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BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF *STREPTOCOCCUS CRISTA* STRAINS ISOLATED FROM HUMAN DENTAL BIOFILM BY MEANS OF ARBITRARY PRIMERS – PCR (AP-PCR)

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ABSTRACT

Streptococcus ssp are important components of the dental biofilm and *Streptococcus crista* is considered to be an interesting model of bacterial interactions taking place in this biofilm. In the present work, *S. crista* strains were isolated from the dental biofilm of Brazilian individuals and studied with respect to their biological characteristics and their molecular profile by means of AP-PCR techniques, using the RR2, 434, OPR2, OPR8, and OPR13 primers. Results allowed us to build a similarity dendrogram. Analysis of the similarity dendrogram allowed the separation of the studied strains into similarity groups. All isolates presented fibril tufts by Transmission Electron Microscopy (TEM). These isolates were able to bind to salivary amylase and to adhere to mouth epithelial cells. Some strains displaying fibril tufts and positive adherence were not able to co-aggregate with *Fusobacterium nucleatum*, suggesting that different adhesin groups are present in these strains.

Key words: biological characterization, molecular characterization, dendrogram analysis, biofilm

INTRODUCTION

The human oral cavity is considered to be the ecological niche with the widest biodiversity known to date. Approximately 700 species responsible for the formation of a biofilm on the dental surface can be found there (29). It is believed that the physical nature of the dental biofilm should facilitate the exchange of signals among the bacterial cells present in the oral cavity (45). The dental plaque is a microbial biofilm formed by organisms tightly bound to each other and to the solid substratum by means of an exopolymer matrix, in which the microorganisms are embedded. It is known that such microorganisms are involved in the etiology of the most common diseases of the oral cavity (36). Also, the presence of salivary proteins and bacterial cellular debris should help the adhesion of these bacteria to the enamel (33).

Among the bacterial species present in the dental biofilm, the *Streptococcus mitis* group (17,32) is considered to be important for its formation. *Streptococcus crista* is a subspecies belonging to the *S. mitis* group (10,13,15), and its biochemical characterization is considered to be difficult because of the phenotypic variation exhibited by individuals of the same species (30,31). *S. crista* are a Gram positive cocci that cannot produce catalase; hydrolyze arginine; or decompose manitol, sorbitol, inulin, and raffinose. They grow in sculine bile agar, can degrade threalose and lactose, bind to salivary amylase, and present a hemolysis (3,9,43,44).

Salivary amylase is able to specifically bind to several species of oral streptococci. The amylase-binding capacity of these bacteria is used to differentiate between groups of oral streptococci (9,19,37). *Streptococcus mitis, Streptococcus gordonii, Streptococcus salivarious, Streptococcus anginous,*

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and *S. crista* are bacterial species that are able to bind to this amylase (9,19,37,38). Moreover, the binding-amylase capacity is related to the oral bacterial colonization ability and biofilm formation (4,16,39).

Bacterial adherence to surfaces is critical to their survival in the human oral cavity and has been associated with the formation of the human oral biofilm (4), being Streptococci the major components of this film (16). Bacterial cells express multiple cell-surface adhesins that are responsible for their ability to adhere to various other proteins, including salivary and serous proteins, as well as to epithelial cells and other bacterial cells (16).

On the surface of the human dental biofilm it is possible to find corncob structures that correspond to the specific formation of bacterial co-aggregation consisting of a central rod-shaped or filamentous bacterium surrounded by several other bound streptococci. These co-aggregates are highly ordered structures and usually consist of the gram positive pair *Corynebacterium matruchotii- S. crista* or the gram negative-gram positive pair *Fusobacterium nucleatum- S. crista*, with *C. matruchotii* and *F. nucleatum* being responsible for the central core. The binding capacity between *S. crista* and the other bacterial cells is due to a polar fibril tuft present on the surface of *S. crista* (6,7,13,22,23,28).

Molecular characterization methods have been proposed in order to overcome the phenotypic variation displayed by *S. crista* (30,31). In 1999, Rudney and Larson (32) proposed the use of a technique known as arbitrary primers-PCR (AP-PCR), with primers designated RR2 and 434, as a simple, rapid, and low-cost technique to characterize this bacterium.

