high concentration of antibody may be needed to observe any change in acid production by *S. mutans*.

Various immunological interventions have been tried for the prevention of caries formation, using various surface molecules of *S. mutans* including synthetic, conjugate and DNA vaccines. Although showing promising anticaries effects, but their use was restricted of having cross reactivity with human heart tissues and skeleton muscles[52]. However western blot analysis revealed that antibodies against dextransucrase did not show any cross reactivity with human heart, liver, gall bladder and other mammalian tissues tested as described previously[16]. In the present study various human samples were tested for cross reactivity with dextransucrase antibody by immunohistochemistry which also showed negative results confirming no cross reactivity of dextransucrase antibodies with the human tissues.

In conclusion the present study demonstrates that dextransucrase antibodies show an inhibitory effect on bioflim formation, hydrophobicity, exopolysaccharide synthesis and cell adherence and has no cross reactivity with human samples. Thus the present data further supports the contention that dextransucrase is a potential candidate as a useful anticariogenic agent. Further studies on developing the monoclonal antibodies or sub unit fragments using dextransucrase protein may be of interest to explore the anticariogenic potential for clinical or physiological aspects.

Declarations

Acknowledgement

Author Contributions Statement

S.A.R wrote the main manuscript and performed experiments, A.M contributed in concept design and/or critical analysis and interpretation of the data. S.C.S provided culture facilities and reviewed the article for important inputs. A.B helped in analysis of immunohistochemistry, A.M.R reviewed the articles. L.S helped in bacterial culture experiments.

Compliance with Ethical Standards

The study was carried out in compliance with the ARRIVE guidelines and approved by Central Animal Ethics Committee Panjab University Chandigarh (IAEC no. PU/IAEC/S/16/52). The experiments were

performed in compliance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Prepaid slides of human tissues were obtained from Department of Experimental Medicine and Biotechnology Postgraduate Institute of Medical Education and Research Chandigarh in compliance with the standards of institutional ethical committee.

Competing interests

All authors declare that there is no conflict of interest with the contents of this article.

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Figures
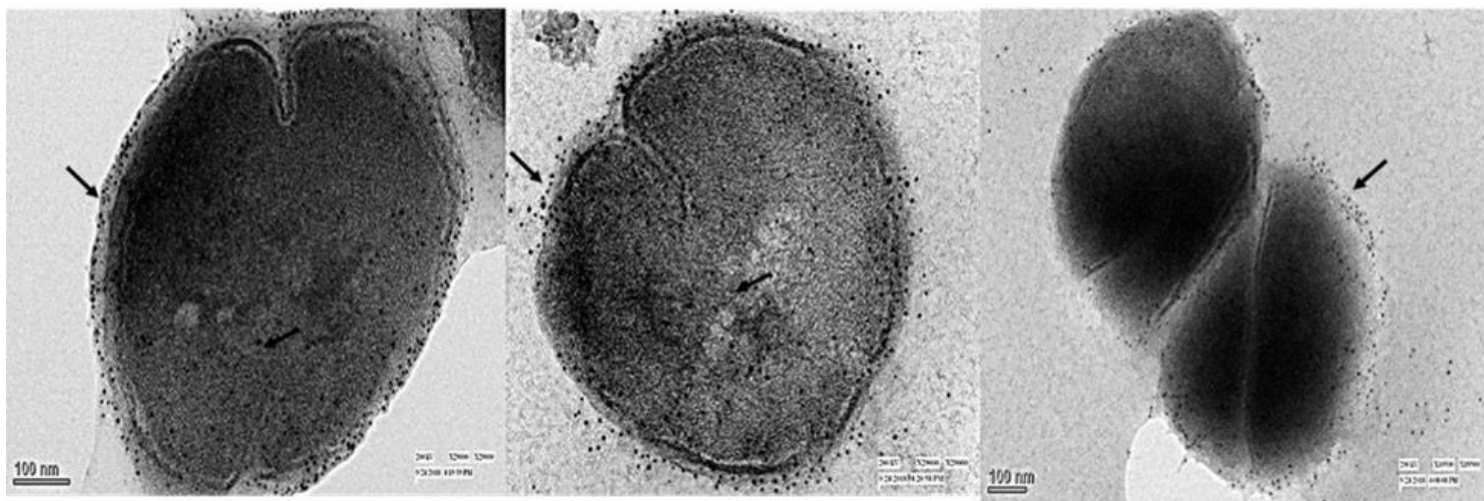
Figure 1

