*Oral Microbiology Immunology 2004: 19: 188–195*
*Printed in Denmark. All rights reserved*



Oral Microbiology and Immunology

# Salivary IgA reactions to cell-surface antigens of oral streptococci

**M. L. Lundin Wallengren, K. Hamberg, D. Ericson**
Department of Cariology, Faculty of Odontology, Malmö University, Sweden

*Lundin Wallengren ML, Hamberg K, Ericson D. Salivary IgA reactions to cell-surface antigens of oral streptococci.*
*Oral Microbiol Immunol 2004: 19: 188–195.* © Blackwell Munksgaard, 2004.

**Background:** In the immunoblot technique, using whole bacteria cell extracts as antigens, both intra- and extracellular antigens are detected, which gives a large number of immunoglobulin A (IgA) reactions (immunoblot bands) when incubated with saliva. It is important to distinguish which immunoblot bands represent bacterial cell-surface antigens, since these antigens could be involved in adhesion mechanisms and be available for blocking *in vivo*.
**Methods:** Bacterial extracts of *Streptococcus mutans, Streptococcus sobrinus, Streptococcus parasanguis* and the streptococcal antigen I/II were separated using SDS–PAGE. The antigens were detected with saliva in Western blot. Untreated saliva and saliva in which cell-surface reactive IgA had been absorbed with whole bacteria cells were analyzed.
**Results:** Approximately half the number of the bands were absent for saliva absorbed with homologous cells, compared to untreated saliva. The absorption pattern was almost identical for *S. mutans* and *S. sobrinus* but not for *S. parasanguis*. Salivary IgA reactive against streptococcal antigen I/II was absorbed by *S. mutans* cells, to a lesser extent by *S. sobrinus* cells, and not at all by *S. parasanguis* cells.
**Conclusion:** It is likely that the bands that were absent after absorption represented cell-surface antigens. For *S. mutans* and *S. sobrinus*, these bands were probably the streptococcal antigen I/II.

Key words: cell-surface antigen; oral streptococci; salivary IgA; Western blot

Marie Louise Lundin Wallengren, Department of Cariology, Faculty of Odontology, Malmö University, Centre for Oral Health Sciences, SE-205 06 Malmö, Sweden
Tel.: +46 406658541;
e-mail: Marie-Louise.Wallengren@od.mah.se
Accepted for publication January 15, 2004

Mutans streptococci are a group of related oral bacteria that is regarded as an important etiologic agent for dental caries in humans (19, 21). Although mutans streptococci are found in almost all individuals, there are large variations in levels of colonization (38). Not only environmental factors (6, 9, 18) but also immune and non-immune host factors may play an important role in the acquisition of mutans streptococci (5, 11, 26, 32, 36, 37). The immune response against mutans streptococci has been studied extensively, especially in the search for a caries vaccine (13, 34). A specific immune defense against mutans streptococci is thought to depend upon salivary antibodies (2). The major salivary immunoglobulin (Ig) is secretory IgA (S-IgA), which is generated by the common mucosal immune system (25). These antibodies may control the colonization by mutans streptococci by reducing the initial adherence of the bacteria to saliva-coated tooth surfaces (14) as well as neutralizing extracellular enzymes (35), though their role in the acquisition and regulation of the normal oral microbiota is still controversial (24). Widerström and coworkers (41, 43) reported that the salivary IgA antibodies react with a number of proteins from mutans streptococci and also that the reaction profile is unique for each individual, though there is a certain familial pattern.

In an earlier study by our group, the salivary IgA reactions to extracts of strains of oral streptococci were investigated in individuals with different human leukocyte antigen (HLA) profiles (22). Individuals expressing a certain HLA class II allele, *DRB1*04*, revealed fewer and less intense immunoblot bands to antigen extracts of mutans streptococci, compared to individuals with other HLA profiles. However, the results of the immunoblot analyses were extensive: 12–15 immunoblot bands were detected for each bacterial extract. Immune response patterns revealed by Western blotting are often complex and their interpretation is complicated by limited knowledge of the identity of detected

antigens (4, 36). The preparations of bacterial antigens for this type of assay include many steps and it is likely that most preparations contain both antigens exposed on the bacterial surface *in vivo* and antigens not available for reactions in the oral cavity.

The aim of this study was to determine which immunoblot bands may represent cell-surface antigens, since these antigens could mediate attachment of the bacteria to the tooth surface and be available for reactions with salivary IgA *in vivo*. It was of particular interest to identify the streptococcal antigen (SA) I/II. This antigen has attracted a great deal of attention (10, 15, 23) as it interacts with salivary glycoprotein in the pellicle to promote adhesion of *Streptococcus mutans* to the tooth surface (13) and has been tested as a possible target in the search for an effective caries vaccine (25, 30, 34).

## Material and methods

### Subjects

Eight apparently healthy non-smoking subjects, 40–49 years old, five females and three males, agreed to participate in the study. All subjects were low-caries active without any open cavities. The Ethical Committee at Lund University, Sweden, approved the study.

### Saliva

Approximately 30 ml of whole paraffin-stimulated saliva was collected from each subject, and the collecting time measured. To achieve the approximately 30 ml saliva necessary, saliva had to be collected on two, or even three (subjects E and G), different occasions. All the saliva samples, however, were collected in the morning and in early autumn. For 1 h prior to the collection, the subjects refrained from eating, drinking and smoking. The saliva samples were stored at –20°C until analyzed. Before use, the samples were thawed, and then clarified by centrifugation at 1520 ***g*** for 10 min at 4°C. The total amount of salivary IgA was measured by the modified method (3) for immunobead enzyme-linked immunosorbent assay of Sack and co-workers (33). The protein concentration of the saliva samples was determined using Bio-Rad Laboratories protein assay (Richmond, CA).

### Bacteria

Salivary levels of mutans streptococci were estimated by the Strip mutans® test (Orion Diagnostica, Espoo, Finland) according to the manufacturer's instructions (17). The test strips were then stored at –20°C. For sampling and cultivation of bacteria from each subject, two typical colony-forming units of mutans streptococci were picked from each strip with a sterile toothpick, added to 1 ml of Todd-Hewitt broth each and grown overnight at 37°C. The two broths were then transferred to one bottle with 100 ml Todd-Hewitt broth and grown overnight in an atmosphere of 95% $N_2$ and 5% $CO_2$. The broth was divided into 10 sterile tubes, centrifuged at 1520 ***g*** for 10 min at 4°C and the supernatant discarded. The pellets were washed repeatedly in phosphate-buffered saline, pH 7.2, and then collected into one tube with 5 ml phosphate-buffered saline. The optical density at 650 nm was measured at a dilution of 1 : 10 and set to ~ 5.0 using phosphate-buffered saline. The bacteria samples were stored at –20°C in 110-μl portions before use.

