

Uncultivable Bacteria in the Oral Cavity: A Review

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ABSTRACT

Uncultivable bacteria are those bacteria, which cannot be grown *in vitro* using the current culture methods. The presence of various uncultivable bacteria has been found in healthy oral cavity as well as in various lesions of the oral cavity using molecular methods like 16SrRNA technique. Many new approaches to cultivate these bacteria are currently underway with the most popular approach for cultivation is mainly aimed at providing a growth environment which is similar to the natural environment of the bacterium. As culture is essential to determine the pathogenesis of bacteria, the current information with respect to most of the uncultivable microorganisms is at present, incomplete.

Keywords: Bacteria, Co-culture, Human Oral Microbiome Database, Minitrap, Uncultivable bacteria

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INTRODUCTION

Oral microorganisms were among the first to be discovered by the humans. From birth, the oral cavity is exposed to the many different microorganisms present in the environment. The oral flora comprises a diverse array of organisms and includes bacteria, fungi, mycoplasma, protozoa and possibly a viral flora which may persist from time to time. Bacteria by far are the predominant group of organisms.

It is estimated that 99% of the bacteria in the environment are yet to be grown *in vitro*, whereas in the oral cavity, 40-60% of the bacteria remains uncultivable the term "uncultivable bacteria" denotes those bacteria which are unable to be grown using the existing laboratory culture methods. The reasons for uncultivability can be many. It may be due to the inability to impart all the growth requirements of the culture medium, presence of an inhibitory bacteria, absence of a coexistent microorganism in the culture media, slow growth of certain bacteria etc.^{1,2}

There has been a rise in interest toward uncultivable microorganisms in the past decade. Currently, the information on uncultivable microorganisms in various oral lesions are largely obtained using 16SrRNA which

is based on the principle that DNA sequences found in 16SrRNA is unique for a particular group of bacteria, but varies with different species. It is done by extracting DNA material after which polymerase chain reaction (PCR) is performed for amplification followed by performing radiolabeling technique to aid in visualization of sequence. To integrate the available data of each of the 600 species of microorganisms of the oral cavity which are identified by 16SrRNA technique, Dewhirst *et al.* created Human Oral Microbiome Database (HOMD). Additional data obtained from each species of microorganisms with time can be incorporated in HOMD. It also helps in comparing different studies done on same species, as well as different species. Though 16SrRNA technique can accurately identify the presence of a specific bacterium, a proper understanding of physiology, and host interaction of these microorganisms requires their cultivation in laboratory. The most accepted method is growing these bacteria in a simulated environment which has obtained much success. The other methods include longer culture time, addition of specific growth factors, co-culturing with other bacteria etc.^{1,2}

Uncultivable microorganisms are becoming important in the field of pharmacological research as well. At present, the development of new drugs is made via newly cultivated previously uncultivable microorganisms, to

which the bacteria is less tolerant and so, will be more effective. One such antibiotic was developed in 2014, which was named teixobactin was found to be effective against methicillin-resistant *Staphylococcus aureus* and various other pathogens in culture medium.³

MOLECULAR BIOLOGY OF BACTERIA

All the bacteria seen in the environment has organelles as that of a human cell though the concentration and structure of each organelle varies. They have a cell wall made of murein, which provides rigidity. Inside this is the cell membrane which controls the movement of substances in and out of the cell. The genetic material is present within a nucleoid. Ribosomes are numerous in number and may be attached to the rough endoplasmic reticulum or may be present free in the cytoplasm. They are present in the form of 30S and 50S subunits.

EVIDENCE FOR UNCULTIVABLE BACTERIA

The first evidence regarding uncultivable bacteria came when the numbers of bacteria observed through a microscope were significantly higher than the cultivable bacteria, and this was termed "The great plate count anomaly."⁴ These uncultivable cells were initially thought to be dead cells, later, many of these cells were found to be metabolically active though they couldn't be cultured.

Current Techniques for Detection of Uncultivable Bacteria

The current information on uncultivable bacteria is obtained by molecular methods, largely by 16SrRNA gene sequencing. The principle is that the genetic code present in 16S ribosomal RNA, which is present in 30S ribosome subunit, varies with each type of bacteria. This technique is done by initially extracting genetic material from the bacteria followed by performing PCR which amplifies specific DNA sequences. This is followed by electrophoresis which separates the proteins after which radiolabeling technique is used to detect the specific gene sequences. The other methods such as immunological methods, electrophoresis methods, chromatographic methods, ATP bioluminescence are also available at present but were found to have a limited success when microorganisms were unknown.^{1,2,5-7}

HOMD has classified oral microorganisms into 13 phyla namely Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, SR1, Synergistetes, Tenericutes, and TM7 out of which 96% of the microorganisms belongs to Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, and