Immuno-gold micrograph of S. mutans cells. Electron-dense immunogold particles (black arrow heads) are associated with the bacterial surface and a thin distribution of immuno-gold particles are present in the cytosolic region. Images were taken at magnification X29000 bar, 100 nm. The sections were examined using a Tecnai, G 20 (FEI) transmission electron microscope operated at an accelerating voltage of 200 kV.
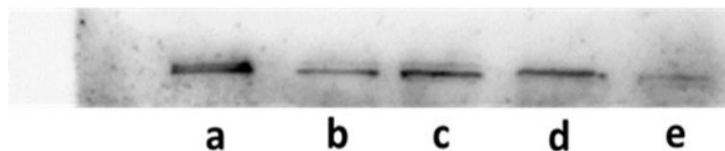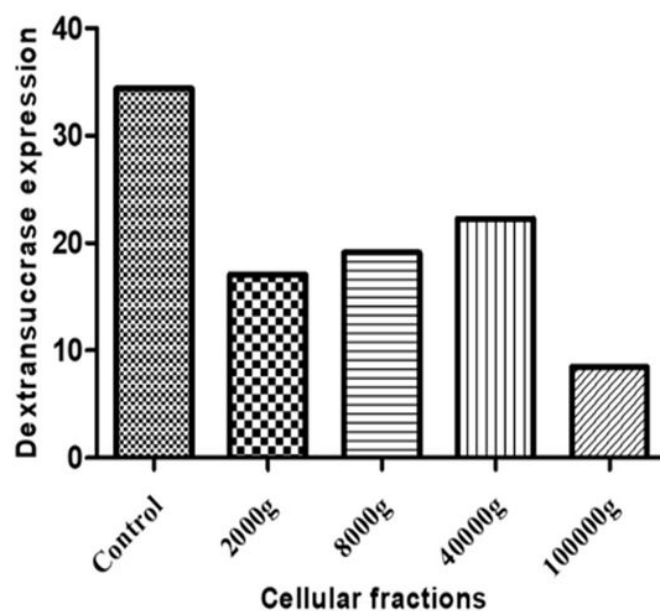
Figure 2