The colony morphology of the fresh isolates was verified by spreading 50 μl of the broth on mitis salivarius bacitracin agar plates and incubated at 37°C in 95% $N_2$ and 5% $CO_2$ for 48 h. The fresh isolates were also identified by polymerase chain reaction using a simplified method for identification of *S. mutans* and *Streptococcus sobrinus* developed by Carlsson and Hamberg at our laboratory (28). Primers for amplification of sequences on the dextranase genes, designed by Igarashi and coworkers (16) for *S. mutans* and synthesized by BMC, Lund University, Sweden, and for *S. sobrinus* (GenBank accession no. M96978) were used. The sample preparation was from a Todd-Hewitt broth culture, optical density = 1.0, added to commercially available polymerase chain reaction-beads (Pharmacia Biotech Ready-To-Go 27-9553). It was confirmed that all the selected fresh isolates were *S. mutans*.

In addition, reference strains of *S. mutans* serotype c (strain KPSK2, Carlsson 1967), *S. sobrinus* serotype g (strain OMZ65, Guggenheim 1968) and *Streptococcus parasanguis* (strain NT62, Carlsson 1968), as well as fresh isolated *S. mutans* strains from two other different individuals, were cultivated as described above.

### Absorption

The 110-μl portions of each bacteria-sample were thawed and carefully mixed with 1.5 ml of diluted (1 : 2) saliva. The saliva-bacteria samples were then placed on an Orbital shaker at room temperature for 30 min. The samples were then centrifuged at 1520 ***g*** for 10 min at 4°C and the supernatant frozen at –20°C until analyzed.

### SDS–PAGE and Western blot analysis

SDS–PAGE and Western blot assays were performed in accordance with the methods described previously (22, 41). Briefly, *S. mutans* serotype c (strain KPSK2), *S. sobrinus* serotype g (strain OMZ65) and *S. parasanguis* (strain NT62) and SA I/II were used. The SA I/II (also designated as SpaP, PAc, protein B, P1) from *S. mutans* was kindly provided by Professor Lehner and co-workers at Guy's and St. Thomas's Hospital, London, UK (31). Antigen extract from whole cells was prepared as described by Renneberg and coworkers (29) and as modified by Widerström and coworkers (43). Briefly, the cells were washed in phosphate-buffered saline, suspended in HEPES in Tris and mechanically broken by sonication three times for 5 min at 80 W in an ice-chilled cup and centrifuged for 15 min at 5000 ***g***. The protein concentration of the supernatant was determined by Bio-Rad Laboratories protein assay. The antigen extracts were frozen at –20°C until analyzed.

Prior to the electrophoretic analysis the antigens were thawed and adjusted to the same protein concentration (1 mg/ml) by dilution in 2% SDS sample buffer (0.06 M Tris-HCl at pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.0012% bromphenol blue) and boiled for 5 min. As molecular weight markers, Bio-Rad Laboratories high molecular weight markers were used. The electrophoretic separation of the antigens was carried out in a 7.5% SDS–PAGE for 55 min at 190 V (20). The proteins were then transblotted to Immobilon membrane (hydrophobic polyvinylidene difluoride membrane by Millipore Intertech, Bedford, MA) (39).

Western blot analysis (39) was performed to identify salivary IgA antibodies to *S. mutans, S. sobrinus* and *S. parasanguis* and SA I/II. Briefly, the membranes were placed in an incubator and rinsed repeatedly with Tris buffered saline (TBS), pH 7.4, and non-specific binding sites were blocked with 5% non-fat milk in TBS. The membranes were then repeatedly washed in 0.1% non-fat milk in TBS and incubated overnight. Lane 1, the standard, was incubated with 0.1% non-fat milk in TBS, lane 2 with mouse anti SA I/II monoclonal antibody 4-10A (IgG) and lanes 3–9 with 1.5 ml diluted (1 : 2) saliva per well according to the outline below. The mouse anti SA I/II monoclonal

*Table 1.* Strip mutans score, saliva secretion rate, total IgA and protein, percent IgA of total protein and saliva sampling occasion used for each Western blot

| Subjects and sampling occasions | Strip mutans class[a] | Saliva secretion rate ml/min[b] | Total IgA mg/100 ml saliva[c] | Total protein mg/100 ml saliva[d] | % IgA/protein[e] | Western blot |
|---|---|---|---|---|---|---|
| A 1 | 3 | 0.82 | 3.92 | 44.67 | 8.77 | KPSK2 OMZ65 SA I/II |
| 2 | | 1.28 | 4.79 | 93.93 | 5.09 | Nt62 |
| B 1 | 0 | 1.54 | 2.29 | 65.58 | 3.50 | KPSK2 OMZ65 |
| 2 | | | 3.01 | 65.54 | 4.59 | SA I/II Nt62 |
| C 1 | 0 | 1.06 | 3.37 | 65.25 | 5.16 | KPSK2 OMZ65 SA I/II |
| 2 | | 1.50 | 3.48 | 54.45 | 6.38 | Nt62 |
| D 1 | 3 | 2.36 | 2.93 | 48.89 | 5.99 | KPSK2 OMZ65 SA I/II |
| 2 | | 3.00 | 2.91 | 58.35 | 4.30 | Nt62 |
| E 1 | 2 | 1.13 | 3.40 | 44.24 | 7.69 | OMZ65 |
| 2 | | 1.41 | 3.33 | 45.90 | 7.25 | SA I/II |
| 3 | | 1.61 | 4.04 | 60.33 | 6.70 | KPSK2 Nt62 |
| F 1 | 3 | 1.65 | 2.74 | 49.94 | 5.49 | KPSK2 OMZ65 SA I/II |
| 2 | | 1.40 | 2.36 | 50.13 | 4.70 | Nt62 |
| G 1 | 0 | 0.89 | 2.52 | 49.48 | 5.09 | KPSK2 OMZ65 |
| 2 | | 0.80 | 2.91 | 48.89 | 5.95 | SA I/II |
| 3 | | 0.68 | 3.57 | 44.01 | 8.11 | Nt62 |
| H 1 | 0 | 0.80 | 7.42 | 45.84 | 16.19 | KPSK2 OMZ65 SA I/II |
| 2 | | 0.93 | 5.89 | 36.23 | 16.25 | Nt62 |

