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Identification of the Salivary Microbiota of Patients with Oral Cancer in Antofagasta- Chile by Molecular Diagnosis of the 16S rRNA Gene

Identificación de la Microbiota de Saliva de Pacientes con Cáncer Oral de Antofagasta por Diagnóstico Molecular del Gen ARNr 16S

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ABSTRACT: Oral cancer, is part of several cancers that affect head and neck, and that according to numerous studies shows an increase of approximately 3-5 % of all cancers. In the last 10 years there has been an increased interest in saliva for its use as a diagnostic tool, due to its easy access, and non-invasive sample collection, unlike blood samples. Studies of metagenomics to the microbiota of oral cancer patients have shown the presence of some groups in greater proportion, it is postulated that they could be used as markers especially in the saliva of these patients. In Antofagasta the number of patients with this type of cancer increases each year. However, there are no studies on the changes that affect the oral microbiota in these patients. The objective of this study is to contribute to the knowledge of the microbiota present in the saliva of patients with oral cancer in the region of Antofagasta, limiting this study exclusively to the group of cultivable anaerobic bacteria. The strains identified are related to the group of *Streptococcus mitis / oralis, S. anginosus* and *S. gordoni*, only one strain is grouped near the cluster that includes *S. australis* and *S. parasanguinis*. 36.4 % of the molecularly identified isolates, are closely related to the *S. anginosus* species described in the literature in oropharyngeal tumors. The same percentage is obtained for the taxa related to the cluster that includes *S. mitis*, proposed in other studies as a diagnostic indicator in subjects with oral squamous cell carcinoma.

KEY WORDS: oral microbiota, saliva, oral cancer, microbiological indicators.

INTRODUCTION

The International Classification of Diseases of the World Health Organization (WHO) defines oral cancer as neoplasia originating in the lips, oral cavity, oropharynx, nasopharynx and hypopharynx. Every year more than 575,000 new cases of oral cancer are diagnosed in the world. As for age, 90 % of oral cancers are diagnosed in people over 40 years of age, and more than 50 % in individuals over 65 years. An increase in the incidence of this disease in people under 40 has recently been observed, which could be associated with an infection related to the Human Papilloma Virus. In reference to histology, 90 % of oral carcinomas are of oral squamous cell carcinoma type. Survival at 5 years is 40-56 %, although it varies according to the type of cancer: 70 % labial cancer and 30 % other oral locations (Ochsenius, 2003; García-García & Bascones Martínez, 2009). Oral and pharyngeal cancer It is the eighth in frequency within the male population, whereas in Chile it represents between 3 and 6 % of all cancers that the population suffers, being more frequent in men than in women. Chile does not currently have cancer incidence and prevalence registries at the national level since 1993, are being registered in Valdivia, since 1998 in Antofagasta and since 2003 in Concepción.

Saliva is a mirror of the human health and a reservoir of analytes from systemic sources that reach the oral cavity through various pathways. The composition of saliva reflects levels of hormonal,

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immunological, toxicological and infectious disease markers. Consequently this fluid provides a source for the monitoring of oral and also systemic health (Zimmermann & Wong, 2008). Most of the bacteria in saliva are attached to exfoliated human epithelial cells (Dawes, 2003). In addition to its clinical importance as a diagnostic indicator of oral cancer (Mager *et al*., 2005) and possibly other diseases, the human salivary microbiome may provide insights into human population structure and migrations.

Metagenomics studies conducted to determine the biodiversity of genera and species predominant in the microbiota of patients with oral cancer have demonstrated the presence of some groups in greater proportion, it is postulated that they could be used as markers that indicate an alteration in the mucosa and other areas sampled, especially the saliva of these patients. The saliva counts of 3 oral bacteria species were found to be diagnostic indicators of OSCC: *Capnocytophaga gingivalis*, *Prevotella melaninogenica* y *Streptococcus mitis* (Mager *et al*.).

In Antofagasta the number of patients with this type of cancer increases every year, however there are no studies on the changes that affect the oral microbiota of these patients. The objective of this study is to contribute to the knowledge of the microbiota present in saliva of patients with oral cancer in the region of Antofagasta, limiting this study exclusively to the group of cultivable anaerobic bacteria.

MATERIAL AND METHOD

Samples and culture: Samples were obtained from saliva of patients with oral cancer that were collected at the Cancer Clinic of the Hospital Clínico de Antofagasta. Samples of saliva were obtained in sterile tubes with the prior consent of patients who were instructed to brush their teeth without toothpaste or mouthwash on the day of sampling. The tubes were refrigerated for storage at 4 ° C.