(a & b). Western blot analysis of the different fractions of S. mutans cells obtained after differential centrifugation. (a) Control (b) 2000g (c) 8000g (d) 40000g (e) 100000g represent the cell fractions obtained after centrifugation at different rcf of the sonicated cells of S.mutans. 2(a) Western blot showing expression of different fractions and 2(b) showing quantification of signal intensity in different fractions. Signal intensity of western blotting results in this manuscript was measured by Image J. Full-length blots are presented in Supplementary file Fig. S 2(a, b & c).
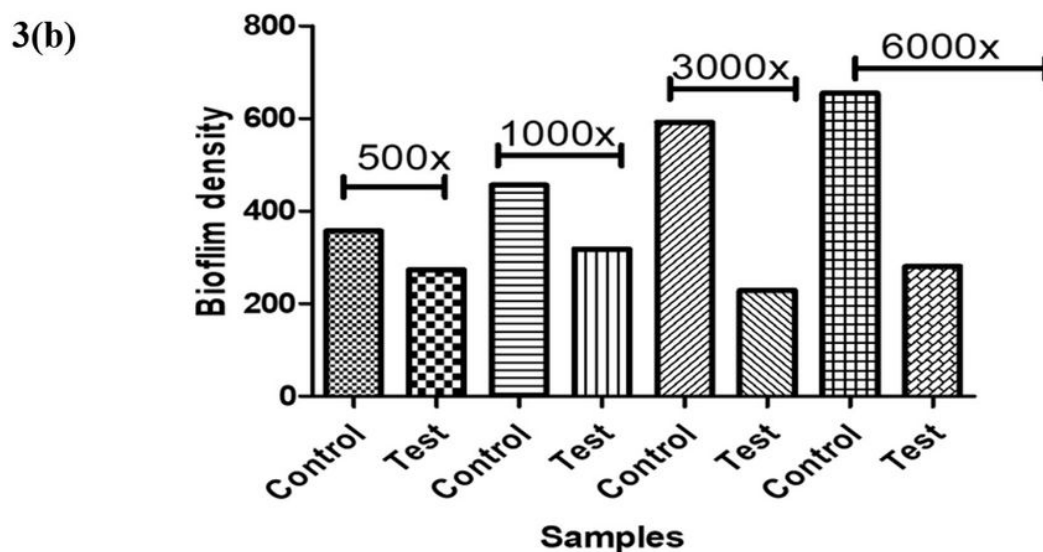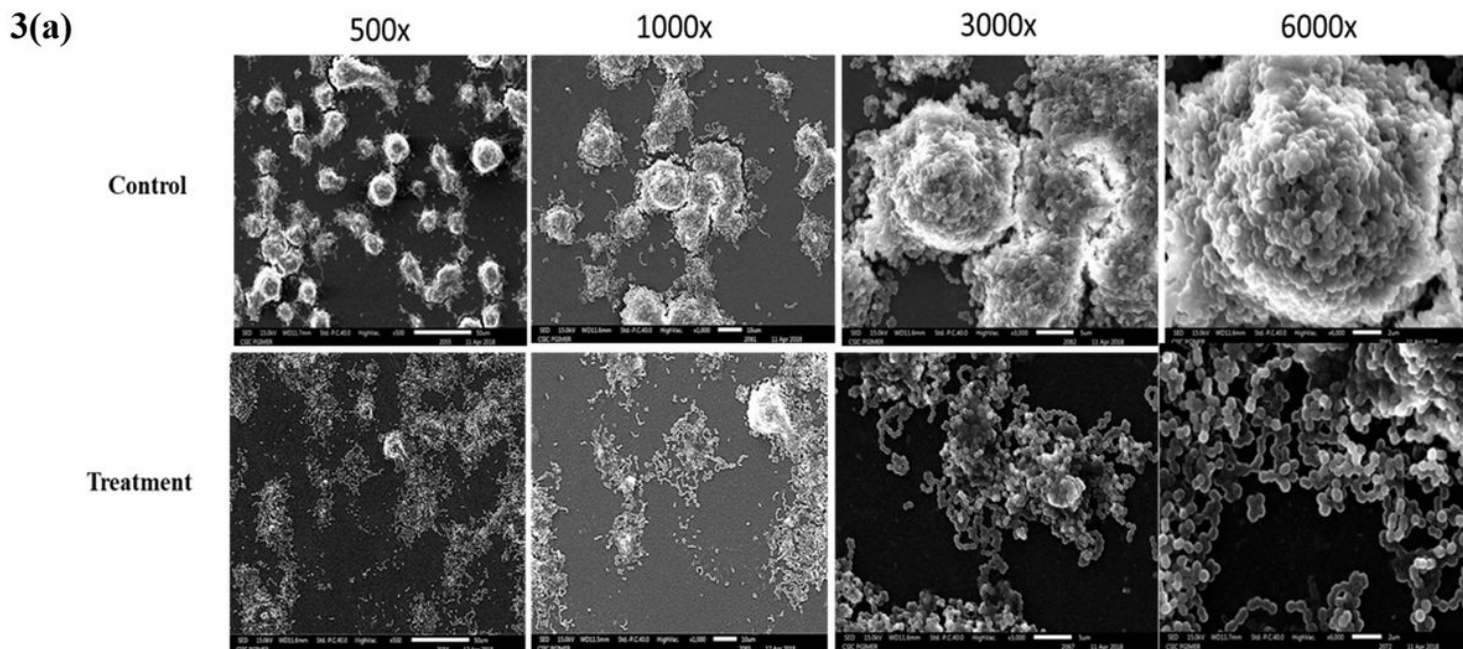
Figure 3

