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Could the salivary microbiota be an individual signature?

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Abstract

DNA is one of the important pieces of evidence at the crime scene. There may be cases where the DNA is damaged or insufficient. In this case, the criminal can be identified by qualifying microorganisms as evidence. In recent studies, it has been shown that the detection of microbiota communities on the surface of the human body can bring a new perspective to forensic science. In our study, we investigated the importance of the microbiota in the saliva in terms of identification in forensic sciences, by using phenotypic and morphological features as opposed to genotypic identification, and by checking how unique and characteristic features of these determined microorganisms are compared to other people. 60 different swab samples, including the mouth and bitten right index finger, were cultivated in 4 different media (Blood, Chocolate, Endo, and Chrom) from 30 subjects. All reproducible microorganisms found in the saliva of individuals were investigated. According to the data obtained from the mouth and bitten finger; It was determined that alpha-hemolytic streptococci were the most dominant species among these microorganisms. In addition, Neisseria sp bacteria in the salivary microbiota of individuals have been shown to have a significant relationship with smoking and Candida sp with oral hygiene. We believe that this study will present a different perspective to the literature in cases where the DNA procedure obtained from the bite marks of suspects in the criminal investigation is degraded or insufficient, or to obtain a complementary result in addition to genotypic approaches.

Keywords: Saliva, microbiota, human identification, forensic sciences

Introduction

The main purpose of forensic science is to protect human life and property and to promote peace and harmony in social life. Therefore, it is essential to develop crime detection techniques to reduce crime and ultimately prevent violations of the law [1]. Crime investigation includes studies to determine the etiology and type of evidence rather than classical evidence studies [2]. The evidence found at the crime scene is sometimes visible, macroscopic, and sometimes invisible, microscopic. Forensic microbiology, a new field of study, in which the existence of this micro-level evidence in forensic cases is mentioned, has gained importance in terms of defining microorganisms to identify the guilty person and, on the contrary, to protect innocent people, as a result of different social life [3]. Evidence obtained from the crime scene is obtained through different bioanalytical applications, microscopic, chemical, immunological, and enzymatic applications are used to determine the origin of these biological pieces of evidence [4].

The Human Microbiome Project (HMP) took its first steps in 2007 and preliminary assessments have shown that each region of the body has its microbiota. These microbiota areas have shown that people are closely related to ethnicity and race. In addition, people's lifestyle, geographical location, diet, and style form a unique structure [5].

With the developing technology, forensic microbiology is no longer static and has begun to enter all areas of crime. Bite marks, cigarette butts, saliva stains, and the structure of the teeth found at the crime scene in accidents, murders, rapes, various sexual abuses, and attacks, on the body of the victim or criminal or any object are very important for identification and crime detection [6]. One of them, saliva; is a piece of biological evidence transmitted during oral contact in forensic cases [7]. It is possible to encounter saliva in the bite marks of the victim or the accused in criminal cases, and on many pieces of evidence and objects at the crime

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scene [8-10]. Saliva plays an important role in determining the composition and activity of the oral microbiota through a variety of mechanisms. Molecules mainly from saliva form a conditioning film on oral surfaces, thus providing receptors for bacterial attachment. Oral bacteria sequentially work in concert to catabolize these structurally complex molecules [11]. Oral microbiota varies between individuals and even in different regions of the oral cavity in the same person. According to the effects of different anatomical and physiological structures, oxygen content, nutrient types, temperature, and host defense response, some microorganism species are more dominant and abundant in the oral microbiota [12]. In this environment, known as the oral microbiota, nearly 280 officially defined bacterial species have been isolated in culture. Bacteria are the densest taxonomic group in the oral microbiota and their colonization begins in the postpartum period [13]. Oral bacteria can be cultured in small quantities by aerobic microbiological methods [14]. Besides the few anaerobic bacteria found in the oral cavity, the oral cavity has a large population of organisms that make up the oral microbiome. Large virus families with double-stranded nucleic acids, which make up a small part of the oral microbiota for the Candida genus, are another element called the mycobiome [15,16].

