

# Coculture method for *in vitro* cultivation of uncultured oral bacteria

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## Abstract

**Purpose:** The purpose of the study is to culture uncultured oral bacteria with helper strains using the coculture method from the subgingival plaque samples of chronic periodontitis patients.

**Materials and Methods:** The samples were processed and inoculated on a blood agar medium enriched with hemin and Vitamin K. A helper strain *Propionibacterium acnes* (ATCC 6919) was cross-streaked across the inoculums to facilitate coculture. The plates were then incubated for 7 days with subsequent subculturing and further incubation.

**Results:** Satellite colonies around helper strain showed one colony type of *Porphyromonas gingivalis*, one was of nonpigmented *Prevotella*, three were of *Fusobacterium nucleatum* and five isolates remained unidentified.

**Conclusions:** Coculture could be used effectively as one of the methods in the isolation and *in vitro* cultivation of oral bacteria. Incubation using the anaerobic jar technique was found to be economical and efficient for the growth of anaerobic oral bacteria.

**Keywords:** Coculture, *in vitro* culture, oral bacteria

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## INTRODUCTION

The oral cavity harbors around 771 bacterial taxa. These estimations are based on studies spanning several years using cultural and molecular studies. Of all these species, Human Oral Microbiome Database (<http://www.homd.org>) lists 57% cultivated and officially named, 13% unnamed but cultured and 30% of oral bacterial taxa have not yet been grown *in vitro* culture, known only as uncultivated phylotypes, and their presence is signified by 16s rRNA gene-sequencing studies.<sup>[1,2]</sup>

It is well known that several bacterial taxa that colonize various sites in the oral cavity are associated with health and are implicated in diseases, such as dental caries, periodontitis, endodontic lesions and other odontogenic infections.<sup>[3-5]</sup> In addition, oral bacteria may play a role in the pathogenesis of several systemic illnesses such as malignancy,<sup>[6]</sup> type II diabetes mellitus,<sup>[7]</sup> heart disease<sup>[8]</sup> and preterm labor complications.<sup>[9]</sup>

Since a significant portion of oral bacteria either remains uncultivable or difficult to cultivate, the diversity of the

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**Table 1: *In vitro* growth of uncultured bacteria by coculture**

Sample number	Grown in coculture	Other anaerobes in samples	Microscopic morphology
1	<i>P. gingivalis</i>	Nonpigmented <i>Prevotella</i> ( $50 \times 10^3$ ) Pigmented <i>Prevotella</i> ( $32 \times 10^3$ ) <i>F. nucleatum</i> ( $28 \times 10^3$ )	Short thin Gram-negative bacilli
2	Nonpigmented <i>prevotella</i>	Nonpigmented <i>Prevotella</i> ( $33 \times 10^3$ ) Pigmented <i>Prevotella</i> ( $13 \times 10^3$ ) <i>P. gingivalis</i> ( $6 \times 10^3$ )	Short thin Gram-negative bacilli
3	<i>F. nucleatum</i>	Nonpigmented <i>Prevotella</i> ( $30 \times 10^3$ ) <i>P. gingivalis</i> ( $10 \times 10^3$ )	Long tapered ends
4	Unidentified	Pigmented <i>Prevotella</i> ( $5 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $30 \times 10^3$ ) <i>F. nucleatum</i> ( $18 \times 10^3$ )	Long filamentous Gram-negative bacilli
5	-	<i>P. gingivalis</i> ( $6 \times 10^3$ ) Pigmented <i>Prevotella</i> ( $8 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $19 \times 10^3$ ) <i>F. nucleatum</i> ( $23 \times 10^3$ )	
6	<i>F. nucleatum</i>	Pigmented <i>Prevotella</i> ( $14 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $35 \times 10^3$ ) <i>F. nucleatum</i> ( $26 \times 10^3$ )	Long tapered ends
7	-	Nonpigmented <i>Prevotella</i> $22 \times 10^3$	
8	Unidentified	Pigmented <i>Prevotella</i> ( $12 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $56 \times 10^3$ ) <i>F. nucleatum</i> ( $28 \times 10^3$ )	Long filamentous Gram-negative bacilli
9	Unidentified	<i>P. gingivalis</i> ( $10 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $38 \times 10^3$ ) <i>F. nucleatum</i> $919 \times 10^3$ )	
10	-	<i>P. gingivalis</i> ( $18 \times 10^3$ ) Pigmented <i>Prevotella</i> ( $6 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $28 \times 10^3$ ) <i>F. nucleatum</i> ( $28 \times 10^3$ )	
11	Unidentified	<i>P. gingivalis</i> ( $15 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $26 \times 10^3$ ) <i>F. nucleatum</i> ( $32 \times 10^3$ )	Long filamentous Gram-negative bacilli
12	Unidentified	Pigmented <i>Prevotella</i> ( $16 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $45 \times 10^3$ ) <i>F. nucleatum</i> ( $30 \times 10^3$ )	Long filamentous Gram-negative bacilli
13	-	Nonpigmented <i>Prevotella</i> ( $40 \times 10^3$ ) <i>F. nucleatum</i> ( $28 \times 10^3$ )	
14	-	<i>P. gingivalis</i> ( $11 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $30 \times 10^3$ ) <i>F. nucleatum</i> ( $38 \times 10^3$ )	
15	<i>F. nucleatum</i>	<i>P. gingivalis</i> ( $18 \times 10^3$ ) Pigmented <i>Prevotella</i> ( $6 \times 10^3$ ) Nonpigmented <i>Prevotella</i> ( $32 \times 10^3$ )	Long tapered ends