[a]Strip mutans classes: 0–1 = low levels of mutans streptococci in saliva, corresponding to less than $10^5$ cfu/ml saliva using conventional methods; 2 = high levels of mutans streptococci in saliva corresponding to $10^5$–$10^6$ cfu/ml saliva using conventional methods; 3 = very high levels of mutans streptococci in saliva corresponding to $>10^6$ cfu/ml saliva using conventional methods.
[b]Stimulated saliva secretion, ranging from 0.68 to 3.00 ml/min.
[c]Total IgA mg/100 ml, ranging from 2.29 to 7.42.
[d]Total protein mg/100 ml saliva, ranging from 36.23 to 93.93.
[e]Percent IgA/protein, ranging from 3.50 to 16.25.

antibody, which was included as a reference and control in all blots, was kindly provided by Dr Bleiweis and colleagues (1).

The following day, the membranes were washed, as described above. Biotinylated avidin was added to lane 1, rabbit anti mouse IgG HRP (Dako Corporation, CA) to lane 2 and rabbit alpha-chain specific anti-human IgA HRP (Dako) to lanes 3–9, diluted 1 : 1000 in TBS containing 1% non-fat milk and 0.05% Tween 20. Two hours later the membranes were rewashed as before and stained for 15 min with peroxidase substrate (3-amino-9-ethylcarbazole in acetone; A 5754, Sigma Chemical Co., St. Louis, MO) in a buffer (50 mM $CH_3COONa$, pH 5.0) and 0.015% $H_2O_2$. Finally, the membranes were washed with distilled water. All incubations were performed at room temperature on an Orbital shaker.

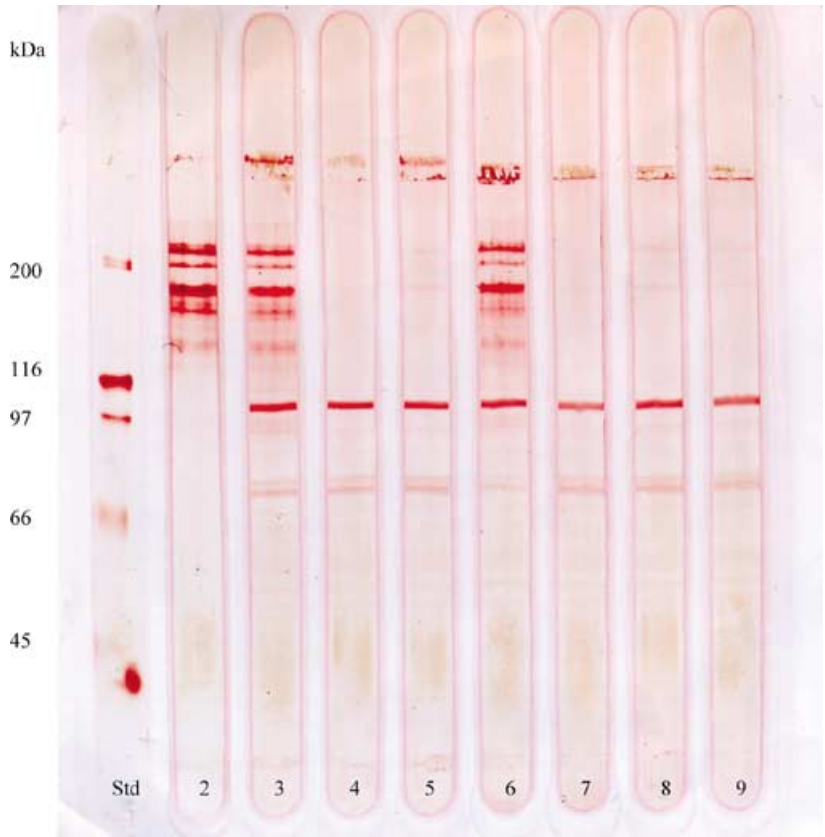


*Fig. 1.* Representative immunoblot analysis of IgA antibody activity in saliva from one subject (D) to antigen extract of *S. mutans*. Lane 1: standard. Lane 2: SA I/II monoclonal antibody shows five to six clear bands. Lane 3: untreated saliva shows bands of ~ 70–200 kDa. Lane 4: saliva absorbed with *S. mutans*. All bands >100 kDa are absent. Lane 5: saliva absorbed with *S. sobrinus*. Bands >100 kDa are almost absent. Lane 6: saliva absorbed with *S. parasanguis*. No absorption of IgA reactive to *S. mutans*. Lane 7: saliva absorbed with ''own'' *S. mutans*. Bands >100 kDa are absent. Lanes 8 and 9: saliva absorbed with ''foreign'' *S. mutans*. Bands >100 kDa are absent.

#### General assay design

Saliva samples from each of the eight subjects were absorbed in a number of assays with different bacteria: reference strains of *S. mutans, S. sobrinus, S. parasanguis*, fresh isolated *S. mutans* from the saliva donor (''own'' *S. mutans*), and fresh isolated *S. mutans* from two different subjects (''foreign'' *S. mutans*). Untreated saliva and the absorbed saliva were then analyzed in Western blots. Four separate immunoblot assays were performed for each subject, using four different antigen

extracts: *S. mutans, S. sobrinus, S. parasanguis* and SA I/II.

For each assay, the Immobilon membranes were incubated as follows:

- Lane 1: standard
- Lane 2: SA I/II monoclonal antibody as reference and control
- Lane 3: untreated saliva
- Lane 4: saliva absorbed with *S. mutans,* KPSK2
- Lane 5: saliva absorbed with *S. sobrinus,* OMZ65
- Lane 6: saliva absorbed with *S. parasanguis,* Nt62
- Lane 7: saliva absorbed with ''own'' *S. mutans*, fresh isolated
- Lane 8: saliva absorbed with ''foreign'' *S. mutans*, fresh isolated
- Lane 9: saliva absorbed with other ''foreign'' *S. mutans*, fresh isolated

#### Immunoblot analysis

As described previously (22), all immunoblot membranes were scanned into a computer program for analysis (The Discovery Series™, Quantity One® SW, PC, Bio-Rad). Briefly, all lanes and all bands in each lane were defined and comparisons between untreated saliva and saliva absorbed with different bacteria cells were made for each bacterial extract and for SA I/II.