(a & b). Scanning electron microscopic (SEM) images of biofilms: 3(a) Biofilms were formed in BHI medium supplemented with 5% sucrose. Biofilm formation on glass coverslips were analysed by Scanning electron microscopic (JSM-IT300 JEOL Japan). The images are captured at 500X, 1000X, 3000X and 6000X with accelerating voltages at 15kV in both treated and control samples. 3(b) Biofilm density measured using ImageJ software. A marked difference was observed in the control and treated samples. The results presented are representative of three independent experiments.
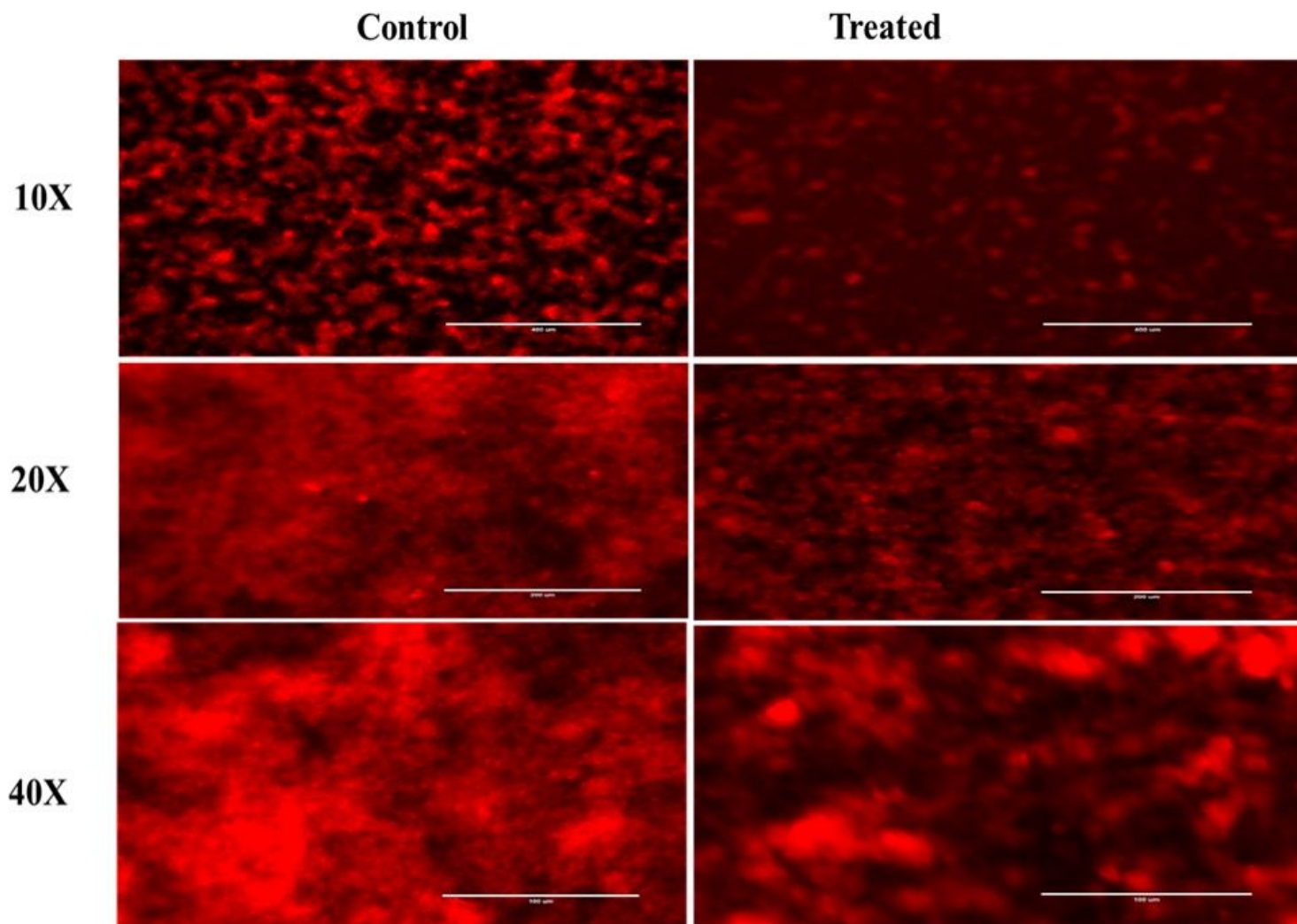
Figure 4