*P. gingivalis*: *Porphyromonas gingivalis*, *F. nucleatum*: *Fusobacterium nucleatum*

oral bacterial community is underestimated using standard cultivation methods. Furthermore, the contribution of these bacteria in maintaining oral microbial ecology as well as their role in oral and systemic health and disease may be overlooked if they remain uncultivable.<sup>[10]</sup> With the development of culture-independent molecular techniques, it has been possible to identify numerous novel phylotypes from both healthy and diseased sites in the oral cavity.<sup>[11,12]</sup>

It must be remembered that despite the availability of sophisticated molecular methods for the evaluation of the oral microbial community, cultural analyses are far from redundant. Only through the isolation and identification of individual bacterial species in pure culture, proper characterization of physiological properties and a full assessment of their virulence potential may be

undertaken.<sup>[10]</sup> Hence, there have been concerted attempts in recent years on developing novel culture methods for growing these bacterial species not cultivated so far *in vitro*.

One of the reasons for the inability of certain bacteria to grow *in vitro* in isolation is their dependence on other bacteria for metabolic products and chemical signals within complex bacterial communities such as dental plaque.<sup>[13]</sup> Hence, efforts to culture uncultivable bacteria have sometimes focused on using coculture with other bacteria to supply growth factors and facilitate the growth of dependent strains.<sup>[14]</sup>

In light of the above information, the present study was designed to use *Propionibacterium acnes* (ATCC 6919) as a standard organism for bacterial coculture for subgingival

plaque samples from patients with chronic periodontitis to isolate not yet cultivated or difficult to cultivate bacteria *in vitro*.

## MATERIALS AND METHODS

The study involved 15 adult participants with chronic periodontitis between the age group of 25 and 50 years, representing both genders. The participants were enrolled after obtaining informed consent. Approval of the institutional ethical committee was obtained before performing the study. Patients with chronic periodontitis were selected according to the classification laid down by the American Association of Periodontology.<sup>[15]</sup> Each patient was checked for a minimum of four sites with a pocket depth of >5 mm and four sites with clinical attachment loss of >3 mm. All participants included in the study had a minimum requirement of 20 natural noncarious or restored teeth present with at least three posterior teeth in two quadrants. Patients with a history of tobacco use, antibiotic usage or periodontal therapy during the past 3 months and those with the systemic disease were excluded from the study.

