Resemblance of salivary protein profiles between children with early childhood caries and caries-free controls


Although prolonged bottle feeding with a carbohydrate-rich content is commonly agreed to be the main etiologic factor for early childhood caries (ECC), in recent years additional endogenous factors, including the composition of saliva, have been suspected as predisposing factors in children for the development of this aggressive form of dental caries. As a basis for investigating the putative involvement of salivary proteins in the etiology of ECC, a qualitative comparison of major salivary protein profiles between children with ECC and caries-free controls was performed. Saliva was collected from 30 children with ECC and, after separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, was compared with saliva from 20 caries-free controls for the general composition of proteins by means of silver staining, glycoprotein staining, and lectin blotting. Gels and blots were analysed using densitometry, and the protein-banding patterns resulting from the individuals’ samples were compared by image analysis for the presence or absence of protein bands. Dendrograms obtained after comparison of all samples showed a high degree of similarity for the experimental groups. In summary, the results attest a uniform expression of the major protein components in children’s saliva, regardless of the clinical manifestation of ECC, and thus pave the way for further detailed investigations of more subtle differences in the salivary proteome.

With the decline of caries prevalence in many countries, it became apparent that a small percentage of children still remain at high risk of acquiring a rampant form of dental caries that has been termed ‘early childhood caries’ (ECC) (1, 2). Although ECC is commonly assumed to be caused by prolonged and night-time bottle feeding, which provides a constant source of carbohydrate in dental plaque, not all children who use a bottle containing sugary solutions necessarily develop this rampant form of caries (3). As a consequence, the contribution of the composition of the oral microbial flora in ECC has been investigated in great detail (4), but the possible involvement of endogenous host-associated attributes in this disease process has so far received relatively little attention.

A potential role of saliva in the pathogenesis of ECC has only quite recently begun to be explored (5–9). Saliva has been extensively probed in the past as a putative indicator of risk for dental caries in humans (10, 11), and the importance of salivary constituents in early childhood is well documented (12–16). Proteins in saliva, in addition to their protective functions, exert important roles through their various interactions with oral bacteria, which include antibacterial activities, bacterial agglutination, and clearance, as well as by providing receptors for bacterial adhesion and subsequent colonization (17). Salivary adhesion receptors for oral bacteria include the group of proline-rich proteins (15, 16, 18) as well as a set of mostly larger glycosylated proteins that are recognized by corresponding lectin-like oral bacterial adhesins (16, 18) and that are detectable by probing with purified lectins of defined specificities (18, 19). Thus, as a basis for investigating a putative involvement of salivary proteins in the etiology of caries, a comprehensive comparison of the major salivary protein patterns, after separation by gel electrophoresis, was performed and compared using qualitative image analysis among children with ECC and caries-free controls.

Material and methods

The study was approved by the Ethics Committee of the Medical Faculty of the University of Regensburg (# 97/96), and parents had provided informed, written consent for the
voluntary participation of their children. The experimental group in this study comprised 30 age- and gender-matched children who had been diagnosed with ECC (the ECC group) according to previously published criteria (3, 20). The experimental group was further subdivided into a group of 22 children, for whom prolonged and night-time bottle feeding with a carbohydrate-rich content was permitted (ECC+ group), and a group of eight children with ECC where no inappropriate bottle usage had taken place according to the feedback from parents’ interviews (ECC− group). Twenty children who were free of carious lesions served as a control group (CF). A detailed caries status was recorded from all 50 children. Additional information from both children and parents, including general health status, previous antibiotic therapy, dietary habits, domestic oral hygiene, and fluoride prophylaxis of the children, were documented.

Saliva
Whole saliva was collected by expectoration into a sterile polypropylene vial at the time-point of caries examination. In earlier studies, it was shown that the relative qualitative composition of salivary proteins remains essentially stable, regardless of day or time-point of sample collection (19, 21, 22). For children who were not able to expectorate into a vial, saliva was collected by manual suction with the use of sterile disposable polypropylene Pasteur pipettes (Plastibrand, Brand, Wertheim, Germany) (19). No intake of food had taken place for at least 2 h before the collection of the saliva sample. Samples were stored on ice and further processed not later than 30 min after collection.