Fluorescence images of bioflim formation by S. mutans in presence and absence of dextransucrase antibody. The images were taken at 10X, 20X and 40X using Evos FL Auto fluorescent microscope and scale bar represents 400μm, 200μm, 100μm in length. The experiment was carried in triplicates.
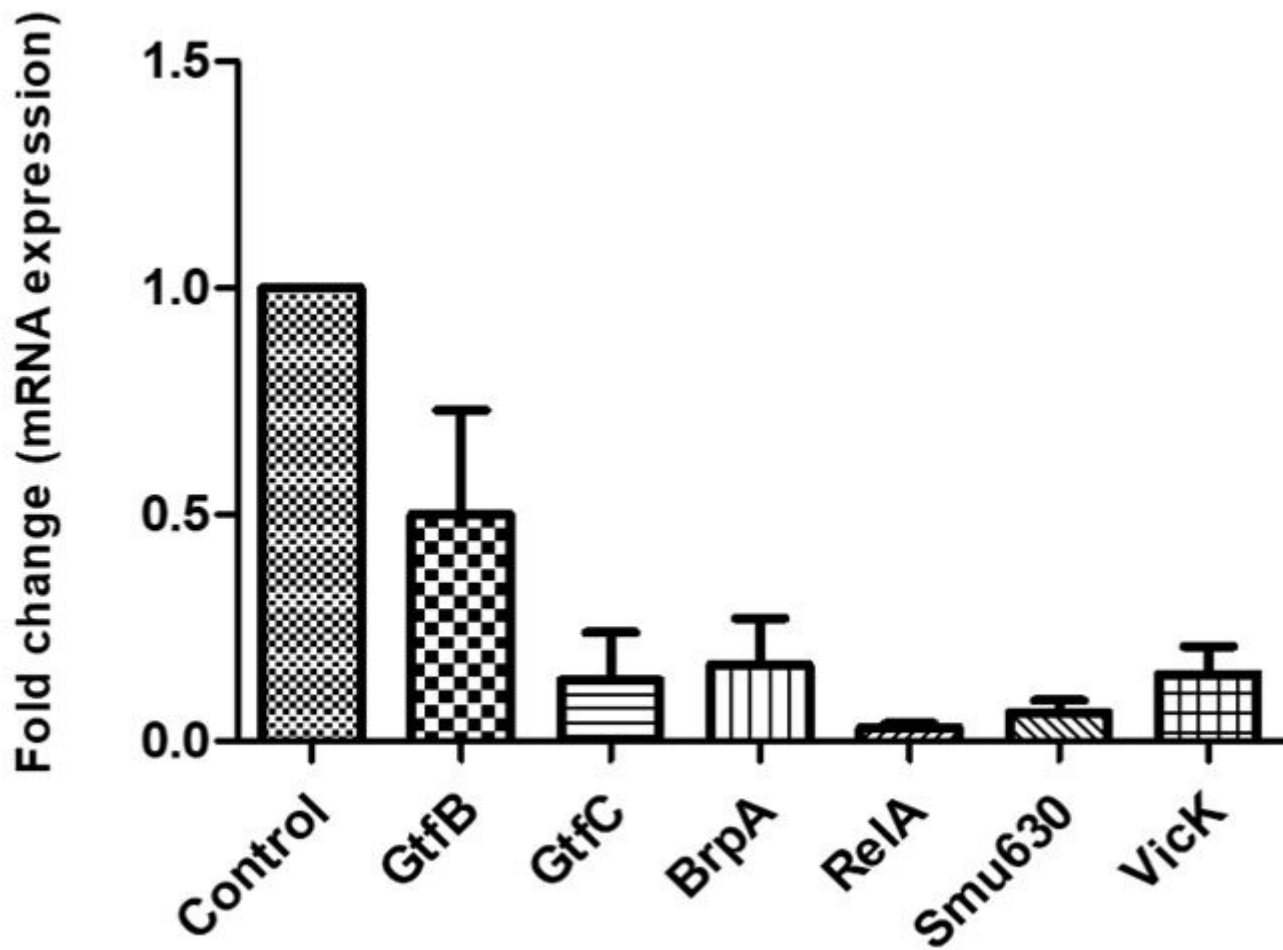
Figure 5