From each participant, the subgingival plaque was collected from the sites with the deepest pocket after carefully stripping off of the supragingival plaque with a sterile curette. The plaque material was transferred to a vial containing reduced transport fluid and immediately sent to the laboratory where they were processed within half an hour of collection. Each plaque sample was vortexed for 10 s, diluted 50-fold in thioglycolate broth with hemin and Vitamin K and inoculated onto multiple plates (at least 3) of blood agar medium enriched with hemin (5 ug/ml) and Vitamin K (1 ug/ml). A helper strain of *P. acnes* (ATCC 6919) was cross-streaked across the inoculum in a thin straight line. The plates were incubated in an anaerobic jar with a modified gas pack system at 37°C for at least 7 days. A periodic examination of the plates was done every 48 h and the nature of colonies if any around the helper strains were noted. Any satellite colonies that grew around the helper strain were Gram-stained for morphology and then subcultured on fresh blood agar plates with and without the helper strain. The plates were again incubated for 7 days anaerobically before the inspection. Some colonies were also transferred to thioglycolate broth with 1% horse serum to observe for growth.

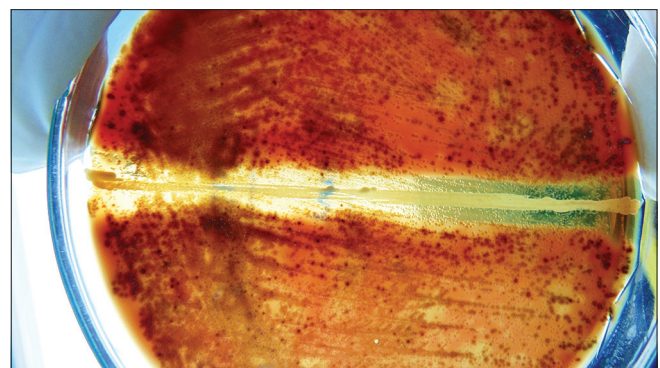
In addition, the plates were also examined to look for the presence of other anaerobic bacteria and their colony characters were noted and morphology was studied by Gram staining. Identification of organisms was done using

phenotypic tests which includes catalase, oxidase, lipase, indole formation, nitrate reduction, gelatin liquefaction and sugar fermentation tests that included glucose, sucrose, maltose, cellobiose, lactose, mannitol, melibiose and xylose.<sup>[16]</sup>

## RESULTS

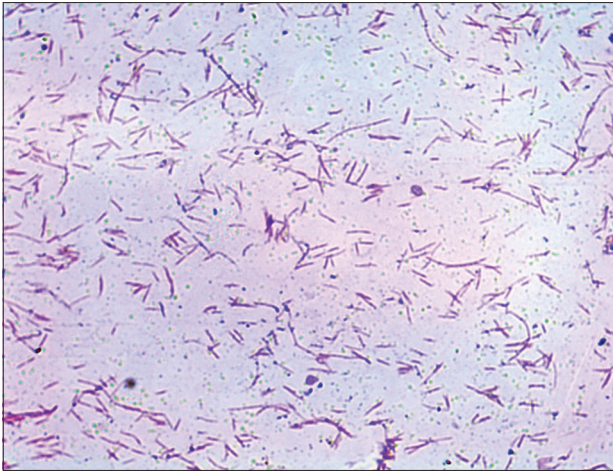
A total of 15 samples from patients were cocultured with *P. acnes* as a helper strain [Figure 1 and Table 1]. When the satellite colonies around the helper strain were studied, it was observed that three colony types were of *Fusobacterium nucleatum* [Figure 2], one was of nonpigmented *Prevotella* [Figure 3], other one was of *Porphyromonas gingivalis* [Figure 4] and five isolates remained unidentified [Figure 5]. There were no satellite colonies in the remaining five samples. Satellite colonies could not be seen when the plates were inspected after 48 h. Minute colonies of varying morphology measuring around 2–3 mm could be observed only after 7 days of incubation. Strains of *P. gingivalis*, *F. nucleatum* and nonpigmented *Prevotella* could be subcultured without helper strain only after the second passage but continued to demonstrate slow growth (after 7 days of incubation). They also showed sparse growth in the liquid medium. The other five strains which could not be identified could be maintained with helper strain for two more subcultures but were lost subsequently because of poor growth.

