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Araştırma/ Research Article

IDENTIFICATION OF AEROBIC BACTERIAL FLORA IN SALIVA OF SUBJECTS WHO APPLY TO THE FACULTY OF DENTISTRY IN ATATÜRK UNIVERSITY BY USING MICROBIAL IDENTIFICATION SYSTEM

ATATÜRK ÜNİVERSİTESİ DİŞ HEKİMLİĞİ FAKÜLTESİNE BAŞVURAN BİREYLERİN AEROBİK BAKTERİYEL FLORASININ MİKROBİYAL TEŞHİS SİSTEMİ KULLANILARAK SAPTANMASI

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ABSTRACT

Introduction: The aim of the present study was to identify the aerobic bacterial flora in the saliva of patient who apply to The Faculty of Dentistry in Atatürk University.

Methods: Unstimulated saliva samples were collected from 20 healthy subjects with Decayed Missed Filled Teeth (DMFT) index values of between 9-15. Total bacterial flora was recovered from saliva samples on two non-selective media, Nutrient Agar and Sensitive Agar. Isolated bacterial organisms were identified based on fatty acid methyl ester (FAME) analysis using the Microbial Identification System (MIS; MIDI Inc., Newark, Del.). Results: A total of 75 bacterial strains were isolated. Of these, 67 were identified as belonging to 34 species of 16 genera, while the remaining strains were unidentified. The most abundant bacterial genus was Streptococcus, followed by Bacillus, Neisseria, Psychrobacter, Enterecoccus, Haemophilus, Staphylococcus, Paenibacillus, Camphylobacter, Micrococcus, Xanthobacter, Helicobacter, Actinomadura, Kocuria, Pseudomonas, and Cellulomonas. To our knowledge, this is the first report in the literature of the identification of the bacterial species Xanthobacter agilis, Actinomadura yumaensis and Psychrobacter-phenylpyruvicus in oral flora. Conclusions: This is the first study to utilize MIS for the identification of aerobic bacteria in saliva. Our results suggest that MIS is an accurate, efficient and relatively rapid method for identifying bacteria in saliva; however, the MIS databases, particularly those of oral bacterial flora, need to be improved.

Keywords: fatty acid methyl ester, Microbial Identification System, oral flora, saliva

ÖZET

Bu çalışmanın amacı, Atatürk Üniversitesi Diş Hekimliği Fakültesine başvuran hastaların tükürük aerobik bakteri florasını tanımlamaktır.

Yöntem: DMFT indeksi 9-15 arasındaki 20 sağlıklı bireyin sitümüle edilmemiş tükürükleri toplandı.Toplam bakteri florası iki seçici olmayan medya, Nutrient Agar ve Sensitive Agar üzerindeki tükürük örneklerinden toplanmıştır. Izole bakteriyel organizmalar, fatty acid methyl ester (FAME) analizine dayanan Microbial Identification System (MIS; MIDI Inc., Newark, Del.) kullanılarak sınıflandırıldı .

Sonuçlar. 75 bakteri suş izole edildi. Bunlardan, 67'sinin , 16 cins ve 34 türe ait olduğu saptanırken Kalan suşlar tanımlanamadı. En çok bulunan bakteri Streptococcus iken takip edenler Bacillus, Neisseria, Psychrobacter, Enterecoccus, Haemophilus, Staphylococcus, Paenibacillus, Camphylobacter, Micrococcus, Xanthobacter, Helikobakter, Actinomadura, Kocuria, Pseudomonas ve Cellulomonas oldu. Bilgilerimize dayanarak bu çalışma, bakteri türlerinden Xanthobacter Agilis, Actinomadura yumaensis ve Psychrobacter-phenylpyruvicus'u ağız florasında tanımlayan ilk literatürdür.

Sonuç: Bu Çalışma;tükürük aerobik bakterilerinin tespiti için MIS'den yararlanılan ilk çalışmadır. Bizim sonuçlarımız MIS'in tükürük bakterinin tespiti için verimli ve hızlı ve doğru bir yöntem olduğunu desteklemektedir; ancak MIS veritabanlarının özellikle ağız bakteri florası olanlarının geliştirilmesi gerekmektedir.