Gene expression profile of some biofilm forming genes of S. mutans: Quantitative Real Time -PCR was performed in triplicates. 16sRNA was used for the normalization of each gene. Values were compared with control taking arbitrary value of 1 to measure the fold change in the expression of genes.Values are mean ± SD of three independent experiments.
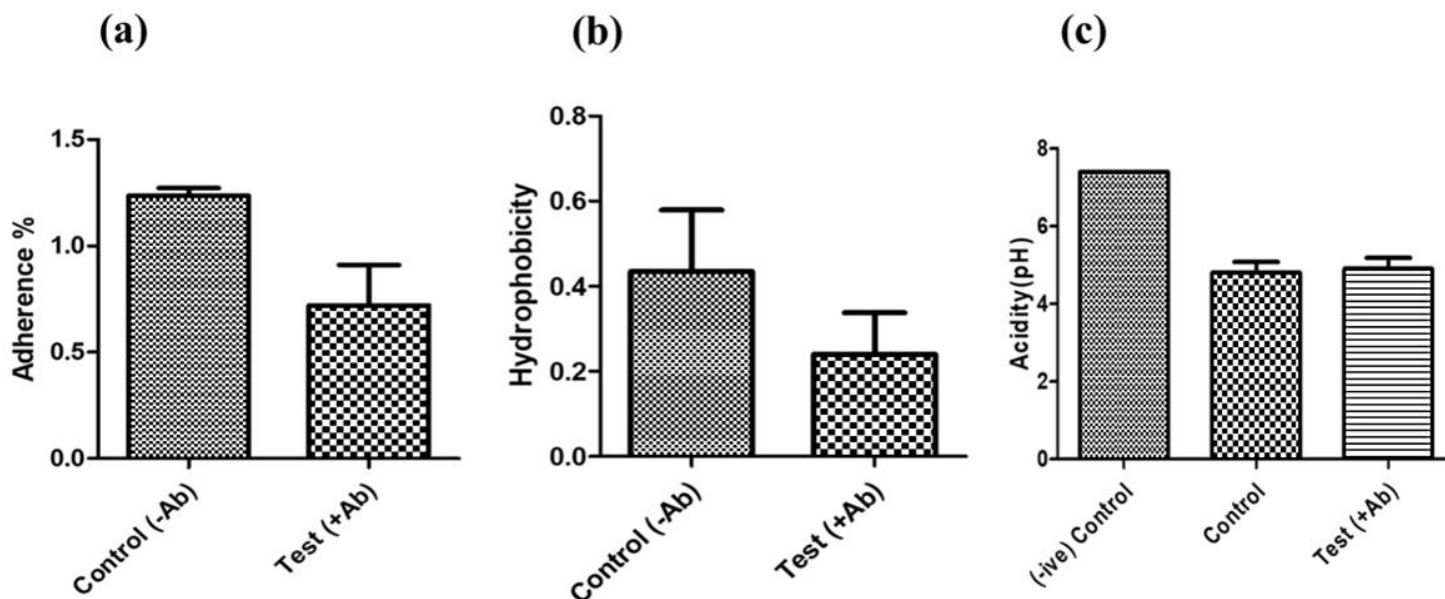
Figure 6