## Results

Western blot analysis revealed characteristic immunoblot profiles for each bacterial extract as well as for SA I/II. Only minor differences in immunoblot profiles, i.e. in the number of bands and band intensity, were noted in the eight individuals, and the significant absorption patterns were almost identical for each subject. The individual differences (Table 1) in Strip mutans® scores, the salivary secretion rate, levels of total IgA and protein, and the percent IgA of total protein did not seem to influence the main results of the Western blots.

A significant observation noted for all bacterial extracts was that when the saliva had been absorbed with homologous cells, almost all bands with high molecular weight, >100 kDa, were absent.

The results of the immunoblot analysis for each bacterial extract, as well as for SA I/II, are described below for *all* individuals. In addition, printouts of representative immunoblots are presented from one subject, D (Figs 1–4) and in one intensity graph visualizing the absorption of IgA in assays with bacterial extract of *S. mutans* (Fig. 5).

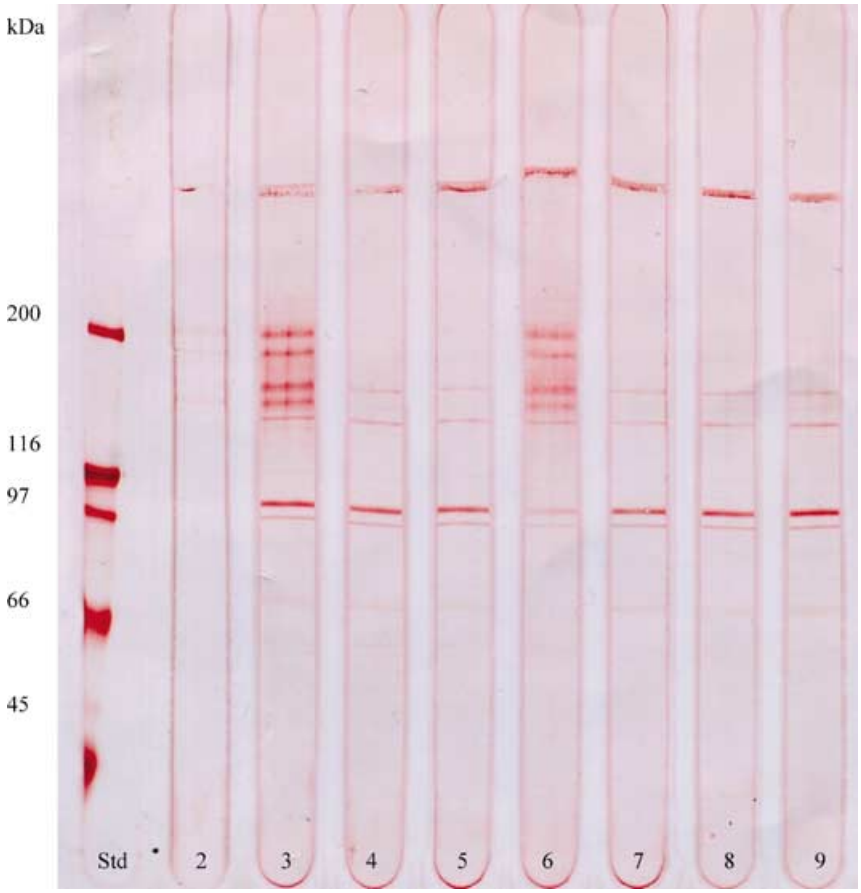


*Fig. 2.* Representative immunoblot analysis of IgA antibody activity in saliva from one subject (D) to antigen extract of *S. sobrinus.* Lane 1: standard. Lane 2: SA I/II monoclonal antibody shows very weak reactions. Lane 3: untreated saliva, bands of 100–200 kDa. Lane 4: saliva absorbed with *S. mutans*. Bands >100 kDa are absent, except two weak bands. Lane 5: saliva absorbed with *S. sobrinus*. Bands >100 kDa are absent, except two weak bands. Lane 6: saliva absorbed with *S. parasanguis*. Almost no absorption of IgA reactive to *S. sobrinus.* Lane 7: saliva absorbed with ''own'' *S. mutans*. Bands >100 kDa are absent, except two weak bands. Lanes 8 and 9: saliva absorbed with ''foreign'' *S. mutans*. Bands >100 kDa are absent, except two weak bands.

#### Immunoblot bands in assays using bacterial extract of *S. mutans*, KPSK2 (Figs 1 and 5)

Incubation with the monoclonal antibody to SA I/II (Fig. 1, lane 2) revealed five to six clear bands with a molecular weight of approximately 130–200 kDa. For untreated saliva (Fig. 1, lane 3) 12–15 clear bands were noted. When the saliva had been absorbed with homologous (here *S. mutans*) cells (Figs 1 and 5, lane 4), all bands with a molecular weight over 100 kDa, approximately five to six bands, were absent. When the saliva had been absorbed with *S. sobrinus* (Fig. 1, lane 5), the band pattern was almost identical to that as after absorption with *S. mutans* (Fig. 1, lane 4), though some very weak, high molecular bands could be seen. There were no changes in the immunoblot profile following absorption with *S. parasanguis* (Fig. 1, lane 6) compared with untreated saliva. When the saliva had been absorbed with ''own'' *S. mutans* (Fig. 1, lane 7) or ''foreign'' *S. mutans* (Fig. 1, lane 8 and 9), all high molecular bands were absent or very weak, i.e. the same profile was noted as when absorbed with homologous cells or *S. sobrinus*. All eight saliva samples revealed one very intense band at ~100 kDa that was not influenced by any absorption.