The purpose of this work was to study *S. crista* isolates obtained from the dental biofilm of individuals from the state of São Paulo, Brazil, with regard to their biological characteristics and molecular profiles, by means of the AP-PCR technique, as described by Rudney and Larson (32), and by using the OPR2, OPR8, and OPR13 primers, as described by Williams *et al.* (43). Our aim was to differentiate isolates from standard strains and from strains described by other groups.

MATERIALS AND METHODS

Studied strains

Bacterial growth was obtained from the mouth biofilm of 12 individuals (patients of the Dental Clinic of the São Francisco University, Bragança Paulista, SP), by using the method described by Lancy *et al.* (22), Correia *et al.* (6), and Mouton *et al.* (28). Strains of *S. crista* (CR3, CR311, and CC5AWT) (13) and *Streptococcus parasanguis* (PSH1a and PSH1b) (obtained from the Microbiology Dept. of the Dental School of the Pennsylvania State University, USA); *Streptococcus sanguis*, *S. mitis*, *Streptococccus oralis*, and *S. gordonii* (Microbiology Dept., ICB, University of São Paulo, Brazil); *Streptococcus suis* 2 and *Streptococcus suis* 527 (Veterinary Medicine School of Saint-Hyacinthe of Montreal University, Canada); and *F. nucleatum* (ATCC 10953) were used as controls.

Biochemical identification of the isolates

Biochemical tests for the mouth-isolated bacteria were accomplished as described by Beighton *et al.* (2,3), from cultures grown in rich liquid medium (Brain Heart Infusion - Difco). Acid production from mannitol, sorbitol, inulin, raffinose, lactose, and threalose; aesculin and arginine hydrolysis; as well as H_2O_2 production were determined as described by Beighton *et al*. (2,3). Carbohydrate fermentation reactions were determined in basic medium cosisting of Purple Broth Base (Difco) (24g) and thioglycollate medium in the absence of dextrose or an indicator (Difco) (12g), with carbohydrates added to a concentration of 0.5% before autoclaving.

The amylase salivary test was performed as described by Douglas *et al.* (9), in order to eliminate those isolates that were not able to bind to this enzyme and thus could not be *S. crista*. Bacteria grown in the brain heart infusion broth (Difco) (20 mL) overnight at 37ºC was harvested by centrifugation, washed once in 50mM phosphate buffer (pH6.5), and suspended in whole unstimulated saliva (50 µL) previously colleted on ice and cleared by centrifugation at 4ºC for 10 min. at 20,000 X g. The suspension was incubated at room temperature for 30 min., and the bacteria was sedimented by centrifugation at 10,000 X g for 5 min. at 4ºC. Samples (10 µL) of the supernatant were assayed for amylase activity by diffusion from a 3.5 mm – diameter well punched in a starch agarose gel. The gel consisted of 1% agarose and 1% soluble starch (Merck) in 50 mM tris-hydrochloride buffer (pH7.5). The gel was poured on a glass plate to a thickness of approximately 2 mm. After incubation for 3 to 18h at 37ºC in a humid chamber, starch hydrolysis was visualized by staining the gel with lugol iodine. Wells showing no enzymatic activity or significantly reduced enzymatic activity were scored as positive, while wells showing a zone of starch hydrolysis identical to that seen with a control saliva sample were scored as negative.

Positive colonies were further confirmed by using the Vitek (bioMerieux) kit and the BBL Crystal (Becton Dickinson) kit.

Genomic DNA extraction

Bacterial genomic DNA was extracted as described by Ausubel *et al.* (1) and quantified as described by Sambrook *et al.* (34).