Anahtar Kelimeler: yağ asidi metil ester, Mikrobiyal Tanımlama Sistemi, Ağız florası, tükürük

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INTRODUCTION

The oral cavity provides two types of surfaces for bacteria colonization – soft tissue and hard tooth enamel/exposed root surfaces – thus supporting the growth of different bacterial communities[1,2.] Because the microorganisms present in saliva are removed from different surfaces of the mouth, saliva flora can provide an overview of the bacteria in the mouth of an individual [2].

Recent studies have focused on the importance of saliva analysis and the correlation of the data obtained from this analysis with caries levels. Many studies have shown a strong relationship between salivary flora and caries[3-7].

These studies generally employ selective media for the isolation of bacterial flora from saliva[3,4-7] and use traditional microbiological tests (morphological, physiological, biochemical and pathological) for identification purposes[8,9]. These conventional methods are usually complex, time-consuming, susceptible to errors in interpretation and lacking in discriminatory power[10-16].

Recent advances in molecular biology and biotechnology are creating exciting possibilities with regard to the development of rapid, accurate and standardized methods for detecting and identifying unknown microorganisms. Over the past two decades, an automated gas chromatography system has been developed that uses a computer interface and software with databases of fatty acid methyl ester (FAME) profiles of yeast, fungi, bacteria and actinomycetes. The Sherlock Microbial Identification System (MIS) developed by MIDI (Microbial ID, Newark, DE) has been used for microbial identification and characterization. Previous studies in different fields have demonstrated that MIS fatty acid analysis is rapid, accurate, inexpensive and simple to perform. Results obtained using this fully automated system have strongly agreed with those obtained from DNA-based techniques[12] . However, to date, MIS has not been used for the identification of oral bacterial flora isolated from saliva samples.

The aim of the present study was to isolate, identify and characterize total aerobic bacterial flora present in saliva samples of Turkish subjects who apply to The Faculty of Dentistry of Atatürk University with DMFT indexes between 9-15 using MIS FAME analysis.

MATERIALS AND METHODS

Subjects

Twenty patients (9 males, 11 females) ranging in age from 18 to 35 years (mean age: 25 years) were selected for inclusion in the study. Individuals with underlying medical disorders or those who had taken any medication within the previous 4 weeks were excluded. Dental caries indexes were recorded at the beginning of the study using WHO criteria[20], and those with DMFT indexes between 9-15 were included in the study.

Saliva samples

Analysis was conducted on unstimulated saliva samples. Samples were obtained between 8 a.m. and 12 p.m. Subjects were instructed not to drink, eat, smoke, or clean their teeth for 2h before the sampling.

Isolation of bacterial flora and culture conditions

Saliva samples were collected in sterilized eppendorf tubes, and tenfold serial dilutions of the samples were prepared in saline solution. Bacteria were isolated by spreading diluted samples (100µl) in small petri dishes containing Nutrient agar (NA) and Sensitive agar (SA) (Acumedia, Baltimore, Maryland, USA). All plates were incubated at 37°C for 3-5 days. Following incubation, bacterial colonies were purified by sub-culturing on the same media used for isolation. In total, 75 bacterial strains were isolated and stored for further study (Table 1). Four bacterial strains from the American Type Culture Collection (ATCC) were used as reference cultures (Table 1). Bacterial strains were maintained for long-term storage in nutrient broth with 15% glycerol at ¯86°C.

Extraction and analysis of FAMEs and identification of bacterial species

FAMEs from whole-cell fatty acids from bacterial strains were prepared and analyzed according to the method described by the MIS manufacturer (MIDI, Newark, DE). Approximately 40 mg of living cells from each sample were harvested and added to 1 mL 1.2M NaOH in 50% aqueous methanol with 5 glass beads (3 mm dia) in a screw-cap tube and incubated in a water bath at 100°C for 30 min. The saponified samples were cooled at room temperature for 25 min, acidified

and methylated by adding 2 mL 54% 6 NHCl in 46% aqueous methanol and incubated at 80°C for 10 min in a water bath. Following rapid cooling, methylated fatty acids were extracted with 1.25 mL 50% methyl-tert butyl ether (MTBE) in hexane. Each sample was mixed for 10 min, and the bottom phase was removed with a Pasteur pipette. The top phase was washed with 3 mL 0.3M NaOH, mixed for 5 min and then removed for analysis. Following the base wash, the extract (FAMEs) was cleaned in anhydrous sodium sulfate and then transferred to a GC sample vial for analysis.