#### Immunoblot bands in assays using bacterial extract of *S. sobrinus*, OMZ65 (Fig. 2)

For the monoclonal antibody to SA I/II (lane 2), three to four very weak bands with a molecular weight of approximately 160–200 kDa were noted. For untreated saliva (lane 3) 8–10 bands were observed, most of them with a molecular weight over 100 kDa. Absorption with *S. mutans*

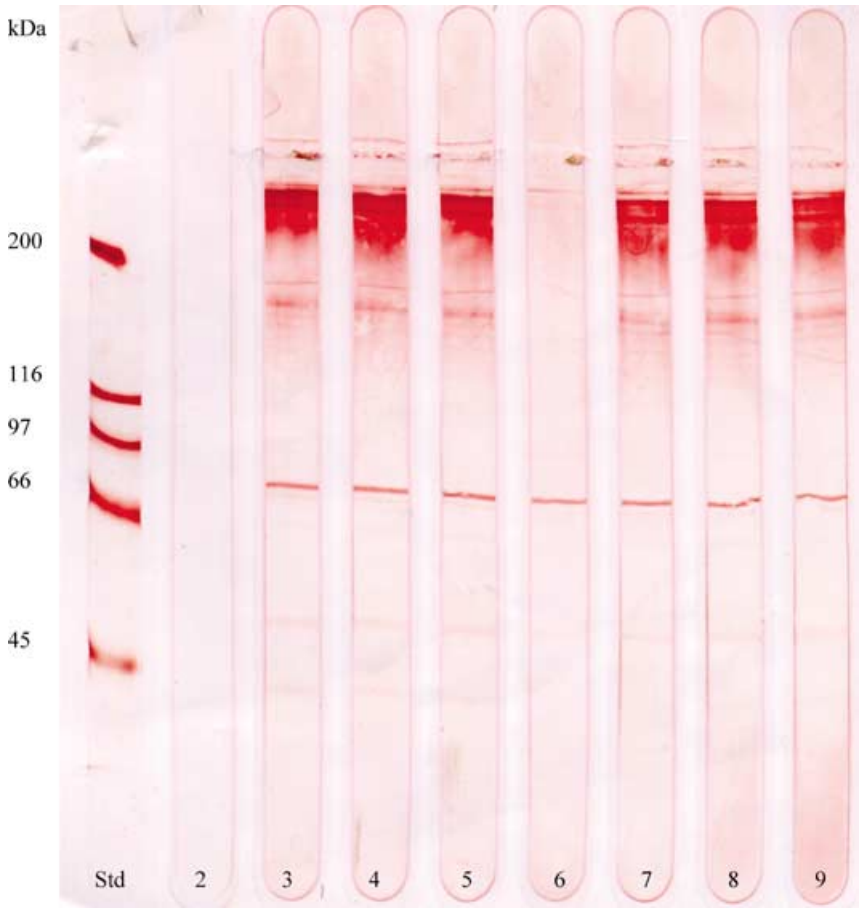


*Fig. 3.* Representative immunoblot analysis of IgA antibody activity in saliva from one subject (D) to antigen extract of *S. parasanguis.* Lane 1: standard. Lane 2: SA I/II monoclonal antibody shows no reactions at all. Lane 3: untreated saliva, all bands, except one at 70 kDa, are of very high molecular weight, 150–280 kDa. Lane 4: saliva absorbed with *S. mutans.* Lane 5: saliva absorbed with *S. sobrinus.* Lane 7: saliva absorbed with ''own'' *S. mutans*, lanes 8 and 9: saliva absorbed with ''foreign'' *S. mutans*. Lanes 4–5 and 7–9 show an identical profile to lane 3. Lane 6: saliva absorbed with *S. parasanguis*. One band at 70 kDa persists.

(lane 4) as well as homologous cells (lane 5) resulted in almost identical immunoblot profiles, i.e. two to four bands at 160–200 kDa were absent compared to the immunoblot profile of untreated saliva. Absorption with *S. parasanguis* (lane 6) gave almost no changes in immunoblot profile compared with untreated saliva. Saliva that had been absorbed with ''own'' *S. mutans* (lane 7) or ''foreign'' *S. mutans* (lane 8 and 9) showed the same immunoblot profile as when absorbed with *S. mutans* or homologous cells. Also for *S. sobrinus,* all eight saliva samples revealed one intense band at ~100 kDa that was not influenced by any absorption.

#### Immunoblot bands in assays using bacterial extract of *S. parasanguis*, Nt62 (Fig. 3)

This bacterial extract was included as a control. No reactions were noted for the monoclonal SA I/II antibody (lane 2). Approximately eight rather intense bands were observed for all saliva samples, untreated as well as absorbed (lanes 3–5, 7–9), except when the saliva had been absorbed with homologous bacteria (lane 6), in which case all bands but one were absent. The persisting band was one intense band of approximately 70 kDa.

#### Immunoblot bands in assays using purified SA I/II (Fig. 4)

When incubated with the SA I/II monoclonal antibody (lane 2), two to three intense bands with a molecular weight of 150–200 kDa were observed. In most immunoblots there was also one weaker band of approximately 100 kDa. The same immunoblot pattern was noted for untreated saliva (lane 3). When the saliva had been absorbed with *S. mutans* (lane 4) there was only one weak band at 200 kDa, as compared to absorption with *S. sobrinus* (lane 5), where one more intense band at 200 kDa could be detected. Absorption with *S. parasanguis* (lane 6) gave no changes in immunoblot profile compared to untreated saliva (lane 3). The 200 kDa band persisted for all individuals except for one (E), irrespective of any absorption.

## Discussion

Western blot assays are used worldwide to analyze immune responses to different bacteria (12). However, when bacterial extracts of whole cells are used, it is not clear whether detected antigens are internal or cell-surface antigens. It is of interest to find out which immunoblot bands represent cell-surface associated antigens, since they probably represent important adhesins, or other targets for salivary antibodies *in vivo*. Also when working with a larger number of samples, it is essential to focus on the most relevant antigen reactions.

In this study, when the immunoblot pattern of untreated saliva and of saliva that had been absorbed with whole bacterial cells was compared, it was obvious that specific, cell-surface reactive, salivary IgA had been absorbed by the whole cells (e.g. Fig. 1, lanes 4, 5, 7–9; and Fig. 5). It was of particular interest to identify the immunoblot bands that might represent SA I/II, since these antigens are considered important for adhesion and colonization (10, 15, 23). In assays using bacterial extract of *S. mutans, S. sobrinus* or purified SA I/II, it was likely that the bands of high molecular weight that were absent after absorption represented the SA I/II, as the absent bands were at the same positions as the bands noted for the monoclonal SA I/II antibody in the same blot (Figs 1, 2 and 4). It was also observed that almost all subjects revealed strong IgA reactions to antigens at these positions, which may indicate that they are important antigens.