AP-PCR and RAPD assay

The AP-PCR assay was acomplished as described by Menard and Mouton (27), with the modifications proposed by Rudney and Larson (32), using the RR2 (5´-AAG AGA GGAG CTA GCT CTT CTT GGA-3´) and 434 (5´-GCA CAA CAG TTC CCT GAC TTG CAC-3') primers. Each tube contained sterile

mili-Q H_2O (50 µL); 10 X "PCR Buffer" (5 µL) (Gibco-BRL); 10 mM "dNTP mixture" (1 µL) (Gibco-BRL); 50 mM $MgCl_2$ (1.5 µL) (Gibco-BRL); DNA (1 µL); Taq-DNA polymerase (5U) (0.25 µL) (Gibco-BRL), and 11 µg/mL RR2 primer or 8.25 µg/mL 434 primer. Tubes were cycled through denaturation at 94ºC, annealing at 30ºC, and extension at 72ºC. Each step lasted 5 min. in the case of the first 4 cycles. Denaturation and annealing were shortened to 1 min., and extension to 2 min. for 35 more cycles, ending with a final extension step of 5 min.

The RAPD assay was accomplished as described by Williams *et al.* (43), with the OPR2 (5'CACAGCTGCC3'), OPR8 (5'CCCGTTGCCT3'), and OPR13 (5'GGACGACAAG3') primers. Each tube contained sterile mili-Q H_2O (50 µL); 10 X "PCR Buffer" (5 µL) (Gibco-BRL); 10 mM "dNTP mixture" (1 µL) (Gibco-BRL); 50 mM $MgCl_2$ (1.5 µL) (Gibco-BRL); DNA (1 µL); Taq-DNA polymerase (5U) (0.25 µL) (Gibco-BRL) and one of the primers (1 µL). The tubes containing either OPR 2 or 8 were cycled through denaturation at 94ºC, annealing at 33ºC, and extension at 72ºC. Each step lasted 1 min., for 45 cycles. The tubes with OPR 13 were cycled through denaturation at 94ºC, annealing at 30ºC, and extension at 72ºC. The two first steps lasted 1 min. and the last step lasted 2 min., for 45 cycles.

All primers were purchased from Operon Technologies and the reactions were run in an MJ Research Thermo Cycler.

AP-PCR fingerprinting analyses

The AP-PCR fingerprinting analysis of the amplified DNA fragments obtained after agarose gel electrophoresis was recorded. The presence of a given band was coded as 1 and the absence of a given band was coded as 0 in a data matrix, analyzed using the Program NTSYSpc, version 2.10m (Copyright c 1986-2000 Appied Biostatistics Inc.). Similarity dendrograms containing all the isolates were built.

Transmission Electron Microscopy (TEM) studies

TEM studies were carried out in order to visualize the fibril structures. These studies were performed as described by Handley *et al.* (13) in a 906 Carl Zeiss electron microscope. The strains were grown overnight at 37ºC in a brain heart infusion broth. The cells were harvested and washed three times in a saline 3% – sucrose 1% solution, and then negatively stained in uranyl acetate 2%, according to Handley *et al.* (13).

Adhesion to *in vitro* cultivated HEp-2 and KB cells and to oral epithelial cells

Adhesion to *in vitro* cultivated HEp-2 (human larynx epithelial cells) (ATCC HB-8065) and KB (derived from human oral epithelial carcinoma) (ATCC CCL-17) cells was accomplished as described by Scaletsky *et al.* (35). Adhesion to oral epithelial cells was carried out as described by St. Geme and Cutter (41). The *in vitro* adhesion model was performed to investigate the interaction of *S. crista* with KB, HEp-2, and epithelial cells, which, to the best of our knowledge, has not been reported in previous studies. Results concerning adhesion to all cells were observed under light microscope with magnification of 1,000X. The strains EPEC 2348/69 and *Escherichia coli* K12 HB101 were used as control strains for the adhesion studies with HEp-2 cells, and *E. coli* K12 HB101 and *Porphyromonas gingivalis* ATCC33277 were used as control in the case of KB cells.