Table 1 Identification of bacterial strains isolated from saliva samples of Turkish subjects by MIS on the basis of FAME analysis.

Species of Bacteria	Origin [a]	No. of Samples	Range of similarity Indices (%)
Isolated strains			
Streptococcus bovis	Saliva	8/20	11- 28
Streptococcus oralis	/this	6/20	13-31
Psychrobacter-phenylpyruvicus	study	5/20	≤ 10
Bacillus licheniformis	"	5/20	31-71
Streptococcus sanguis	"	4/20	≤ 10
Neisseria sicca	"	4/20	42-59
Bacillus circulans	"	3/20	35-63
Haemophilus parainfluenzae	"	3/20	14-24
Neisseria flavescens	"	3/20	21-22
Streptococcus anginosus	"	2/20	13-60
Paenibacillus macerans	"	2/20	≤ 10
Bacillus coagulans	"	1/20	13
Micrococcus luteus	"	1/20	21
Enterecoccus hirae	"	1/20	24
Enterecoccus mundtii	"	1/20	16
Enterecoccus solitaius	"	1/20	21
Neisseria subflava	"	1/20	65
Neisseria-meningitidis	"	1/20	43
Neisseria gonorrhoeae	"	1/20	20
Xanthobacter agilis	"	1/20	27
Streptococcus canis	"	1/20	14
Staphylococcus haemolyticus	"	1/20	34
Staphylococcus capitis	"	1/20	35
Camphylobacter fetus	"	1/20	11
Actinomadura yumaensis	"	1/20	19
Kocuria varians	"	1/20	28
Pseudomonas aeruginosa	"	1/20	15
Cellulomonas hominis	"	1/20	≤ 10
Bacillus-megaterium	"	1/20	≤ 10
Enterecoccus ovium	"	1/20	≤ 10
Streptococcus mutans	"	1/20	≤ 10
Helicobacter cinaedi	"	1/20	≤ 10
Staphylococcus kloosii	"	1/20	≤ 10
Camphylobacter jejuni	"	1/20	≤ 10
Unidentified	"	8/20	nowwwmatch
Reference strains	"		
Bacillus substilis		1	78
Enterococcus faecalis	ATCC-	1	83
Staphylococcus aureus	6633	1	63
Pseudomonas fluorescens	ATCC-	1	72
	29212		
[a]Origin=Sources of bacterial strains isolated;	ATCC-		
ATCC= American Type Culture Collection,	29213		
USA.	ATCC-		
	49838		

FAMEs were separated using gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) in a fused-silica capillary column (25m x 0.2mm) with cross-linked 5% phenylmethyl silicone. The operating parameters for the study were set and controlled automatically by the computer program. Chromatograms with peak retention times and areas were produced on the recording integrator and electronically transferred to the computer for analysis, storage and report generation. Peak naming and column performance was achieved using a calibration standard mix (Microbial ID 1200-A) containing nC9-nC20 saturated and 2&3 hydroxy fatty acids. Cellular fatty acids were identified on the basis of equivalent chain-length data. FAME profiles of each bacterial strain tested were identified by comparing commercial databases (CLIN 40 and TSBA 40) with the MIS software package. Identities of the bacterial strains were confirmed using the conventional techniques, including morphological, physiological and biochemical identification tests [8].