In immunoblots using bacterial extract of *S. mutans,* the profiles for saliva absorbed with *S. mutans* and for saliva absorbed with *S. sobrinus* were almost identical, and the absent immunoblot bands were at the same positions as the bands observed for the SA I/II monoclonal antibody (Fig. 1, lanes 2, 4 and 5). This finding may support the view that SA I/II of *S. mutans,* PAc, and the SA I/II of *S. sobrinus,* PAg (30) may have both structural and antigenic similarities. However, two weak reactions were observed for saliva absorbed with *S. sobrinus* compared with saliva absorbed with *S. mutans* (illustrated in Fig. 5 and indicated by two

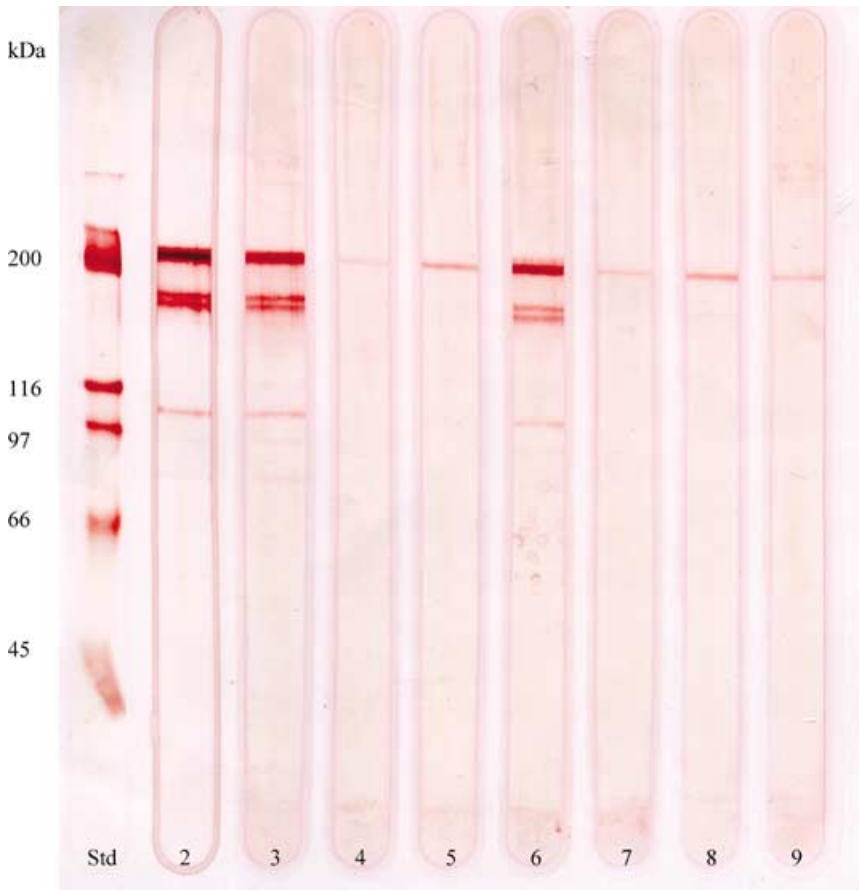


*Fig. 4.* Representative immunoblot analysis of IgA antibody activity in saliva from one subject (D) to SA I/II. Lane 1: standard. Lane 2: SA I/II monoclonal antibody, intense reactions of 150–200 kDa and one weaker band at 100 kDa. Lane 3: untreated saliva, the same profile as in lane 2. Lane 4: saliva absorbed with *S. mutans*. Only one band, though weak, persists, at 200 kDa. Lane 5: saliva absorbed with *S. sobrinus*. Lane 7: saliva absorbed with ''own'' *S. mutans*. Lanes 8 and 9: saliva absorbed with ''foreign'' *S. mutans*. Lanes 5 and 7–9 show one band at 200 kDa. Lane 6: saliva absorbed with *S. parasanguis*. This has the same profile as lanes 2 and 3.

arrows). Diverse immunoblot profiles were also noted for the monoclonal SA I/II antibody, in assays with bacterial extracts of *S. mutans,* serotype c*,* and *S. sobrinus,* serotype g (Figs 1 and 2, lane 2). A possible explanation for these differences could be that oral streptococci of serotype c, e and f possess both antigen I and II determinants, whereas serotype a, d, and g possess a determinant related to antigen I but not related to antigen II, as already been pointed out by Russell and coworkers in 1980 (31). When focusing on the monoclonal SA I/II antibody, in assays using bacterial extract of *S. mutans*, it was remarkable to note that instead of the expected two or three bands, as many as five or six bands were detected (Fig. 1, lane 2). These bands probably represent degradation products in the preparations of *S. mutans* that the monoclonal SA I/II reacted with.

Generally, it appears that the whole bacteria cells did not absorb any salivary IgA reactive to antigens with a molecular weight below 100 kDa. All bands that persisted after absorption were considered to likely represent reactions with intracellular antigens, which implies that our antigen preparations may merely contain intracellular fragments with molecular weights below 100 kDa. In contrast to our findings, other groups have reported important antigens in the low molecular weight regions. Chia and co-workers (7) demonstrated, in immunoblots using extracellular- as well as cell wall-associated preparations of *S. mutans*, two antigens of approximately 63 and 60 kDa as the predominant reactive antigens recognized by salivary IgA. Perrone and her group (27) have also observed a significant 66 kDa fimbrial protein of unknown identity in all their preparations. Antigens of these sizes were most likely also seen in the present assays, though they were not influenced by any absorption (Fig. 1, lanes 3–9). Different antigen preparation methods, dissimilar concentration of antigen antibody, as well as host genetic diversities probably contributed to the differences between those studies and our results. In a comparison of Asian and Swedish children and their salivary IgA immunoblot profiles to *S. mutans,* Bratthall and colleagues (4) suggested that the antibody reactions might be genetically influenced.

All saliva in immunoblots with bacterial extract of *S. mutans* or *S. sobrinus* revealed one or two intense bands at $\sim$ 100 kDa that persisted irrespective of any absorption. It is not known which protein might correspond to this strong band, though it was seen frequently in our preparations and is therefore of interest for future studies.

*S. parasanguis*, Nt62, a non-mutans streptococcus, was included in this study as a reference. In the immunoblot assays using antigens separated from *S. parasanguis* (Fig. 3), the immunoblot profile of untreated saliva was identical to the profiles of saliva that had been absorbed with *S. mutans* or *S. sobrinus*. However, in saliva absored with homologous bacteria, immunoblot bands of high molecular weight were absent. This implies that no antigenic similarities between *S. parasanguis* and *S. mutans* or *S. sobrinus* were observed in this study. In contrast, Cole and coworkers (8) found that salivary IgA antibodies were directed to antigens shared by all four species (*Streptococcus mitis, Streptococcus oralis, S. mutans* and *Enterococcus faecalis*) investigated in their study. These discrepancies may be due to different ratios of bacteria density and protein concentrations; too high a bacteria density might result in non-specific reactions.