Co-aggregation tests

The co-aggregation tests were performed as described by Cisar *et al.* (5), with the modifications suggested by Handley *et al.* (12), to determine the aggregation model exhibited by the studied bacteria. *F. nucleatum* ATCC 10953 was anaerobically grown in brain heart infusion broth (Difco) at 37ºC, for 5 days. *S. crista* strains were grown on a complex medium containing 0.5% tryptone, 0.5% yeast extract, and 0.05% Tween 80, buffered to pH7.5 with K_2HPO_4. The cultures were harvested by triple washing in co-aggregation buffer (0.25M potassium phosphate pH8.0 supplied with 0.025M sodium chloride and 0.02% sodium azide). The washed suspensions were adjusted to an optical density of 1.5 at 660 nm. Aliquots (0.2 mL) of the *S. cristas* and the *F. nucleatum* suspensions were mixed on a rotamixer, left at room temperature for 1h, mixed again, and flicked gently with the finger a few times before being scored for co-aggregation. Control tubes were set up with co-aggregation buffer (0.2 mL). The tubes were allowed to stand at room temperature overnight, mixed the next day for at least 10s, and scored again. Scores for the degree of co-aggregation ranged from "zero" to "four +" rating as follows: 0 – no visible aggregates in suspension; 1+ - small uniform co-aggregates in suspension; 2+ - definite co-aggregates easily seen but suspension remains turbid without immediate settling of co-aggregates; 3+ - large co-aggregates formed with some settling, although the majority remained in suspension; 4+ - large co-aggregates which settle very rapidly leaving a clear supernatant.

RESULTS

In this work, eight *S. crista* strains (C1 to C8) were isolated from the dental biofilm of twelve women aged between 20 and 40, and identified by means of the Vitek (bioMerieus) and the BBL Crystal (Becton Dickison) identification kits.

All the isolates presented fibril tufts on the polar extremities of the cells when analyzed by TEM (Fig. 1-a). They also adhered to mouth epithelial cells but not to HEp-2 or KB cells cultivated *in vitro* (Fig. 1-b). Strains C1 to C8 were able to bind to salivary amylase as do the standard strains. Co-aggregation test results are shown in Table 1.

The AP- PCR assay, using the RR2 and 434 primers (Fig.3 and 4), and the RAPD assays, using the OPR primers, were able to amplify several DNA fragments shown in Table 2.

Table 1. Co-aggregation level exhibited by the control strains PSH1a, PSH1b, CR3, CR311 and the isolated strains.

Strain	PSH1a[a]	PSH1b[a]	CR3[b]	CR311[b]	CC5AWT[b]	C1[c]	C2[c]	C3[c]	C4[c]	C5[c]	C6[c]	C7[c]	C8[c]
Co-aggregation Level	0	0	3	3	2	0	0	0	0	0	3	3	0

([a]*S. parasangui* strain types, [b]*S. crista* strain types, [c]Clinical isolate).