Electrophoresis and staining of proteins and glycans
The total protein concentrations in salivary samples were determined with the use of the bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL, USA). Salivary proteins were denatured under reducing conditions, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (0.75 µg per lane) on 4–20% gradient gels (Invitrogen, Karlsruhe, Germany), and visualized by means of an ammoniacal silver stain kit (SilverXpress; Invitrogen) or transferred to nitrocellulose (Invitrogen), containing 6 µg of total salivary protein per lane. Glycosylated proteins were stained on nitrocellulose transfers according to a modification of the hydrazide method (21). Glycans were further detected by blotting with biotinylated lectins from Canavalia ensiformis [concanavalin A (ConA)], Canavalia ensiformis [concanavalin A (ConA)], Arachis hypogaea [peanut agglutinin (PNA)], and Lotus tetragonolobus agglutinin (LTA) (EY Laboratories, San Mateo, CA, USA) or by overlay with biotin-tagged bacteria, including Streptococcus gordonii DL1, S. gordonii M5, and Streptococcus oralis 34 followed by development with avidin-D-alkaline phosphatase, as previously described (18).

Image analysis and statistical evaluation
Gels and blots were scanned using a desktop scanner (Sharp Color Image Scanner, Model JX-330; Amersham Biosciences, Freiburg, Germany) fitted with a film scanning unit (Sharp Film Scanner Unit Model JX-3F6; Amersham Biosciences) and using the software LABSCAN UTILITY v. 2.0 (Amersham Biosciences). The resulting TIFF files were analyzed using the IMAGE MASTER 1D software 2.0, as previously described (21). For the analysis of similarity between the individual patients’ banding patterns, lanes were scored for the presence or absence of bands. The comparison of patterns was performed using the IMAGE MASTER 1D database software, v. 4.0 (Amersham Biosciences). Similarity coefficients were calculated by the software according to the method of Pearson. Dendrograms were drawn on the basis of the unweighted pair group method using the arithmetic average (UPGMA). Reference samples from one healthy adult individual, not belonging to the study population, were run on both sides of each gel. Descriptive statistical methods were used for age and gender distribution of experimental groups. For each group, caries data were summarized for each tooth of the dentition by calculating the proportion of caries-affected teeth (in per cent) based on the total number of teeth.

Results
Patient and clinical data
The age distribution among experimental groups was similar, with age medians ranging from 3.5 to 4.0 yr. In the ECC group, all incisors of the upper jaw were affected by caries, whereas the incisors of the lower jaw remained mostly caries-free (Fig. 1). This was most evident in the ECC+ group (Fig. 1B), whereas the ECC− group showed a more equal distribution of caries in the upper dentition including also the posterior teeth, but no involvement of the lower incisors (Fig. 1A). Standardized interviews with the parents of each child did not
reveal any other factors correlating with the clinical groups in addition to the ones already known, including dietary habits and socio-cultural background. No correlation to prolonged breastfeeding was apparent, even in the ECC− group (data not shown).

**Protein profiles in saliva**

To obtain a comprehensive overview of protein components in the saliva of children from the above-indicated clinical groups, a series of different gel and blot stains was performed. Occasional difficulties in obtaining the samples as a result of patient anxiety or low compliance in the clinical setting meant that it was not possible to standardize salivary flow or amount of saliva collected. Nevertheless, the concentrations of total protein in whole saliva were similar in all experimental groups, with medians between 1.0 and 1.1 mg/ml. The resulting banding patterns of salivary proteins from all children, after SDS-PAGE and silver staining, are depicted in Fig. 2A. The similarities between the individual children's salivary protein profiles were graphically depicted in a dendrogram (Fig. 2B), showing an overall correlation coefficient of 0.93. On this high level of correlation, no clustering could be delineated that effectively separated the experimental groups ECC+ from ECC−, or ECC from CF. At such a high similarity level, relative influences not caused by differences in the intrinsic banding pattern of the samples may play a greater role.