Fusobacteria whereas TM7, SR1, and Chloroflexi still don't have a cultivable representative.^{8,9} According to HOMD, there are approximately 220 oral taxa that are yet to be cultivated.¹⁰

Reasons for Uncultivability

The possible reasons for the uncultivability of certain bacteria may be:

- The inability to include all the nutritional and environmental requirements of their natural environment.¹¹
- Inhibitory effect produced by other bacteria toward a specific bacteria.¹²
- Bacteria in a phase of dormancy during which they are unable to divide.¹³
- Coexistence with another microorganism can be a possibility in which a microorganism degrades complex substrates which can then be used by other bacteria to fulfill its nutritional demand. Thus, absence of the coexistent microorganism in the culture will cease the growth of the dependent bacteria.²
- Most of the culture media currently under use is nutrient rich the fast growing species may outgrow and mask the slow growing species. Sometimes, excess nutrients may be toxic to certain bacteria which will affect its growth in culture.¹⁴
- Disruption of bacterial intercommunication systems due to separation of bacteria on solid culture media.⁶
- Some bacteria are slow growing which may require weeks for culture.¹⁵

Uncultivable Bacteria Associated with Oral Health and Oral Disease

In a study conducted by Aas *et al.* to evaluate the presence of uncultivable bacteria in different sites of healthy oral cavity using 16SrRNA technique, they found a significant presence of uncultivable bacteria in all sites. Most of the sites showed 50% of the population of uncultivable bacteria the least concentration of 30% was obtained from the maxillary anterior vestibule.

The concentration of uncultivable microorganisms varies in different lesions of the oral cavity. About 55% of bacteria present in root canals in endodontic infections had no cultivable representative which mainly includes HOT-272 phylotype. Others mainly belong to the genera of Prevotella, Olsenella, Treponema, Megasphaera, TM7, Lachnospiraceae, Solobacterium. About 33-50% of bacteria associated with dental caries are yet to be cultivated, most of which belongs to the phyla Propionibacteria, Bifidobacterium and Atopobium. About 27% of bacteria associated with osteoradionecrosis were uncultivable,¹⁶ with periodontal diseases; 40-60% of bacteria are uncultivable which mainly includes

species belonging to phyla Prevotella, Desulphobulbus, Treponema, Selenomonas, Synergistetes and SR1. Phylum TM7 and genera Dialister is found to be associated with halitosis. The percentage of uncultivable microorganisms associated with acute dental abscess and noma is estimated to be 40% and 37% respectively.^{2,16,17}

Significance of Uncultivable Bacteria

Various studies using 16SrRNA techniques have revealed that about 50% of the bacteria associated with various diseases of the oral cavity are uncultivable. This is a matter of concern because, without culture, information obtained about the pathogenesis, virulence and resistance of any bacteria is incomplete which in turn makes the knowledge on oral disease incomplete.²

Current Approaches to Cultivate Unculturable Bacteria

- The most widely preferred approach is by growing the bacteria in its natural environment using specialized devices like diffusion chamber which has a semi-permeable barrier which allows nutrients from the natural environment to enter but prevents bacteria from passing through the barrier. This was successful in cultivating marine uncultivable microorganisms.¹⁸ A modified diffusion chamber for the oral cavity called "Minitrap" was designed which is attached to a palatal device and placed in an individual's mouth for 48 hrs and during this period the bacteria gets entrapped in the agar medium and the growth occurs in the agar medium by diffusion of nutrients from the natural environment. Thirty new species have been grown using this technique.^{14,19}
- Addition of certain growth factors or signaling molecules were found to aid in the growth of specific bacteria. It has been shown that the addition of LQPEV, which is a 5-amino acid peptide to the culture media will induce the growth of *Psychrobacter* sp. strain MSC33, which is otherwise uncultivable.¹¹
- Using a culture media which is less rich in nutrients will prevent the overgrowth of the faster growing bacteria.²⁰
- Co-culturing bacteria with other feeder bacteria will aid in the growth. Recently it was found that *Fretibacterium fastidiosum* can be cultivated by co-culturing with *Fusobacterium nucleatum*.²¹
- Certain bacterium requires weeks for it to be grown in culture medium. So adequate time, when provided, can aid in the growth of these bacteria.⁷

Isolation of the specific bacteria from the faster-growing bacteria by serial dilution is also done before culture as this prevents interference from other bacteria. The culturability of SAR11 strains was improved by this method.²²⁻²⁴

CONCLUSION

Uncultivable doesn't mean the pathogens are nonexistent. They represent a vast area which is relatively unexplored. Though they are believed to play an important role in the disease process, their exact role in various oral lesions is not properly understood, largely because of uncultivability. So new techniques of cultivation and/or modification of the conventional cultivation methods are necessary which will help in better understanding of these bacteria and will in turn help in understanding the pathogenesis of many oral lesions.

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