In our study, it was planned to define the presence of Indicator microbiota in a human biological fluid (saliva), which is very important for forensic sciences, by using traditional (conventional) methods and to compare the microorganisms in the mouth and transferred from the oral microbiota to the bitten object. Determining whether these microorganisms can be used for identification in forensic sciences, also aims to provide a new perspective in terms of taking the microorganisms (bacteria) existing in the saliva into account in criminal cases and bio-crimes.

Materials and Methods

The study was approved by the İstanbul University-Cerrahpasa Ethics Committee (approval date and number: 2019/170548). Thirty different individuals, 12 (40%) men, and 18 (60%) women, aged between min.10 and max.60, participated in our study. Swap samples were taken from the mouths of these people who participated voluntarily. Then, the right hand was asked to bite the index finger for 5 seconds, two different module materials were taken by taking another swab from the bitten finger. A total of 60 swab samples were cultivated on 4 different (Blood Agar, Chocolate Agar, Chrom Agar, MacConkey Agar) media that selectively and enriches microorganisms. Then Gram and Simple staining procedures were applied. All stained microorganisms were examined separately under a binocular microscope (10x100) magnification objective 100 immersion eyepieces. Some microorganisms that we could not diagnose with simple biochemical test methods were identified with the MALDI-TOF MS automated system, which is one of the advanced diagnostic test methods. Different biochemical (Catalase, Coagulase, VogesProskauer (VP), KOH, and Citrate) tests were applied to distinguish microorganisms from each other. In our study, the objectives and needed materials and materials were determined and the necessary ethics committee permission was obtained.

Collection of Samples

The samples of this study we conducted were collected voluntarily from the employees of Istanbul University-Cerrahpaşa Faculty of Medicine, patient relatives, and students, and all volunteers were included in our study by signing the informed consent form as well as filling out the questionnaire we prepared. First, swabs were taken from the mouths of each participating individual, and then the subjects were asked to bite their right index finger for five seconds (Figure 1).

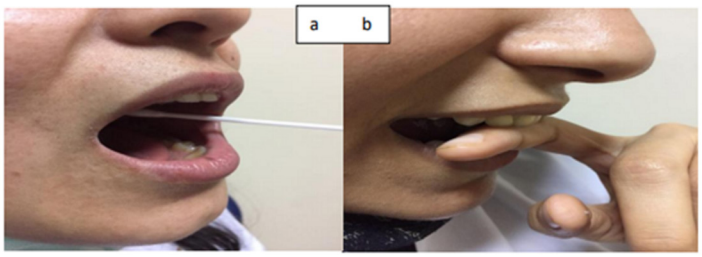

Figure 1. Oral and finger biological samples (a,b)

Characteristics of Collected Samples

Thirty different individuals, 12 men, and 18 women participated in our study. Swap samples were taken from the mouths of these people who participated voluntarily. Then, the right hand was asked to bite the index finger for 5 seconds, two different module materials were taken by taking another swab from the bitten finger. Swabs were placed in sterile containers to prevent any contamination. It was transferred to the laboratory environment by applying the cold chain method. The labeling process was done separately for each sample as the name of the person they belonged to, the date the swap was taken, and the code number of the person. Swaps from all volunteers took place at certain time intervals. Important conditions such as age and gender distribution of the people participating in the study, smoking consumption information, chronic disease, presence of oral implants, and frequency of tooth brushing during the day were determined.

Culture process

Before planting on swab samples taken from volunteers known to have microorganisms in them, the study room was also kept closed to prevent air currents in the planting room. Sowing swap samples and the medium to be sown were placed in a place where they could be easily taken and left, and sowing was done on different selective and enriching mediums.

Microorganism determination methods and Gram Staining method

Microorganisms were kept at certain temperatures and their development was ensured. For this process, the plates on which microorganisms can grow were kept at 36.6°C in a device called an incubator for 12-36 hours. Then, the Petri dishes containing the medium were processed for culture evaluation. The first phenotypically macroscopic evaluations were discussed. In this evaluation, the characteristics of the colonies (shape, margins, height, color, and structure) were defined.

Staining was performed to make microorganisms visible under the microscope and to obtain better information about their morphology and histology. Simple staining was preferred for the morphological examination of microorganisms. Although both morphology and Gram reaction can be determined by Gram

staining, simple staining is always preferred for morphological examinations. Gram (+) and Gram (-) characteristics of bacteria were determined by using the Gram staining method with the latest preparations.