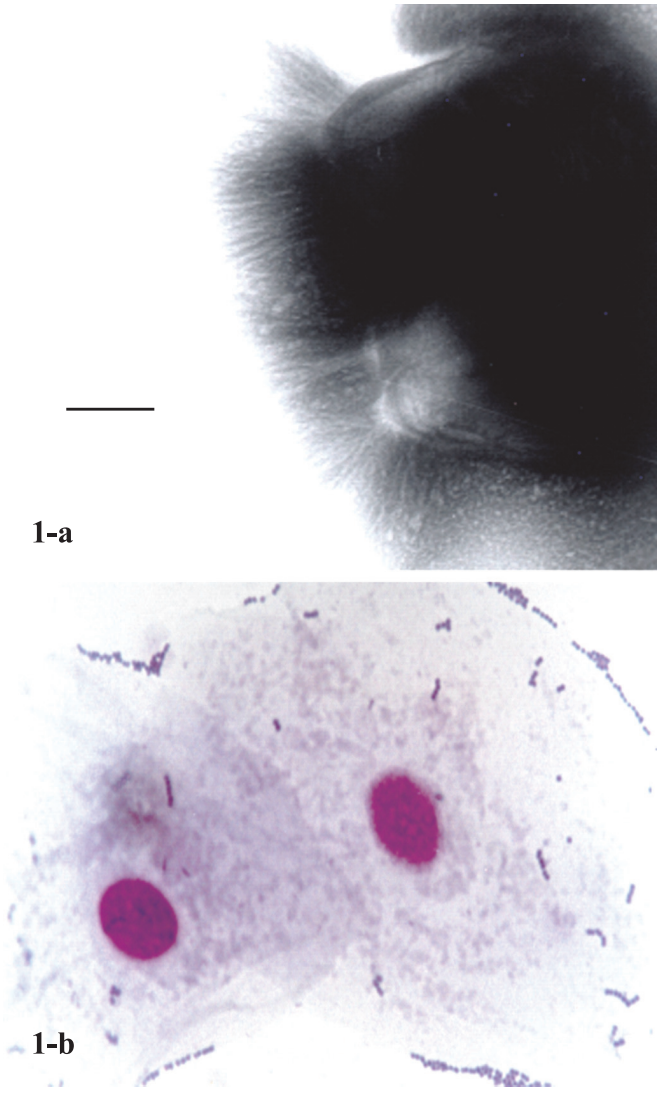

Figure 1. 1-a – C8 strain negatively stained, showing lateral tufts of fibrils on one side of each cell. The cells are in division process. Bar = 92 nm. 1-b – Adherent strain C1 in scraped oral cells. Magnification: 1000X.

Analysis of the above data allowed us to build different similarity dendrograms. The similarity dendrogram obtained by using data from the RR2 and 434 primers (Fig.2-a) indicated 100% similarity between strains CR311 and C1 (fingerprinting 1); C3, C4, and C5 (fingerprinting 2); C7 and C8 (fingerprinting 3). Strain CR3 had 98% similarity with fingerprinting 1 and strain C2 displayed 97% similarity with fingerprinting 2. Concerning these three major fingerprintings, there was 94% similarity between fingerprintings 1 and 2; 39% among fingerprintings 3 and 1 and 2. Strain C6 had closer similarity (56%) with fingerprintings 1 and 2 than with fingerprinting 3 (39%). By using these primers, all strains exhibited only 22% similarity with the standard strain CC5AWT.

The dendrogram obtained by using the OPRs and RR2 and 434 primers together (Fig.2-b) showed a similar pattern, but with a different strain distribution. In this way, strains CR311 and C1 were identical, and strains CR3, C4, C5, and C2 had a high degree of similarity. The same was observed for strains C7 and C8. Strain C3, which was considered to have a high degree of similarity (100%) with strains C4 and C5 and a high degree of similarity with strain C2 when the RR2 and 434 primers were used (Fig.2-a), was then considered to have a smaller degree of similarity (56%) (Fig.2-b). All strains had only 24% similarity with the control CC5AWT.

DISCUSSION

The purpose of this work was to biologically characterize different *S. crista* strains obtained from the dental biofilm of different adult individuals seen at the Dental Clinic of São Francisco University, Bragança Paulista, SP, Brazil by means of molecular techniques, and to compare the results with those published by other groups (3,6,7-9,13,18,19,32,44). To the best of our knowledge, no other research group has published in Brazil, any characterization of these types of strains regarding their molecular profile.

Salivary amylase assays were carried out to differentiate *S. crista* from other *Streptococcus* groups, once the amylase-binding capacity is specific to some oral *Stretpcocci* (9). The results obtained by other authors (9,13,44) and us showed that all the *S. crista* strains have the ability to bind to salivary amylase. This enzyme is one of the major components of the acquired pellicle, and the amylase-binding streptococci are the predominant amylase–binding bacteria (ABB) in the human biofilm (reviewed by 14,38). Besides helping in the identification, these data could suggest that the ability to bind to amylase may play an important role in the oral colonization by streptococci.