Potential bacterial – IgA – proteases did not influence the results of this study, as might be expected in assays like these (14, 40). This was confirmed by the fact that the immunoblot profiles of saliva absorbed with *S. mutans* and reacted with *S. parasanguis,* and *vice versa*, did not vary from the profiles of untreated saliva. Furthermore, these observations indicate that non-specific absorption of salivary IgA was not seen in our assays.

One may argue that the use of saliva collected at different occasions could have influenced the results of this investigation. However, comparisons were made only within separate immunoblot assays where saliva collected at the same occasion was used. Moreover, Widerström and colleagues (42) have demonstrated that, in such a short period of time, variations in specific IgA reactivity should not be expected, even though the total IgA varied.

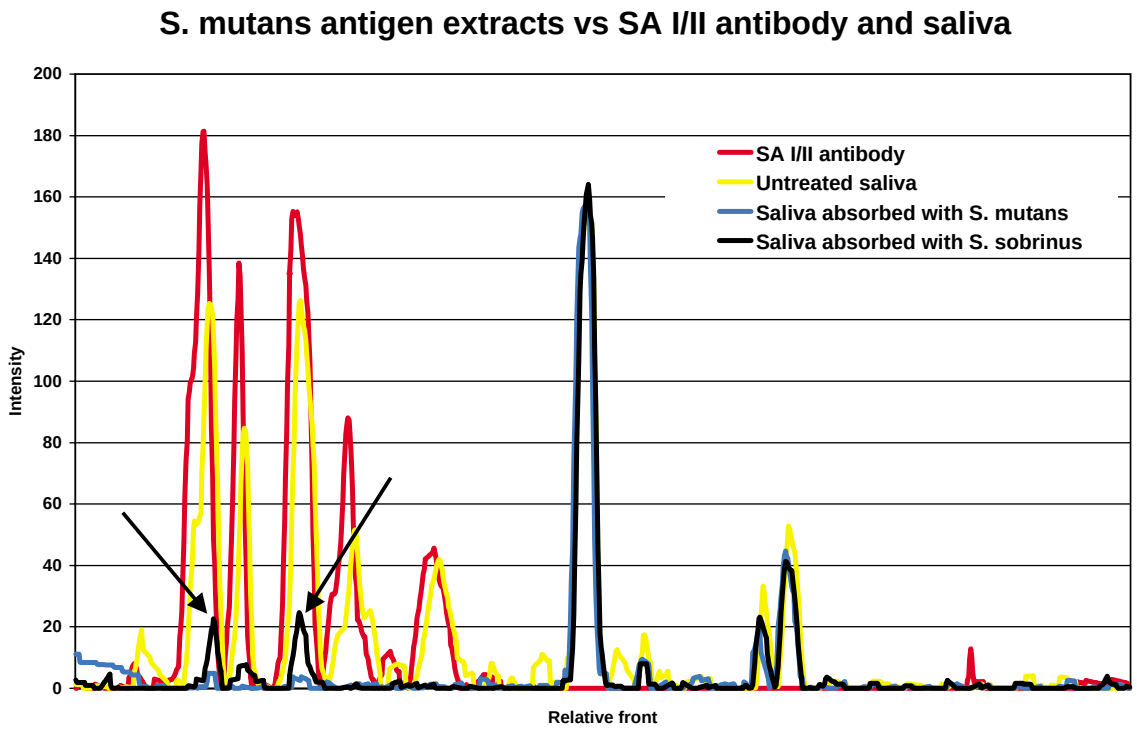


*Fig. 5.* Graph of assay with *S. mutans* antigen extract, illustrating the antibody activity of the SA I/II monoclonal antibody, untreated saliva, saliva absorbed with *S. mutans*, and saliva absorbed with *S. sobrinus*. For SA I/II and untreated saliva, intense reactions with high peaks were observed. Saliva absorbed with *S. mutans* showed barely detectable IgA activity, i.e. no peaks, at the positions where SA I/II antibody and untreated saliva showed intense activity. Saliva absorbed with *S. sobrinus* also showed only weak salivary IgA reactions at the same positions, though two minor peaks were noted, indicating more IgA activity than saliva absorbed with *S. mutans* (see arrows).

To conclude, the results from this study seem to highlight the significance of five to six high molecular weight bands, which narrows down the search for relevant surface antigens. This finding will enable future studies involving large populations to be conducted using the immunoblot technique with whole bacteria cells as antigens, since one could focus on these bands. It is also likely that these bands represent important cell-surface antigens that may provide protection for colonization.