Saliva samples were cultured on blood agar and chocolate agar prepared with BHI base agar (OXOID BRAIN HEART INFUSION AGAR Code: CM1136), and chocolate agar and chocolate agar with agar enriched with Thioglycolate broth (OXOID THIOGLYCOLLATE BROTH USP-ALTERNATIVE Code: CM0391), cultures were performed under laminar flow hood. Samples were incubated at 37 ° C for 48-72 hours in an anaerobic chamber with Gaspak (BD GasPak ™ EZ Anaerobic System Sachets COD: 270304). Following incubation, all colony types on the plates were subcultured for purity, the cellular morphologies of Gram-stained smears were recorded, and isolates were stored at 80°C using Microbank cryovials (Pro-Lab Diagnostics) prior to identification by 16S rRNA gene sequence analysis.

Selection of colonies: The macroscopic observation of the colonies was performed considering the criteria of: size, shape, elevation, margin, surface, color, consistency and hemolysis. Microscopic observation of each of the strains was carried out by Gram Stain and observed in magnification 100x .

Molecular identification of isolates. Genomic DNA was extracted from suspensions of each of the bacterial isolates: colonies was resuspended in 200 mL of sterile H_2O milli Q and Buffer PBS in eppendorf tubes, 200 mL of TEG Alkaline Buffer was added to each tube (Tris HCl 10 mM [SIGMA], EDTA 1 mM y 50 mM de glucose pH 8.0 [MERCK]). Subsequently each sample was sonicated with pulses of 10 s, at a 60 of amplitude, for which the tubes were conserved in ice. Lysozyme 1 mg / ml was added and left for 30 minutes at 37 °C in a 20 mL water bath (50 mg / mL). Then the 20 mL RNAase (20 U / mL) was added and allowed to act for 1 hour at 37 °C, finally adding 12.5 mL Proteinase K [Invitrogen] (25 mg / mL) for 1 hour at 56 °C. It was then washed with phenol [MERCK]; Chloroform [MERCK]; Isoamylic (25: 24: 1) (1 volume) [MERCK] was centrifuged at 13000 rpm for 5 minutes at 4 °C (washed to a clean interface [2 times]). Then washed one time with chloroform (0.8 volume) and centrifuged at 13000 rpm for 5 min. The product was precipitated with Na 3 M acetate (0.1 volume) [MERCK] and 2.5 volume of cold ethanol [SIGMA] at -20 °C. Left overnight at – 24 ºC. The next day the sample was centrifuged at 13000 rpm for 10 min at 4 °C. 500 µl of 70 % ethanol was added and centrifuged at 13.000 rpm for 5 min. The supernatant was removed and the pellet resuspended in sterile H_2O (80 µl).

PCR amplification of 16S rRNA genes was performed in a reaction volume of 100 µL, consisting of 100 mM each deoxynucleoside triphosphate, 50 mM $MgCl_2$, 5 U/µl Taq DNA polymerase, 10 x PCR buffer (all reagents from Invitrogen), between 100-200 ng of extracted DNA as template, and 20 pmoles/µl each primer. The primer pair used were 27F (5-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3) and 1492R (5-CACGGATCCTACGGGTACCTTGTTACGACTT-3),

specific for the domain Bacteria (Dymock *et al*., 1996) (synthesized by Invitrogen). The reaction started by keeping the samples in the Thermocycler (PT-100 programmable Thermal controller, MJ researche, Inc.) for 5 min at 80 °C. Polymerase enzyme was then added and PCR cycles were started. Denaturation was performed at 95 °C for 1 min, primer annealing was performed at 55 °C for 1 min, and extension was performed at 72 °C for 2 min. After which 30 cycles were carried out under the same conditions. In the final cycle, extension was performed at 72 °C for 10 min. Purification of PCR products was performed using the Marligen kit for purification of PCR products, the purification protocol was developed according to the manufacturer's instructions. The 16S rRNA gene PCR products were partially sequenced using the 357F primer (5-CTCCTACGGGAGGCAGCAG-3) (Lane, 1991), ABI Prism BigDye Terminator cycle sequencing ready reaction kits (Perkin-Elmer), and an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems).