MALDI-TOF MS

MALDI-TOF MS (Matrix-assisted laser desorption ionization time of flight mass spectrometry) is an inexpensive, fast, and accurate new identification method currently used for the identification of microorganisms. Protein profiles of microorganisms are obtained by passing through the electromagnetic field after the protein and large organic macromolecule are ionized. Microorganisms are defined based on species and genus, according to the compatibility of the graphical images of these profile spectra with the reference organisms in the database in the system. The isolate was transformed into ionized molecules by giving a laser beam inside the device. Flying molecules were collected with the help of a detector, resulting in only a single ionized species with a single charge. Therefore, microorganism typing was performed with Biotyper. The process was completed in an average of 13-15 minutes and the species identification of microorganisms was made with automated systems.

Biochemical tests

Microorganisms vary considerably in terms of biochemical activities. Simple biochemical tests were preferred to test their biological properties such as enzyme activity and benefit from amino acids and carbohydrates, resulting in a short time and being inexpensive. Catalase, Coagulase, Voges-Proskauer (VP), KOH (Potassium hydroxide), and Citrate tests were used to identify gram-negative/positive bacteria.

Results

Evaluation of mouth and bitten finger materials

It was determined that Gram-positive alpha-hemolytic streptococci were the microorganisms that showed the most growth (22 of 30 (72.6%) in total) in the mediums in which the oral swab materials were cultivated.

In the second module, the microorganism species that reproduced on the plaque of finger materials taken from the same individuals were Gram-positive alpha hemolytic streptococci in 13 of the 30 subjects (43.3%). In addition, proportional decreases were observed in the distribution of all microorganisms in the materials taken from the fingers compared to the samples taken from the mouth.

Evaluation of module materials

The types of microorganisms that reproduce on all plaques and the distribution of 2 different modules taken from 30 different samples according to the materials and the presence of these microorganisms are shown separately in both (mouth and bitten finger) modules. When all plates were examined, the samples containing the most intense microorganisms were the plates on which mouth swabs were planted. In our study, the data obtained by inoculating swab samples from 30 individuals on two different materials onto medium plates were diagrammed (Figure 2). The most dominant bacteria in the salivary microbiota proportionally are Streptococci. These bacteria are carried to objects and human skin by saliva with the act of biting.

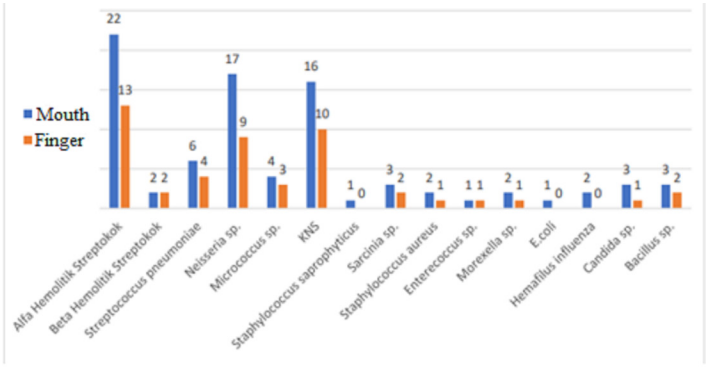

Figure 2. Distribution of growing microorganism species by module materials

Although the distribution is according to gram staining characteristics, the distribution of finger microorganisms is less than oral microorganisms (Figure 2).

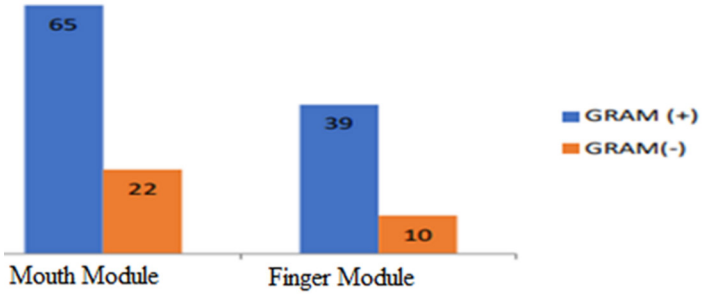

Figure 3. Distribution of growing microorganism species according to Gram staining characteristics

When all plates were examined, it was shown that the samples containing the most intense microorganisms were the plates on which mouth swabs were planted. Microorganisms are seen at a lower rate in the plates on which bitten finger swabs are planted (Table 1).