Table 2. Fragment size (bp) of the amplicons obtained by using the OPR2, OPR8, OPR13, RR2, and 434 primers

Primer/ strain	OPR2	OPR8	OPR13	RR2	434
S. suis 2 [a]	2238	1757; 1121; 670; 364	1867; 563	ND	ND
S. suis 527 [a]	1141; 825	3820; 1757; 1343; 1114; 670; 364	563	ND	ND
S. sanguis [a]	1480; 1415; 1296; 602; 556	2076; 1907; 1688; 1121; 975; 717; 508	2499; 843; 677	822; 340	539
S. mitis [a]	1701; 894	1989; 1688; 1343; 1121; 1010; 605; 526; 508	1503; 1228; 563	ND	ND
S. oralis [a]	1480; 825	2076	2948; 1329; 449	ND	ND
S. gordonii [a]		1561; 1502; 1343; 424	3542; 2499; 1954; 1635; 399	ND	ND
PSH1a [a]	1701	2269; 822; 717; 337	1137; 1053; 907	ND	ND
PSH1b [a]	1701	2269; 822; 717; 337	449	ND	ND
CR3 [b]	2446; 1701; 652	975; 822; 717	2499; 1635; 1503; 1329; 1137; 629	822; 448; 382; 269	1609; 946; 510
CR311 [b]	2446; 1701; 652	2076; 1343; 1121; 911; 670; 605; 564	2499; 1635; 1503; 1329; 1137; 629	822; 448; 382; 269	1609; 946; 510
CC5AWT [b]	1009; 3125	1907; 1121; 975; 670	2499; 1635; 1329	954; 822; 625; 573; 506; 398	1609; 946; 587; 430
C1 [c]	2446; 1701; 652	2076; 1343; 1121; 911; 670; 605; 564	2499; 1708; 1635; 1503; 1329; 1137	822; 448; 382; 269	1609; 946; 510
C2 [c]	2446; 1701; 652	1343; 1121; 975; 911; 822; 717	2499; 1329; 1137; 629	822; 448; 382; 269	1609; 946; 510
C3 [c]	2446; 1701; 652		563; 449	822; 448; 382; 269	1609; 946; 510
C4 [c]	2446; 1701; 652	1343; 975; 911; 822; 717	2499; 1635; 1503; 1329; 1137; 629	822; 448; 382; 269	1609; 946; 510
C5 [c]	2446; 1701; 652	975; 822; 717	2499; 1503; 1329; 1137; 629	822; 448; 382; 340; 269	1609; 946; 510
C6 [c]	1701; 652	1343; 1121; 975	2499; 1635; 1303; 1329; 1137	822; 382; 315; 259	1114; 946; 510
C7 [c]	1701; 652	822; 717	2499	822; 550; 398; 269; 259	1114; 946; 888; 555
C8 [c]	1701; 652	911; 822	2499	822; 550; 398; 269; 259	1114; 946; 888; 555

([a]*Streptococcus* strain types; [b]*S. crista* strain types; [c]Clinical isolate, ND not determined).

To the best of our knowledge, the adhesion assays accomplished in this work have not been used with *S. crista* before. These assays were carried out by us because, if positive, they could suggest the presence of adhesion in the strains. Our results were positive for adhesion in the case of mouth epithelial cells only, but not in the case of HEp-2 or KB cells. This suggests that mouth epithelial cells could be used in adhesion assays to study the possible *in vivo* interaction