Bioinformatic analysis of 16S rRNA sequences and phylogenetic tree construction. This gave reliable sequences of at least 600 nucleotides in length, which were compared to all GenBank DNA sequence entries using the FASTA sequence homology search program (http://www.ebi.ac.uk/services/index.html) (Pearson, 1990). The phylogenetic tree was based on the Neighbor-Joining (Saito & Nei, 1987) and Maximum-Parsimony (Fitch, 1971) methods, after multiple alignments of the data by CLUSTAL and Muscle using the MEGA 5.05 program.

RESULTS AND DISCUSSION

Within the studied samples a great variability of growth was observed between them, which could indicate how it can affect the oral cancer, growth of bacteria, and the maintenance of the bacterial microbiota. It was possible to cultivate 40 colonies of the total of the analyzed samples, of which 92.5 % corresponded to gram + and only 7.5 % to gram. In the Gram-positive group, 79 % presented morphological characteristics similar to the *Streptococcus genus*. In this group of strains studied, alpha-, beta- and gamma-type hemolysis (Fig. 1).

Analysis of the 16S rRNA gene determined that the strains studied corresponded to the genus *Streptococcus*, however the similarity determined by percentage of identity, comparing them with the sequences of reference strains deposited in the databases indicates a similarity less than 99 % with related species of the genus *Streptococcus*. (Table I). In addition, the phylogenetic tree (Fig. 2) groups these clusters separately from the taxa described, which shows an evolutionary distance that could correspond to new species within the genus *Streptococcus*.

In our study the strains identified were related to the group of *Streptococcus mitis / oralis*, *S. anginosus* and *S. gordoni*, only one strain is grouped near the cluster that includes *S. australis* and *S. parasanguinis.*

It is important to note that in the nucleotide databases there are a large number of sequences for taxa related to the *Streptococcus mitis / oralis* group which differ only in a small number of bases. It should be noted that in the analyzed samples, 36.4 % of the molecularly identified isolates are closely related to the *S. anginosus* species described in the literature in oropharyngeal tumors (Shiga *et al*., 2001; Morita *et al*., 2003). Likewise our results indicate that the same percentage is obtained for the taxa related to the clusters that group *S. mitis*, proposed as a diagnostic indicator in subjects with oral squamous cell carcinoma.

The phylogenetic analysis of the strains in the study identifies the isolates within the genus *Streptococcus*, although differences are observed between them. Two clusters were obtained, the first grouping the strains (9 MB, 11MB, 12MB and 18MB) and was closely related to the species *Streptococcus anginosus* DSM 20563; The second cluster grouped

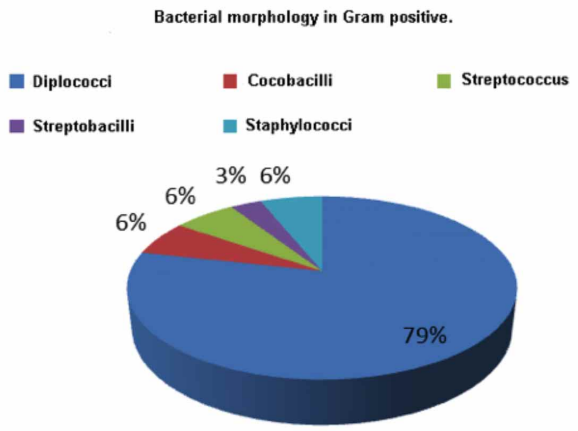

Fig. 1. Differential percentages of bacterial morphology in Gram positive.