A high similarity of profiles was also found when transfers of gels containing all samples were stained for general glycosylation of proteins using the hydrazide method, or by lectin blotting for the presence of specific glycan motifs that could serve as potential bacterial-recognition sites with probes for mannose (ConA), α-fucose (LTA), or terminal Galβ-1-3GalNAc (PNA) (18) (Fig. 3). These lectins also stained some of the high-molecular-weight glycoproteins, including MUC5B, MUC7, or the salivary agglutinin gp-340, which were otherwise visible only poorly or not at all after silver staining as a result of to their extensive glycosylation. Bacterial overlays performed in addition, with strains of oral streptococci exhibiting well-characterized adhesion-binding properties (18), did not reveal any further
differences in the general profiles of salivary bacterial receptors (data not shown). All of these additional analyses resulted in similarly high correlation coefficients, ranging from 0.93 to 0.98 (data not shown).

**Discussion**

A particularly aggressive form of dental caries, namely ECC, was exploited as a model to search for differences in the composition of salivary proteins that might identify any components that could serve as putative indicators of caries risk in children. Following visualization of the banding pattern of salivary proteins after separation by SDS-PAGE, as well as by probing for putative bacterial glycoprotein receptor molecules by lectin blotting or bacterial overlay, a high degree of similarity among individuals became apparent.

The clinical situation of the dentition within the ECC group was in good accordance with previous reports (2, 20), in that most prominently the anterior teeth of the upper jaw were affected by caries, whereas the lower incisors remained mostly unaffected. These findings support the importance of saliva as a crucial protective factor in this disease process, in that a blockade of salivary flow around the upper incisors during bottle feeding (23) promotes the initiation of this disease, whereas the lower incisors, constantly being flushed by submandibular-sublingual saliva, remain unaffected. Although in some reports prolonged breastfeeding during sleep was also suggested to be involved in the pathogenesis of ECC (24), no occurrence of such breastfeeding habits within the ECC group became apparent (data not shown).

The lack of unifying general patterns in the overall salivary protein profiles that significantly distinguished the clinical groups could lie in the fact that the salivary samples were taken from individuals where ECC was already clinically manifest, and not before or immediately at the onset of disease, emphasizing the future need of longitudinal intrafamilial (25) or twin-comparison studies. It is further possible that potentially existing differences in the saliva surrounding the upper incisors (23), the teeth most at risk in ECC, could have been missed. It is also possible that SDS-PAGE analysis may not be sensitive enough to detect putative subtle differences among some less-abundant proteins. Nevertheless, the initial analysis by simple one-dimensional gel electrophoresis is still valuable, because in two-dimensional gels, despite their higher resolving power, some of the high-molecular-weight components of saliva, as well as strongly basic and acidic protein species, cannot adequately be resolved (22). Based on the present results, it is now indicated to test specifically for differential expression of relevant salivary proteins in relation to ECC by employing advanced proteomics techniques (22, 26, 27). Additional complexity can be foreseen because the protective effect of saliva probably involves redundancy of function provided by interactions between many proteins. In summary, although a role of salivary proteins in the etiology of ECC has not yet been proven, the present comprehensive survey of major salivary protein profiles provides a first necessary basis for future advances in this field that may eventually help to identify diagnostic markers beneficial in the early recognition of children at risk for this devastating form of caries.

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**References**


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**Fig. 3.** Glycoprotein and bacterial counter-receptor profiles in whole saliva of three representative children. Whole saliva from patient #6 [early childhood caries-positive (ECC+)], patient #17 [early childhood caries-negative (ECC−)], and patient #29 [caries free (CF)] were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a blotting membrane, and stained for general glycosylation using the hydrazide method or, more specifically, by blotting with lectins from *Canavalia ensiformis* [concanavalin A (ConA)], *Lotus tetragonolobus* agglutinin (LTA), or *Arachis hypogaea* [peanut agglutinin (PNA)]. The sizes of molecular-weight standards (M.W.) are indicated to the left in kilodaltons (kDa).