## References


1. Ayakawa GY, Boushell LW, Crowley PJ, Erdos GW, McArthur WP, Bleiweis AS. Isolation and characterization of monoclonal antibodies specific for antigen P1, a major surface protein of mutans streptococci. Infect Immun 1987: **55**: 2759–2767.
2. Brandtzaeg P. Salivary immunoglobulins. In: Tenovuo J, ed. Human saliva: clinical chemistry and microbiology, **Vol. II**. Boca Raton, FL: CRC Press, 1989: 1–54.
3. Bratthall D, Ellen RP. Determination of immunoglobulin A in saliva by immunobead enzyme-linked immunosorbent assay: comparison with single radial immunodiffusion. J Clin Microbiol 1982: **16**: 766–769.
4. Bratthall D, Serinirach R, Hamberg K, Widerström L. Immunoglobulin A reaction to oral streptococci in saliva of subjects with different combinations of caries and levels of mutans streptococci. Oral Microbiol Immunol 1997: **12**: 212–218.
5. Carlén A, Bratt P, Olsson J, Strömberg N. Agglutinin and acidic proline-rich protein receptor patterns may modulate bacterial adherence and colonization on tooth surfaces. J Dent Res 1998: **77**: 81–90.
6. Carlsson P, Struzycka I, Wierzbicka M, Iwanicka-Frankowska E, Bratthall D. Effect of a preventive program on dental caries and mutans streptococci in Polish schoolchildren. Community Dent Oral Epidemiol 1988: **16**: 253–257.
7. Chia JS, Chang WC, Yang CS, Chen JY. Salivary and serum antibody response to *Streptococcus mutans* antigens in human. Oral Microbiol Immunol 2000: **15**: 131–138.
8. Cole MF, Bryan S, Evans MK, et al. Humoral immunity to commensal oral bacteria in human infants: salivary secretory immunoglobulin A antibodies reactive with *Streptococcus mitis* biovar 1, *Streptococcus oralis, Streptococcus mutans*, and *Enterococcus faecalis* during the first two years of life. Infect Immun 1999: **67**: 1878–1886.
9. Dasanayake AP, Roseman JM, Caufield PW, Butts JT. Distribution and determinants of mutans streptococci among African-American children and association with selected variables. Pediatr Dent 1995: **17**: 192–198.
10. van Dolleweerd CJ, Chargelegue D, Ma JK. Characterization of the conformational epitope of Guy's 13, a monoclonal antibody that prevents *Streptococcus mutans* colonization in humans. Infect Immun 2003: **71**: 754–765.
11. Douglas CW. Bacterial-protein interactions in the oral cavity. Adv Dent Res 1994: **8**: 254–262.
12. Egger D, Bienz K. Protein (western) blotting. Mol Biotechnol 1994: **1**: 289–305.
13. Hajishengallis G, Michalek SM. Current status of a mucosal vaccine against dental caries. Oral Microbiol Immunol 1999: **14**: 1–20.
14. Hajishengallis G, Nikolova E, Russell MW. Inhibition of *Streptococcus mutans* adherence to saliva-coated hydroxyapatite by human secretory immunoglobulin A (S-IgA) antibodies to cell surface protein antigen I/II: reversal by IgA1 protease cleavage. Infect Immun 1992: **60**: 5057–5064.
15. Hajishengallis G, Koga T, Russell MW. Affinity and specificity of the interactions between *Streptococcus mutans* antigen I/II and salivary components. J Dent Res 1994: **73**: 1493–1503.
16. Igarashi T, Yamamoto A, Goto N. Direct detection of *Streptococcus mutans* in human dental plaque by polymerase chain reaction. Oral Microbiol Immunol 1996: **11**: 294–298.
17. Jensen B, Bratthall D. A new method for the estimation of mutans streptococci in human saliva. J Dent Res 1989: **68**: 468–471.

18. Köhler B, Bratthall D, Krasse B. Preventive measures in mothers influence the establishment of the bacterium *Streptococcus mutans* in their infants. Arch Oral Biol 1983: **28**: 225–231.
19. Krasse B. From the art of filling teeth to the science of dental caries prevention: a personal review. J Public Health Dent 1996: **56**: 271–277.
20. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970: **227**: 680–684.
21. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986: **50**: 353–380.
22. Lundin Wallengren ML, Ericson D, Hamberg K, Johnson U. HLA-DR4 and salivary IgA reactions to oral streptococci. Oral Microbiol Immunol 2001: **16**: 45–53.
23. Ma JK, Smith R, Lehner T. Use of monoclonal antibodies in local passive immunization to prevent colonization of human teeth by *Streptococcus mutans*. Infect Immun 1987: **55**: 1274–1278.
24. Marcotte H, Lavoie MC. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol Mol Biol Rev 1998: **62**: 71–109.
25. Michalek SM, Katz J, Childers NK. A vaccine against dental caries: an overview. BioDrugs 2001: **15**: 501–508.
26. Nyvad B, Kilian M. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. Caries Res 1990: **24**: 267–272.
27. Perrone M, Gfell L, Fontana M, Gregory R. Antigenic characterization of fimbria preparations from *Streptococcus mutans* isolates from caries-free and caries-susceptible subjects. Clin Diagn Lab Immunol 1997: **4**: 291–296.
28. Redmo Emanuelsson IM, Carlsson P, Hamberg K, Bratthall D. Tracing genotypes of mutans streptococci on tooth sites by random amplified polymorphic DNA (RAPD) analysis. Oral Microbiol Immunol 2003: **18**: 24–29.
29. Renneberg J, Persson K, Christensen P. Western blot analysis of the antibody response in patients with *Listeria monocytogenes* meningitis and septicemia. Eur J Clin Microbiol Infect Dis 1990: **9**: 659–663.
30. Russell MW. Immunization against dental caries. Oral Maxillofacial Surg Infect 1992: **2**: 72–80.
31. Russell MW, Bergmeier LA, Zanders ED, Lehner T. Protein antigens of *Streptococcus mutans*: purification and properties of a double antigen and its protease-resistant component. Infect Immun 1980: **28**: 486–493.
32. Russell MW, Hajishengallis G, Childers NK, Michalek SM. Secretory immunity in defence against cariogenic mutans streptococci. Caries Res 1999: **33**: 4–15.
33. Sack DA, Neogi P, Alam K. Immunobead enzyme-linked immunosorbent assay for quantitating immunoglobulin A in human secretions and serum. Infect Immun 1980: **29**: 281–283.
34. Smith DJ. Dental caries vaccines: prospects and concerns. Crit Rev Oral Biol Med 2002: **13**: 335–349.
35. Smith DJ, Taubman MA, Ebersole JL. Salivary IgA antibody to glycosyltransferase in man. Clin Exp Immunol 1985: **61**: 416–424.
36. Smith DJ, King WF, Akita H, Taubman MA. Association of salivary immunoglobulin A antibody and initial mutans streptococcal infection. Oral Microbiol Immunol 1998: **13**: 278–285.
37. Stenudd C, Nordlund Å, Ryberg M, Johansson I, Källestål C, Strömberg N. The association of bacterial adhesion with dental caries. J Dent Res 2001: **80**: 2005–2010.
38. Togelius J, Bratthall D. Frequency of the bacterium *Streptococcus mutans* in the saliva of selected human populations. Arch Oral Biol 1982: **27**: 113–116.
39. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979: **76**: 4350–4354.
40. Tyler BM, Cole MF. Effect of IgA1 protease on the ability of secretory IgA1 antibodies to inhibit the adherence of *Streptococcus mutans*. Microbiol Immunol 1998: **42**: 503–508.
41. Widerström L, Bratthall D, Hamberg K. Immunoglobulin A antibody activity to mutans streptococci in parotid, submandibular and whole saliva. Oral Microbiol Immunol 1992: **7**: 326–331.
42. Widerström L, Bratthall D, Hamberg K. Immunoglobulin A antibodies to mutans streptococci in human saliva and serum comparing fresh and subcultivated strains and activity in repeated saliva samples. Oral Microbiol Immunol 1994: **9**: 278–283.
43. Widerström L, Hamberg K, Bratthall D. Intrafamilial similarity in immunoblot profiles of salivary immunoglobulin A antibody activity to oral streptococci. Oral Microbiol Immunol 1995: **10**: 26–34.