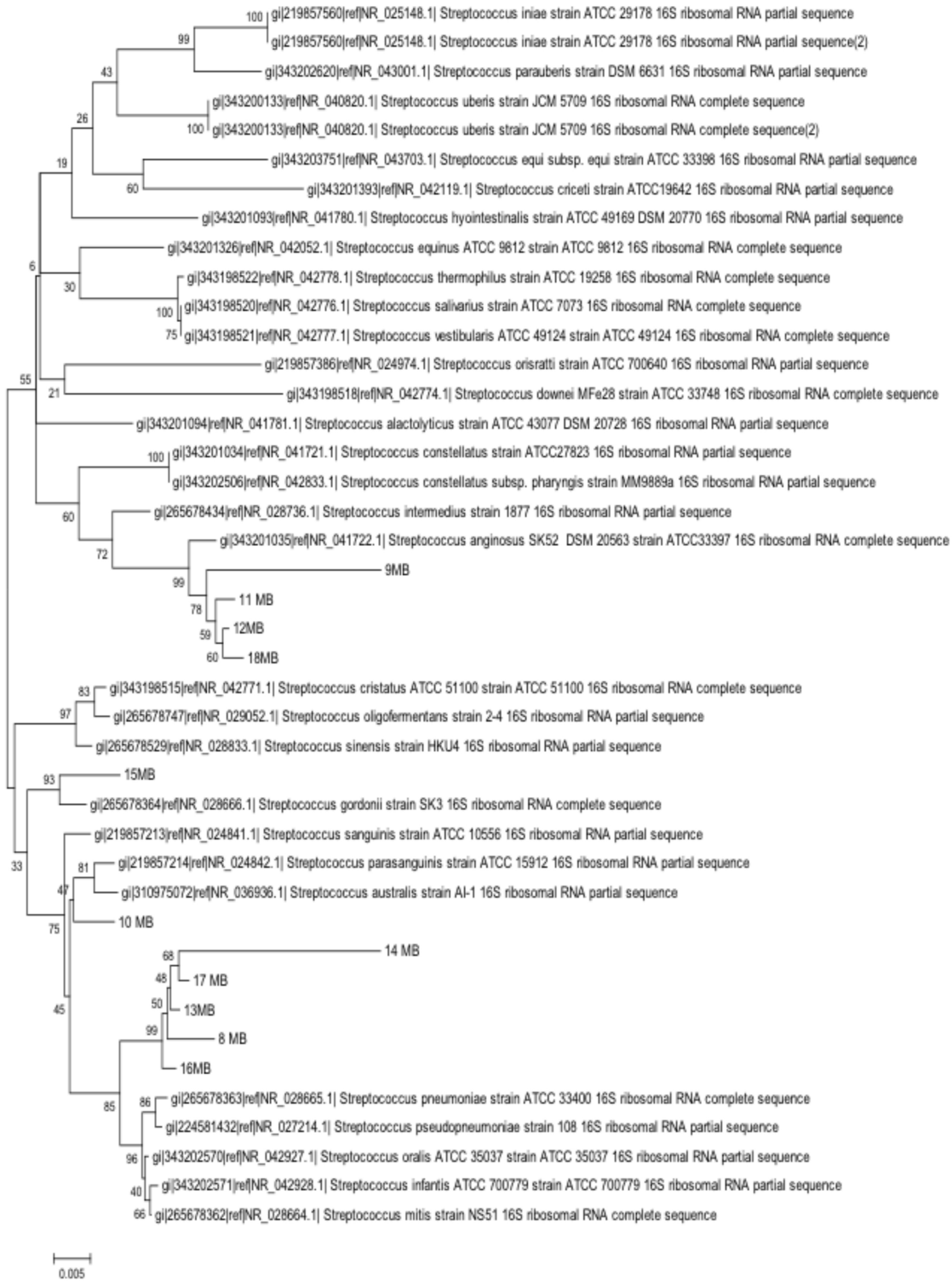

Fig. 2. Phylogenetic tree based on the comparative sequence analysis of the 16S rRNA gene showing position of the strains under study compared to other species of the genus *Streptococcus* described and deposited in the databases and crop collections. Alignment was performed with Clustal, the distance matrix was determined by the Neighbor-joining method (Saitou and Nei, 1987), using the MEGA 5.05 program (Tamura *et al*., 2011). Numbers in the nodes indicate bootstrap values (1000 replicas). Bar indicates 0.005 substitutions per nucleotide position.

Table I. Description of the maximum identity obtained with reference strains deposited in the database, applying the similarity search tool Blastn of the NCBI.

Strain	First Match en Blastn	Maximum identity
8MB	*Streptococcus oralis* ATCC 35037	96%
	S. mitis strain NS51	96%
	S. pseudoneumoniae strain 108	96%
	S. pneumoniae ATCC 33400	96%
9MB	*Streptococcus anginosus* DSM 20563	94%
10MB	*Streptococcus parasanguini* ATCC 15912	98%
	S. australis strain AI-1	98%
11MB	*Streptococcus anginosus* DSM 20563	97%
12MB	*Streptococcus anginosus* DSM 20563	98%
	S. constellatus ATCC 27823	96%
13MB	*Streptococcus mitis* strain NS 51	98%
	S. pneumoniae strain ATCC 33400	98%
14MB	*Streptococcus mitis* strain NS 51	96%
	S.infantis ATCC 700719	96%
	S.oralis ATCC 35037	96%
15MB	*Streptococcus gordonii* strain SK3	99%
	S. sanguinis ATCC 10556	98%
16MB	*Streptococcus infantis* ATCC 700719	99%
	S. pneumoniae ATCC 33400	98%
17MB	*Streptococcus mitis* strain NS 51	97%
	S. pneumoniae ATCC 33400	97%
	S.oralis ATCC 35037	97%
18MB	*Streptococcus anginosus* DSM 20563	98%
	S. constellatus ATCC 27823	97%

