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REVIEW ARTICLE

Exploring the oral bacterial flora: current status and future directions

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OBJECTIVE: The oral cavity forms an indispensable part of the human microbiome, for its unique and diverse microflora distributed within various niches. While majority of these organisms exhibit commensalism, shifts in bacterial community dynamics cause pathological changes within oral cavity and distant sites. The aim of this review was to appraise the current and emerging methods of detecting bacteria of the oral cavity paying particular attention to the cultivation independent methods.

DESIGN: Literature pertaining to cultivation based and cultivation independent methods of oral bacterial identification was reviewed.

METHODS: The specific advantages and disadvantages of cultivation based, microscopic, immunological and metagenomic identification methods were appraised.

RESULTS: Because of their fastidious and exacting growth requirements, cultivation based studies grossly underestimate the extent of bacterial diversity in these polymicrobial infections. Culture independent methods deemed more sensitive in identifying difficult to culture and novel bacterial species.

CONCLUSION: Apart from characterizing potentially novel bacterial species, the nucleic acid sequence data analyzed using various bioinformatics protocols have revealed that there are in excess of 700 bacterial species inhabiting the mouth. Moreover, the latest pyrosequencing based methods have further broadened the extent of bacterial diversity in oral niches.

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Introduction

At the turn of the millennium a new term, 'Human Microbiome' was coined to describe its microbial communities and, in 2007, the US National Institutes of Health (NIH) launched a project termed 'Human Microbiome Project' (http://www.hmpdacc.org/) to characterize the extent and diversity of the microbiome of the human body in a site specific manner and, to identify its link to health and disease (Turnbaugh *et al*, 2007). This essentially includes the totality of microbes, their genetic elements (genomes), and environmental interactions in a defined environment such as the gastrointestinal tract, skin, distal genitourinary tract – and the oral cavity.

The oral cavity forms an indispensable part of the human microbiome, for its unique and diverse microflora distributed in various niches such as the periodontal crevice and pockets, tongue dorsum and other mucosal surfaces. Based on evolutionary genetics, such forms of life are categorized into three domains; bacteria, archaea and eucarya (Figure 1) (Woese *et al*, 1990). The mouth harbors at least six billion bacteria representing more than 700 species (Aas *et al*, 2005), as well as other types of microorganisms, but many of the bacteria have been identified and characterized only recently following the development of novel bacteriological and molecular biology techniques. Because its continuum with the external environment, the population structure of the bacterial flora in the mouth is a dynamic one with changes occurring at immeasurable rates. However, relatively established bacterial biofilm communities are found in the mucosal surfaces of the tongue, buccal mucosa, tooth surfaces, gingival crevices and any artificial surfaces like prostheses and appliances (Samaranayake, 2006c). However, even the population dynamics of these relatively 'stable' bacterial flora is subjected to change by factors such as age, diet and underlying systemic health (Rasiah *et al*, 2005). Preferential bacterial colonization is governed by environmental factors (e.g. nutrient availability and red-ox potentials) and host factors (e.g. the availability of specific binding receptors) (Samaranayake, 2006b). A majority of these organisms exhibit a phenomenon known as commensalism that shares the biological benefits between the host and the organism. However, among this relatively innocuous flora, there exist some organisms with higher virulence attributes and capable

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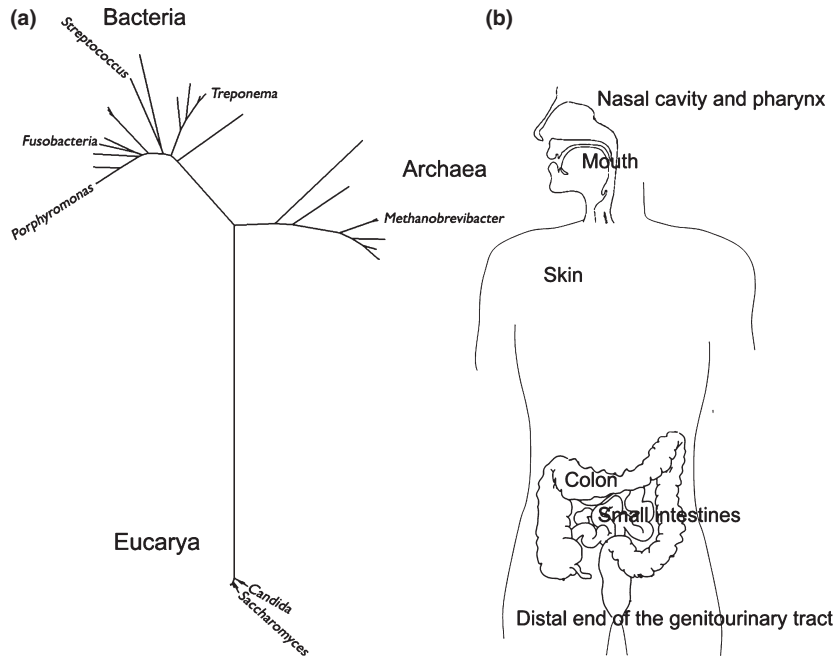