Discussion

Forensic microbiology research is used in almost most of the forensic sciences. crime scene investigation includes studies to determine the etiology and type of the factor other than classical evidence studies.

The clarification of forensic events depends on correct and planned proofing. Depending on the circumstances at the crime scene, the offender may leave a bite mark on foodstuffs, other objects, an assault or murder victim, or a bite mark may be left on an aggressor living by a deceased victim. At the same time, saliva, which is a body secretion, can accumulate in bite marks [17,18].

The factors affecting the growth of microorganisms in the oral cavity were temperature, anaerobiosis, pH, nutrients, host defenses, host genetics, antimicrobial agents, and inhibitors. In addition, there are age, gender, dietary habits, smoking, oral hygiene, antibiotic use, and possibly genetic factors [19,20].

Forensic microbiology, a new field of study, in which the existence of this micro-level evidence in forensic cases is mentioned, has gained importance in terms of defining microorganisms to identify the guilty person and to protect innocent people, as a result of different social life. [11,21].

Table 1. Distribution of microorganism species growing on plaques by module materials

Sample No	Mouth Module	Finger Module
1	Alpha hemolytic streptococcus, *Streptococcus pneumoniae, Neisseria sp.*	Alpha hemolytic streptococcus
2	*Neisseria sp.* KNS	*Neisseria sp.* KNS
3	Alpha hemolytic streptococcus, *Neisseria sp. Sarcinia sp.*	*Neisseria sp.*
4	Alpha hemolytic streptococcus, *Streptococcus pneumoniae,* KNS	Alpha hemolytic streptococcus, KNS
5	*Streptococcus pneumoniae, Neisseria sp. Sarcinia sp. Staphylococcus aureus*	*Streptococcus pneumoniae, Neisseria sp. Sarcinia sp*.
6	Alpha hemolytic streptococcus, Beta hemolytic streptococcus, *Neisseria sp.* KNS	Alpha hemolytic streptococcus, Beta hemolytic streptococcus
7	Alpha hemolytic streptococcus, *Micrococcus sp. Morexella sp.*	Alpha hemolytic streptococcus
8	Alpha hemolytic streptococcus, *Neisseria sp. Candida sp.*	*Neisseria sp. Candida sp.*
9	Alpha hemolytic streptococcus, KNS, *Staphylococcus saprophyticus*	Alpha hemolytic streptococcus, KNS
10	Beta hemolytic streptococcus, *Neisseria sp. Hemafilus influenza*	Beta hemolytic streptococcus, *Hemafilus influenza*
11	*Streptococcus pneumoniae, Neisseria sp.* KNS	Streptococcus pneumoniae, KNS
12	Alpha hemolytic streptococcus, *Neisseria sp. Bacillus sp.*	Alpha hemolytic streptococcus, *Bacillus sp.*
13	Alpha hemolytic streptococcus, KNS, *Morexella sp.*	Alpha hemolytic streptococcus, *Morexella sp.*
14	Alpha hemolytic streptococcus, *Micrococcus sp.*	*Micrococcus sp.*
15	*Neisseria sp.* KNS, Candida sp.	*Neisseria sp.*
16	*Alpha hemolytic streptococcus, Neisseria sp. Staphylococcus aureus*	Alpha hemolytic streptococcus, *Staphylococcus aureus*
17	*Neisseria sp.* KNS, enterecoccus sp.	*Neisseria sp, Enterecoccus sp.*
18	*Alpha hemolytic streptococcus, Sarcinia sp.*	*Alpha hemolytic streptococcus*, *Sarcinia sp.*
19	*Alpha hemolytic streptococcus, Neisseria sp.* KNS,	*Neisseria sp.* KNS
20	*Alpha hemolytic streptococcus*, *Neisseria sp.* KNS, *E.coli*	*Neisseria sp.*
21	*Alpha hemolytic streptococcus, Streptococcus pneumoniae, Neisseria sp.*	*Streptococcus pneumoniae*
22	Alpha hemolytic streptococcus, *Beta Hemolitik Streptokok,* KNS	*Beta hemolytic streptococcus,*KNS
23	*Neisseria sp.* KNS, Hemafilus influenza	*Neisseria sp*. KNS
24	*Alpha hemolytic streptococcus, Neisseria sp. Candida sp. Bacillus sp.*	*Alpha hemolytic streptococcus,* Bacillus sp.
25	*Alpha hemolytic streptococcus, Micrococcus sp.*	*Micrococcus sp.*
26	*Alpha hemolytic streptococcus, Beta hemolytic streptococcus,* KNS	*Alpha hemolytic streptococcus*
27	*Alpha hemolytic streptococcus, Streptococcus pneumoniae*	*Streptococcus pneumoniae*
28	*Micrococcus sp.* KNS	*Micrococcus sp.*
29	*Alpha hemolytic streptococcus, KNS, Bacillus sp.*	*Alpha hemolytic streptococcus*, KNS
30	*Alpha hemolytic streptococcus, Streptococcus pneumoniae, KNS*	*Alpha hemolytic streptococcus,* KNS