the strains (8MB, 13MB, 14MB, 16MB and 17MB) that showed a proximity to a cluster that includes several species of *Streptococcus: S. pneumoniae* ATCC 33400, *S. pseudoneumoniae* ST 108, *S. oralis* ATCC 35037, *S. infantis* ATCC 700779 and *S. mitis* NS51.The strain 15 MB formed a cluster with *S. gordonii* SK3 with a bootstrap of 93 and the strain 10 MB was related to a cluster formed by *S. parasanguinis* ATCC 15912 and *S. australis* AI-1.

The relatively low number of patients and specimens used in this study make it difficult to apply statistical analyses to draw. However, a number of interesting trends are apparent from the results.The detection of *Streptococcus anginosus* in these tissues is also particularly noteworthy because it supports the findings from previous studies in which *S. anginosus* DNA was detected in head and neck carcinoma by PCR (Shiga *et al*.; Morita *et al*.; Sasaki *et al*., 2005). Additionally, the presence of *Streptococcus mitis/ Streptococcus oralis* in both the non-tumorous and tumorous samples is consistent with the previously reported detection of these microorganisms in esophageal carcinoma and control tissues by molecular means (Narikiyo *et al*., 2004).

There are few reports between the possible association between bacterial species and oral carcinoma. In this study 3 the molecular identification corroborated the inclusion of the 11 strains analyzed in the genus *Streptococcus*, which is in agreement with the morphology and Gram obtained in all the strains that were included in the phylogenetic study. The evolutionary distance of the strains under study with the nearest reference strains by similarity to the 16S rRNA gene indicates that these strains probably correspond to new species within the genus *Streptococcus*. The strains identified are related to the group of *Streptococcus mitis / oralis, S. anginosus* and *S. gordoni*, only one strain is grouped near the cluster that includes *S. australis* and *S. parasanguinis*. Finally, 36.4 % of the molecularly identified isolates are closely related to the *S. anginosus* species described in the literature on oropharyngeal tumors. The same percentage is obtained for the taxa related to the cluster that contains *S. mitis*, proposed in other studies as a diagnostic indicator in subjects with oral squamous cell carcinoma.

In Chile, information on oral cancer is scarce, and there are no publications specifically related to the

microbiota associated with patients with this pathology. Therefore, we consider that this study will contribute to the knowledge of the diversity of anaerobic cultivable oral microbiota, detected by molecular techniques from the sequencing of the 16S rRNA gene.

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RESUMEN: El de cáncer oral, es parte de un grupo de cáncer que afecta cabeza y cuello, y que según demuestran numerosos estudios va en aumento corresponde aproximadamente al 3-5 % de todos los cánceres. En los últimos 10 años la saliva ha aumentado su interés para su uso como diagnóstico debido a su fácil acceso y recolección no invasiva, a diferencia de la sangre. Estudios de metagenómica a la microbiota de pacientes con cáncer oral han demostrado la presencia de algunos grupos en mayor proporción, se postula que podrían ser utilizados como marcadores especialmente en la saliva de estos pacientes. En Antofagasta el número de pacientes con este tipo de cáncer aumenta cada año, sin embargo no hay estudios sobre los cambios que afectan a la microbiota oral de estos pacientes. El objetivo de este estudio fue contribuir al conocimiento de la microbiota presente en la saliva de los pacientes con cáncer oral en la región de Antofagasta acotando este estudio exclusivamente al grupo de bacterias anaerobias cultivables. Las cepas identificadas se relacionan con el grupo de *Streptococcus mitis/oralis, S. anginosus* y *S. gordoni*, sólo una cepa se agrupa cerca del cluster que incluye a *S. australis* y *S. parasanguinis*. Un 36,4 % de los aislados identificados molecularmente se relacionan cercanamente a la especie *S. anginosus* descrita en la bibliografía en tumores orofaríngeos y el mismo porcentaje se obtiene para los taxones relacionados al cluster que agrupa a *S. mitis*, propuesto en otros estudios como indicador diagnóstico en sujetos con carcinoma oral de células escamosas.

PALABRAS CLAVE: microbiota oral, saliva, cáncer oral, indicadores microbiológicos.

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