Figure 1 (**a**) Three domains of life (Woese *et al*, 1990), depicted with some microbial species found in the oral cavity. The phylogenetic tree was inferred using the Neighbor-Joining method and drawn to scale, with branch lengths in the same units as those of the evolutionary distances (**b**) Important sites in which the normal flora of the human body (the human microbiome) could be located. The composition of the flora is dependent upon the physiological status of the host and may change on environmental factors

of inflicting disease within and beyond the confines of the oral cavity, such as in central nervous system, skeleton and respiratory system (Parahitiyawa *et al*, 2009). When the status of a particular oral niche is shifted from 'health' to 'disease', consortia of organisms, rather than one single organism have been implicated (Table 1.) (Samaranayake, 2006a; Kuramitsu *et al*, 2007). It is important to note that there is no bacterial species found exclusively in healthy oral niches. This indicates that a balance between pro-healthy and pro-disease bacterial species is operational in oral microbial flora (Jenkinson and Lamont, 2005).

In this review, we summarize the oral conditions where bacteria are considered to be wholly or partly the aetiological factor with focus on existing and developing diagnostic methods. Then, we discuss the 'bigger picture' of the oral microbiome, summarizing the key developments in detection techniques.

Culture-based identification of microorganisms

In routine clinical dentistry, most infections are diagnosed on clinical grounds alone. In rare situations, such as atypical presentations or in ambiguous clinical circumstances, aid is sought from the laboratory. As such, culture-based identification has been the traditional 'gold standard' for the detection of most bacterial and fungal infections (D'Ercole *et al*, 2008). The specimen is grown on a solid, semi-solid or liquid medium under aerobic, microaerophilic or anaerobic conditions. Then organisms are presumptively identified based on colony morphology and staining properties. Additional tests such as motility, demonstration of capsule or fimbriae are helpful. Definitive identification is mostly based on the phenotypic characteristics such as the ability to ferment carbohydrates or to break down other macromolecules. Many of these tests are now miniaturized and are packaged into test panels to allow rapid identification e.g. API and RapidID. Culture of microorganisms is also of pivotal importance for the determination of antimicrobial sensitivity. Furthermore, to understand the virulence attributes of an organism and their expression patterns could only be studied after successful isolation of the organism *in vitro*. However, culture is time-consuming and this may significantly inhibit clinical decision-making. Thus novel molecular techniques have been developed to rapidly identify antimicrobial resistant genes in a bacterial sample (Barken *et al*, 2007). Specimens for microbiological diagnosis include pus (aspirated being ideal), scrapings from mucosal lesions, brushings, discharges and supra- and sub-gingival plaque. However, given the fact that many oral infections are polymicrobial, it is possible that not all the causative organisms in a given niche are identified through this approach. Moreover, as a large proportion of oral infections are caused by fastidious, slow-growing or uncultivable bacteria, it is likely that a large number of the causal bacteria remain undetected.

Direct microscopy

Microscopical examination of a direct cytological or a microbial smear could reveal the presence of certain distinct morphologies of some microbial species cytological or cellular stigmata of such infections. In the diagnosis of oral lesions of syphilis, for example, dark ground microscopy can demonstrate the spiral shaped treponemes. However, there are other spiral shaped organisms (Spirochaetes) that could be present, such as *Treponema denticola, T. vincentii* and *T. pectinovorum*, in which event this method would generate a false positive outcome. However, when microscopy is combined with fluorescent chemical tagged anti *T. pallidum* specific

Table 1 Main oral conditions where infective agents are directly or indirectly implicated (Samaranayake, 2006a; Samaranayake, 2006b)

Condition	*Associated bacterial species*
Dental caries	*Streptococcus mutans*
	Lactobacillus spp
	Actinomyces viscosus
Periodontal disease	
Chronic gingivitis	*Streptococcus sanguis*
	Streptococcus milleri
	Actinomyces israelii
	Actinomyces naeslundii
	Prevotella intermedia
	Capnocytophaga spp
	Fusobacterium nucleatum
	Veillonella spp
Chronic periodontitis	*Porphyromonas gingivalis*
	Prevotella intermedia
	Fusobacterium nucleatum
	Tannerella forsythia
	Aggregatibacter actinomycetemcomitans
	Selenomonas spp
	Capnocytophaga spp
	Spirochaetes
Aggressive periodontitis	*Aggregatibacter actinomycetemcomitans*
	Capnocytophaga spp
	Porphyromonas gingivalis
	Prevotella intermedia
	Tannerella forsythia
Necrotizing ulcerative gingivitis	*Fusobacterium nucleatum*
	Treponema spp
Dentoalveolar infections	
Dentoalveolar abscess	*Prevotella* spp
	Porphyromonas gingivalis
	Fusobacterium nucleatum
	Streptococcus milleri
Ludwig's angina	*Porphyromonas* spp
	Prevotella spp,
	Fusobacterium spp
	Streptococcus spp
Periodontal abscess	*Porphyromonas* spp
	Prevotella spp
	Fusobacterium spp
	Streptococcus spp (especially anaerobic)
	Capnocytophaga spp
	Actinomyces spp
Osteomyelitis	*Tannerella* spp
	Porphyromonas spp
	Prevotella spp
	enterobacteria
Actinomycosis	*Actinomyces israelii*
	Actinomyces bovis
	Actinomyces naeslundii
	Aggregatibacter actinomycetemcomitans
Salivary gland infections	
Suppurative parotitis	Alpha haemolytic streptococci
	Staphylococcus aureus

antibodies, the specificity is increased (Hook *et al*, 1985).