The purpose of creating the Oral Microbiome Database (eHOMD) is to provide comprehensive information on bacterial species. Taxonomic and genomics [22]. Currently, eHOMD contains a total of 775 microbial species. Of all species, 57% are officially named, 13% are unnamed but cultivated, and 30% are known only as uncultivated phylotypes. An important aspect of eHOMD is that it provides a tentative naming scheme for currently unnamed taxa based on 16S rRNA sequence phylogeny [23]. Advances in metagenomics and next-generation sequencing techniques generate rapid sequences and provide comprehensive information on resident microorganisms in the oral cavity [24].

The emergence of new genomic technologies, including next-generation sequencing and bioinformatics, has provided a powerful way to study the microbiome. Oral microbiota varies between individuals and even in the same person in different regions within the oral cavity [25]. The overall species richness of the spectrum of oral microbial species has been demonstrated in terms of both diversity and composition [26]. There are approximately 1.4x108 CFU/ml bacteria in saliva [27]. Mostly, there are *Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, and Saccharibacteri* phyla [28]. Oral bacteria can be cultured in small quantities by aerobic microbiological methods. When the microbiota starts to differentiate from the maternal flora, *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria, and Spirochaetes* groups are first isolated. In addition, an increase is observed in the number of Gram-negative anaerobic bacteria species [29].

Smoking habit is associated with various harmful changes in the mouth. Recent studies describe the toxicology of tobacco smoke, its effects on saliva, oral commensal bacteria, and fungi, as well as oral polymorphonuclear leukocytes. Smoking increases the rate of salivary flow but does not change the composition or the rate of buildup of plaque. Smoking appears to increase anaerobiosis in the oral cavity and suppress the activity of oral leukocytes [30,31]. Analysis of the extracted metagenomes has shown that smoking can alter oral microbial ecology by affecting oral oxygen availability, with consequences for the microbial degradation of xenobiotics [32]. Finally, he observed that the overall oral microbiome composition of ex-smokers was not different from that of never-smokers [31].

The surfaces and biofilms of oral tissues are in constant contact with saliva. The salivary microbiota results from the shedding of biofilm onto the surface of the oral tissue. For these reasons, the microbial profile of saliva resembles that of soft tissue colonization. The salivary microbiota, which can be used as a biomarker, can give good results in some systemic and oral diseases [33].

In the light of all this information; in judicial cases, especially murder, sexual assault, violence, abuse, etc. It is about comparing microorganisms (bacteria) to each other by comparing the presence of Indicator microbiota that can be found in saliva secretion of objects, victims, or criminals, this important human biological fluid in terms of forensic sciences, using conventional microscopic methods. In addition to physical examinations of bite marks, a microscopic approach was tried to be followed in the light of forensic microbiology. Its relationship with smoking is tried to be determined. It is important to evaluate any evidence found at the crime scene that may be related to the crime in terms of exclusion or inclusion as a result of comparison [34].