An attempt was also made to identify few other Gram-negative anaerobes grown in culture plates which showed the presence of *P. gingivalis* in eight samples, pigmented *Prevotella* in nine samples, *F. nucleatum* in 11 samples and nonpigmented *Prevotella* in 15 samples. However, we could not detect *Tannerella forsythia* in any of the samples. It was interesting to observe that in the lone sample that showed *P. gingivalis* as satellite colonies, the organism could not be detected in other areas of the culture plate. A similar phenomenon could also be observed with two out of three samples from which *F. nucleatum* was isolated.

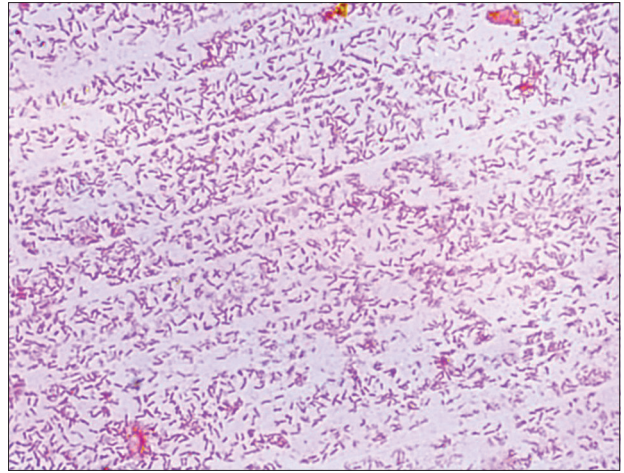


**Figure 1:** Helper strain *Propionibacterium acnes* and satellite colonies

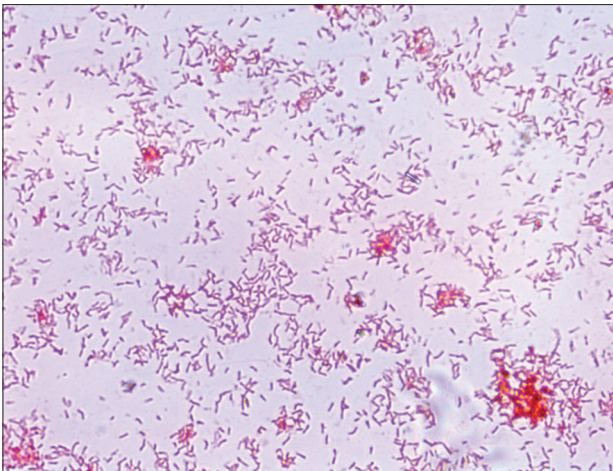




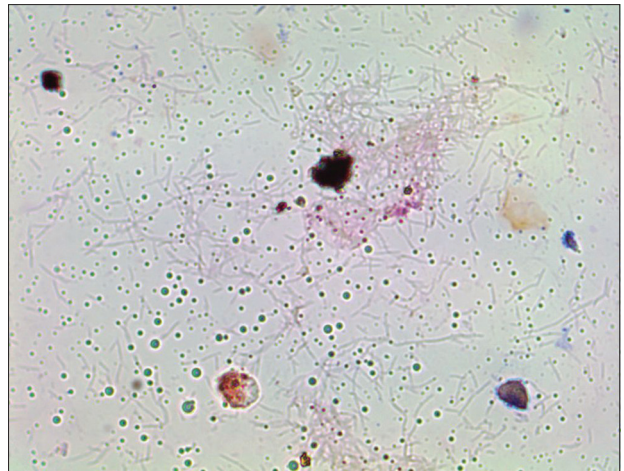
**Figure 2:** *Fusobacterium nucleatum*



**Figure 3:** Nonpigmented *Prevotella*



**Figure 4:** *Porphyromonas gingivalis*



**Figure 5:** Unidentified bacteria

Colonies grown on blood agar plates showed the following characteristics. *P. gingivalis* were minute black-pigmented, circular, hemolytic colonies. *Prevotella intermedia* showed brownish-to-black-pigmented, moist, nonhemolytic colonies. *F. nucleatum* were minute grayish, breadcrumb-like colonies. *T. forsythia* were minute brownish, translucent and moist colonies.

## DISCUSSION

It is a well-known fact that all organisms grow in their natural environment. It is also certain that many bacteria we cannot grow currently will be cultured in the future. The reason why some of these bacteria are not growing in the laboratory is that microbiologists are failing to replicate essential aspects of their environment. However, it is not clear what facet of the environment is not being properly replicated (nutrients, pH, osmotic conditions, temperature, etc.). This has led to the designing of several approaches that mainly depended on bringing the environment to the laboratory.

These attempts demonstrated that there are critical differences between the standard laboratory media that were traditionally being used and the natural environment of unculturable bacteria.<sup>[17]</sup>

During such experiments, it was noticed that some of the bacteria isolated would not grow on a Petri plate unless they were growing close to other bacteria from the same environment. This paved the way for the concept of coculture,<sup>[18]</sup> which was later adapted for isolation of not yet cultivated bacteria from the oral cavity.<sup>[13]</sup>

Several different bacterial species have been used by investigators as helper strains in coculture. One of the most common species used with different types of samples is *P. acnes*.<sup>[19,20]</sup> Other researchers have used bacteria from the oral environment that include *F. nucleatum*, *P. intermedia*, *Streptococcus oralis*, *Parvimonas micro*, *P. gingivalis* and *Actinomyces oris*.<sup>[21]</sup> In the present study, *P. acnes* was used as a helper strain because it is easy to culture and maintain in the