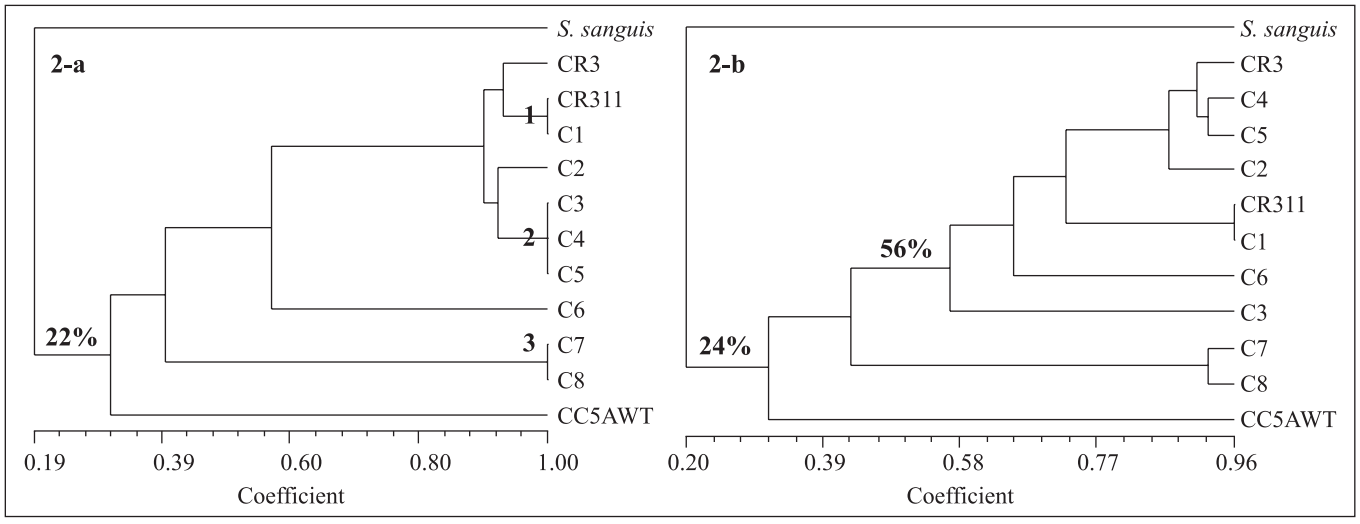

Figure 2. Dendrograms obtained from the AP-PCR analysis. 2-a- Dendrogram obtained by using the RR2 and 434 primers. 2-b- Dendrogram obtained by using primers RR2, 434, OPR2, OPR8, and OPR13.

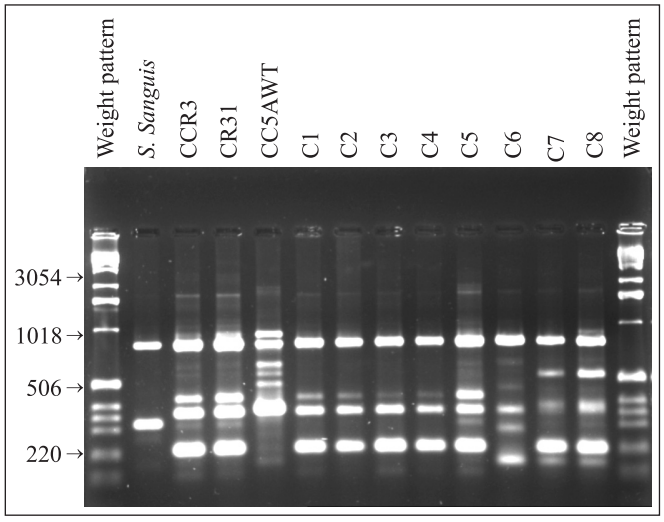

Figure 3. Agarose gel (2%) showing the amplicon patterns obtained with the RR2 primer.

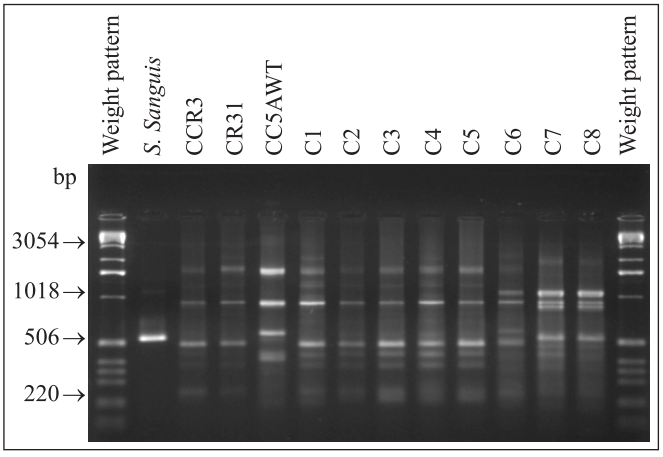

Figure 4. Agarose gel (2%) showing the amplicon patterns obtained with the 434 primer.

of *S. crista* with the mouth and dental environments. The negative results obtained for the other cells could be explained either by the absence of adhesin receptors possibily expressed by this bacterium species or by the fact that the way they were cultivated is not the most appropriate for the expression of these receptors.