In addition, every method that can be used in associating evidence with the suspect is applied for the same purpose. Today, while developing technology enables to obtain detailed information in a shorter time, samples that cannot be cultured and unidentified in the field of microbiology can be detected by molecular methods [35]. Today, as technology has developed, different ideas and techniques are being developed to destroy evidence in planned or unplanned crimes. Associating the bite mark left or owned with personal microbiota has provided a different perspective, versus the erasure of saliva stains or efforts to destroy DNA. Thus, it is aimed to open a window that will enable us to look at the existing bite marks as more visible and conclusive evidence with a complementary study to DNA applications.

Oral microbiota varies between individuals and even in the same person in different regions within the oral cavity. To understand this dynamic and diverse relationship, the morphology and physiology of the oral cavity can be studied in different ecosystems [28].

In this study, we aimed to open a new window for forensic identification by taking advantage of the change and diversity of the salivary microbiota in the mouth of individuals with a changing lifestyle.

According to the reports of many studies, the use of antibiotics is very important, especially in the recent period, they have shown that the use of antibiotics has serious effects on the salivary microbiota [36]. Also, the habit of smoking is associated with various harmful changes in the mouth [37]. The study was conducted to evaluate the transfer of the oral microbiota between individuals during contact with objects and the continuity of microbiota parameters during storage on surfaces. According to the data obtained; It has been shown that *alpha hemolytic streptococci* (22 in mouth plaques and 13 in finger plaques) are the most predominant species of these microorganisms. According to Kreth et al.; The presence of different species in the salivary microbiota is due to various conditions such as nutrition, gender, age, galvanic current in restorative teeth, intraoral pH, and foreign body biting habits [38]. In another study, streptococci are the most dominant bacteria in the oral microbiota, and they are transported to objects with saliva in the act of biting [39].

Fewer types of microorganisms are seen in the plates on which bitten finger swabs are planted.

This shows how long the finger is kept in the mouth and which anatomical areas the mouth touches are closely related. Therefore, in the biting process, a small number of microorganisms were transferred by touching the finger only to the lip, tooth, and tongue tip. For this reason, the retention time of an object or bite mark in the mouth and touching the oral anatomical areas can be a controversial issue in terms of the identification of microbiota transfer [40]. When we look at cigarettes, there are almost no microorganisms, especially in non-smokers.

Neisseria sp constitutes 56.7% of the distribution in the plaques of all growing microorganisms in the study. In this case, the total number of 30 subjects is shown as 8 smokers and 22 non-smokers. While no *Neisseria sp* was seen in smokers, it was seen in 17 of 22 non-smokers. It is not surprising that smoking drastically changes

the microbial ecology of the mouth. Indeed, several other studies have also observed the effects of smoking on oral bacteria. The first in vitro studies using culture-based methods indicated that cigarette smoke had a potent inhibitory effect on the growth of *Neisseria sp*. Jing Wu et al. reported that low *Neisseria sp*. level in smokers is associated with the change in oral ecology caused by smoking and the effect of oxygen deprivation in the oral cavity. This finding suggests that cigarette smoke creates an environment that favors facultative anaerobes over aerobes [31]. In this case, the bite mark left by people in any place, event, or object becomes that person. In other words, a material in which Neisseria sp is not seen reveals that the person smokes. Similarly, the presence of *Candida sp* in material obtained from the crime scene indicates that the person has poor oral hygiene. These cases show that it can be used as a possibility of exclusion in identification. It is thought that some of the conditions in the study, such as the absence of *Neisseria sp* in non-smokers, and whether the remaining behaviors and characteristics (such as age, gender, chronic disease, etc.) are significantly related to microorganisms, are due to the effect of this process.

Conclusion

Differences between habitats and their populations create the potential for the use of microbial populations in a variety of criminal cases, so our study highlights the potential of some forensic microbial populations in detecting links between objects and crime scene/person and cell type identification. About the future of work, with many saliva studies on the microbiota to be done, in terms of being a step towards the use of saliva microbiota with identification in forensic sciences; It can be said that it is also important in terms of being one of the first original works done on behalf of our country.

Conflict of interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical approval
Ethics committee approval was received from the Ethics Committee of Istanbul University-Cerrahpasa for the study(2019-170548).

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