RESULTS

A total of 75 aerobic bacterial strains were isolated. MIS analysis identified 65% (n=49) of these strains, as well as the reference cultures, with similarity indexes ranging from 11%-83%. An additional 24% (n=18) of the isolated strains were identified with low similarity indexes (≤10%) (Table 1). MIS FAME analysis was unable to identify the remaining 11% (n=8) of the isolated strains. With the exception of the unknown bacterial strains, the identity of all bacterial species was confirmed based on conventional tests.[6]

Streptococcus bovis (S. bovis) was found to be the most prevalent bacterial species and was isolated from 40% of the saliva samples tested. The identity and incidences of the other bacterial species isolated were as follows: Streptococcus oralis (S.oralis)(30%); Psychrobacter-phenylpyruvicus, Bacillus licheniformis (25 %); Streptococcus sangius, Neisseria sicca (20%); Bacillus circulans, Haemophilus parainfluenzae, Neisseria flavescens (15%); Streptococcus anginosus, Paenibacillus macerans (10%); and Bacillus-megaterium, Bacillus coagulans, Micrococcus-luteus, Enterecoccus hirae, Enterecoccus mundtii, Enterecoccus solitaius, Enterecoccus ovium, Neisseria subflava, Neisseria-meningitidis, Neisseria gonorrhoeae, Xanthobacter agilis, Streptococcus canis, Streptococcus mutans, Helicobacter cinaedi, Staphylococcus kloosii, Staphylococcus haemolyticus, Staphylococcus capitis, Camphy-

lobacter fetus, Camphylobacter jejuni, Actinomadura yumaensis, Kocuria varians, Pseudomonas aeruginosa and Cellulomonas hominis (5%) (Table 1).

DISCUSSION

A number of studies conducted to determine the relationship between saliva flora and caries have stated that the amounts and types of microorganisms found in saliva may vary among individuals with different DMFT indexes [3-7,18]. In order to focus on the bacterial flora in the saliva of caries, the subjects of the present study were limited to those with DMFT indexes between.[9-15]

Most previous studies have used semi-selective media to isolate oral bacterial flora and conventional tests for their identification.[3,5-7] In order to increase the number of bacterial species collected and respond to nutritional differences in microbial growth, this study employed two non-selective media (NA and SA) to isolate bacteria from saliva samples. In total, 67 bacterial strains comprising 34 species of 16 genera were identified, the most abundant of which was S. bovis. This is an interesting finding, since S. bovis is not generally considered to be a common mouth flora[19], although it has been reported by Crawford and Russel[20] to be present among gingival crevice flora.

Some previous studies have shown nonmutans streptococci such as S. bovis and S. oralis to be prevalent among saliva flora in animals and humans . S. oralis has been shown to be one of the most numerous aciduric-producing bacterial species in dental plaque [21]. In this study, following S. bovis, the second-most prevalent microorganism identified was S. oralis, which has previously been suggested to play an important role in caries development by modifying the dental plaque environment to favor the succession of aciduric species [22]. The results of this study conducted among subjects with high incidences of caries supports this opinion.

In this study, Bacillus species were found in 50% of the saliva samples tested. These species are generally regarded as transient inhabitants of the healthy human oral cavity [1]. However, in an animal study, Rubinstein et al.[23] reported that the Bacillus species elaborate a potent exogenous factor(s) that activate the kallikrein-kinin metabolic pathway in intact oral mucosa, leading to plasma exudation and tissue dysfunction. There has been no information in the literature about the effects of these species on human oral health. The high prevalence of these species in the current study suggests the need for further research into the effects of Bacillus species on human oral mucosa.

To our knowledge, this study represents the first in which Xanthobacter agilis, Actinomadura yumaensis and Psychrobacter phenylpyruvicus have been isolated among oral flora. The role of these bacterial species in oral flora is also unknown and needs to be studied.

This is the first study to utilize MIS for the identification and characterization of oral bacterial flora. Of the total bacterial species isolated using MIS, 24% (Streptococcus sanguis, Streptococcus mutans, Psychrobacter-phenylpyruvicus, Paenibacillus macerans, Bacillus-megaterium, Enterecoccus ovium, Helicobacter cinaedi, Staphylococcus kloosii, Camphylobacter jejuni) were identified with low similarity incidences of less than 10% and 11% of the isolated strains could not be identified. These results suggest that while MIS is an accurate, efficient and relatively rapid method that can be used for the identification of salivary bacteria, the MIS databases, particularly those for oral bacterial flora, need to be improved. This study did not isolate anaerobic bacterial species; therefore, a further study needs to be conducted to determine the composition of anaerobic bacterial flora in saliva.

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