(a). Adherence of S. mutans to the glass surfaces: Effect of dextransucrase antibody on the adherence of S. mutans cells to the glass surface studied in glass tubes in BHI medium supplemented with 5% sucrose for 20 hours at 37°C. Control = without addition of antibody Test = with the addition of antibody. Results are expressed as the means ± SD n= 4. p <0.036 (b). Effect of antidextransuccrase antibody on the cell surface hydrophobicity of S.mutans. A decrease in the cell surface hydrophobicity of S. mutans cells was studied in presence (Test) and absence (Control) of dextransucrase antibody. Values are average of three assays. Data are mean ±SD n=3 p<0.34 (c). Effect of antidextransuccrase antibody on the acid production by S. mutans: Acid production by S. mutans was carried out by measuring the pH of the culture at 0h and 24h of growth at 37°C. No effect was observed on the acid production by S.mutans in presence of dextransucrase antibody. (- ive) Control = Media only, (+ ive) Control = Culture without antibody and Test = Culture with antibody. Data are mean ±SD n = 4.
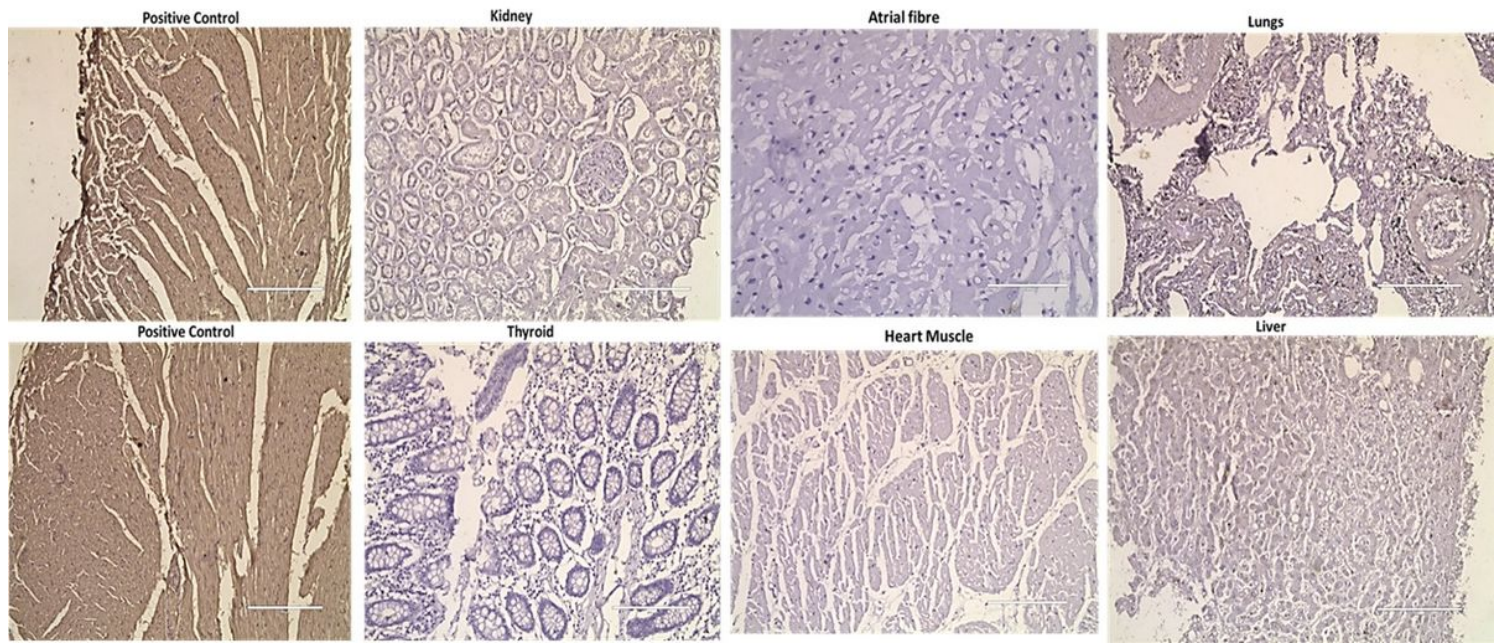
Figure 7

Immunohistochemistry of human tissues viz kidney, thyroid, atrial fire, heart muscle, liver and lung. Primary Antibody was polyclonal dextransucrase antibody (1:50) and Secondary Antibody was Goat anti rabbit IgG-HRP (1:250). Magnifications X=200µm.

Supplementary Files

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