Therefore, because salivary amylase and mouth cells are components of the acquired pellicle, it could be suggested that adherence and amylase-binding are mechanisms used by *S. crista* for biofilm formation. As shown in other works (11,39,47), the biofilm helps cell-cell communication, which is important in the formation and development of the oral bacterial community composed by *S. crista* and other oral pathogens, as suggested by Xie *et al.* (46). However, the exact role of *S. crista* has not been fully elucidated yet.

Complete biolfilm formation involves co-aggregation among the individuals of the bacterial community (20,24,25). Co-aggregation occurs via formation of corncob structures among *S. crista*, *C. matrychotti*, and *F. nucleatum* (6,7,12,13). The presence of corncob structures is a biological characteristic of *S. crista* strains and it is believed that their presence is related to the presence of the fibril tuft (13).

Our results demonstrated that only the *S. crista* control strains and the C6 and C7 strains have the ability to co-aggregate with *F. nucleatum*, despite the fact that all the strains presented fibril tufts when analyzed by TEM. These results indicate that the fibril tuft present in the *S. crista* strains cannot to be responsible for the adhesion capacity observed in the corncob structures and they show that afibrillar adhesins could be present in this bacteria, as already observed for other bacterial strains (21,40,48).

Rudney and Larson (32) proposed the use of amplified DNA fragments with the RR2 (822bp and 316bp) and 434 (964bp and 729bp) primers to characterize *S. crista* strains and to differentiate them from other *Streptococcus* strains found in the mouth.

Our results using both primers described by Rudney and Larson (32), and also both primers together with additional primers (OPRs), indicated a varied degree of similarity among the *S. crista* strains studied herein. The results using the latter method (combination of primers) showed a lower similarity among strains than that obtained when only the RR2 and 434 primers were used.

In this way, although the amplified fragments were not the same as those described by Rudney and Larson (32), we believe that the fragments obtained by us when using the same primers could reflect regional differences among strains. We also believe that the set of primers described herein could be used to better characterize and discriminate *S. crista* from other *Streptococcus* strains. It is also noteworthy that the strains studied herein bear higher similarity with strains from the United Kingdom (CR3 and CR311) than with the one that is considered to be a standard (CC5AWT)(USA). Although we have not studied strains from other Brazilian regions, which could give us different results from those obtained herein and could therefore present a more similar profile to that of CC5AWT, our results could be explained by means of a possibly founder principle occurring in an original strain, which is more likely to be very similar to or identical to strains CR3/CR311 followed by random mutations (26).

Furthermore, the electron microscope and adhesion assays accomplished herein indicated that other not yet described adhesins could be present in certain *S. crista* strains. These adhesins could participate in the adhesion processes present in these strains, being thus involved in the biofilm formation. Studies are presently underway in our laboratory aiming at characterizing these possible adhesins.

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RESUMO

Caracterização biológica e molecular de linhagens de *Streptococcus crista* isoladas do biofilme dental de seres humanos através de iniciadores arbitrários - PCR (AP-PCR)

Streptococcus spp são importantes componentes do biofilme dental sendo *Streptococus crista* considerado um interessante modelo de interações bacterianas que nele ocorrem. No presente trabalho linhagens de *S. crista*, foram isoladas do biofilme dental de indivíduos brasileiros, e estudadas em relação a suas características biológicas e ao seu perfil molecular através da técnica do AP-PCR, usando-se os iniciadores RR2, 434, OPR2, OPR8 e OPR13. Os resultados nos permitiram construir um dendrograma de similaridade. A análise do dendrograma de similaridade permitiu a separação das linhagens estudadas em grupos de similaridade. Todos os isolados apresentaram tufo de fibrilas, quando estudados por Microscopia Eletrônica de Transmissão (MET). Estes isolados foram capazes de se ligar à amilase salivar e de se aderir a células epiteliais bucais. Algumas linhagens, que apresentam tufo de fibrilas e aderência positiva, não foram capazes de coagregar com a *Fusobacterium nucleatum*, sugerindo que diferentes grupos de adesinas estão presentes nestas amostras.

Palavras-chave: caracterização biológica, caracterização molecular, análise de dendrograma, biofilme

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