laboratory and has given better results when compared to many species of oral bacteria.<sup>[22]</sup>

It has been observed by several investigators that *P. acnes* stimulates the growth of several previously uncultivated bacteria, although mechanisms of action are not known. It has also been shown that *P. acnes* has a strong growth-promoting effect on *T. forsythia*,<sup>[14]</sup> probably by releasing cell wall fragments so essential for the growth of this oral microbe.

In the present study, we could observe the growth of coculture-dependent strains in 10 out of 15 clinical samples studied. Among them, five isolates belonged to the established genera. It has been observed by several investigators that even among those anaerobic species which can be cultivated in the laboratory, some strains are dormant and need growth resuscitation.<sup>[22,23]</sup> Probably, that could be the reason why metabolic dependency was observed in these isolates. They could be adapted to the laboratory growth conditions only after 3 subcultures. The remaining five isolates could not be grown without the aid of helper strain, and they continued to show poor and sparse growth even after 7 days of incubation both on the surface of solid media and in broth medium. The strains were lost and could not be maintained for further identification by 16s rRNA genome sequencing.

The growth of some of the commonly detected anaerobes from the plaque samples was noted. All the isolates from these species did not display any type of metabolic dependency and could be grown, purified and identified by phenotypic tests. Further studies could be planned with a greater number of samples and also some other oral bacteria as helper strains.

## CONCLUSIONS

The observations made from this study clearly show that coculture can be used for isolation of both dormant and not yet cultivated bacteria from the oral cavity and the protocol can be adapted by any microbiology laboratory that deals with oral anaerobic bacteria. Furthermore, the use of the anaerobic jar technique, which is economical, compared to other anaerobic culture methods is quite efficient in providing an optimum environment for the growth of these organisms. However, improvisation of the protocol is needed for continuous growth, domestication, subsequent identification and characterization of the uncultured bacteria in the laboratory.

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## Conflicts of interest

There are no conflicts of interest.

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