Recently, an ingenious method for the detection of uncultivable bacterial species from oral niches, based on a microscopical cell sorting chip has been introduced. Taking an uncultivable bacterial species of the phylum TM 7 as a model, Marcy *et al* (2007) have shown that sorting of single bacterial cell is achievable which makes subsequent genome base analysis possible.

Immunological tests

Diagnostic tests based on antigen antibody reactions, despite being widely used to detect viral infections such as those caused by herpesviruses, are not routinely used to diagnose bacterial infections of the mouth. The tests that have been described in the literature are primarily aimed at determining the pathogenesis and possible oral-systemic associations of disease entities such as ischaemic heart disease, diabetes and rheumatoid arthritis. We refrain from a detailed discussion of such conditions as it is beyond the remit of this review. However, there are promising results on the use of enzyme-linked immunosorbent assay (ELISA) of serum for the assessment of periodontal disease severity by measuring the antibody responses to the different serotypes of periodontal pathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Pussinen *et al*, 2002). It has been argued that such tests are as sensitive and specific as detecting the specific organisms using PCR, pointing to the exciting possibility of their large-scale use.

Identification of presumptively novel species

In monitoring the disease progression or treatment outcomes of periodontal disease, the aforementioned traditional microbiological tests remain important (D'Ercole *et al*, 2008). However, when routine microbiological tests based on phenotypic characteristics alone do not yield sufficient information on the identification of a cultured isolate, molecular tests based on DNA probes might be of help. These are of particular value when novel or atypical organisms have been isolated from the oral niches. In combination with phenotypic characterization such as SDS–PAGE of cellular proteins, fatty acid analysis or biochemical profiling, molecular techniques such as DNA–DNA hybridization or sequencing of rDNA is widely used to identify such organisms. Such novel organisms linked to oral infections are listed in Table 2.

Metagenomic detection of the oral microbiome

With the advent of ribosomal RNA gene sequencing, many hitherto unknown aspects of the oral microbiome have been revealed. This, in conjunction with computational algorithms in population biology and bioinformatics, it is now known that there are myriad of bacterial species that could inhabit different oral niches. Furthermore, this has enabled the prediction of yet undiscovered species in the oral microbiome and, it has been conservatively estimated that at present, nearly 40% of the species are yet to be characterized (Paster *et al*, 2001).

The available significant rRNA gene sequencing studies pertaining to the oral microbiome is shown in

Table 2 Novel bacterial species detected from oral cavity in the recent past and their possible disease association (G: new genus, S: new species)

Oral source	*Species (type strain) [G/S]*	*Method of identification (Reference)*	*Association with disease (Reference)*
Dental caries	*Alloscardovia omnicolens* (CCUG 31649(T) LMG 23792(T)) [G]	DNA element-based PCR fingerprinting Analysis of 16S rRNA gene sequences (Huys *et al*, 2007)	Quantity of these organisms are highly associated with higher number of dental caries associated bacteria (Beighton *et al*, 2008)
	Prevotella multisaccharivorax (PPPA20(T)) [S]	16S rRNA sequence analysis Differences in sugar fermentation on API Cellular fatty acid composition analysis (Sakamoto *et al*, 2005b)	Isolated from root dentine and plaque from elderly having root caries (Preza *et al*, 2008)
Endodontic lesions (includes periapical lesions, root canal infections and necrotic pulp)	*Prevotella marshii* (E934) [S] *Prevotella baroniae* (E933) [S] (also seen in number of other oral infections as well as in healthy individuals)	16S rRNA sequence analysis DNA–DNA hybridization tests (Downes *et al*, 2005)	Isolated from endodontic lesions, dental abscesses, isolated from supragingival plaque from individuals with periodontitis as well as from subgingival plaque in periodontally healthy individuals
	Dialister invisus (E725 (T)) [S]	16S rRNA sequencing (Downes *et al*, 2003)	Isolated from periradiculalr lesions (Schirrmeister *et al*, 2009) apical abscesses (Siqueira and Rocas, 2009) 20–30% of infected root canals (Rocas and Siqueira, 2006; Jacin to *et al*, 2007; Rocas *et al*, 2008) Primary (80%) and persistent endodontic infections (Siqueira and Rocas, 2005)
	Mogibacterium diversum (HM-7T) [S] *Mogibacterium neglectum* (P9a-hT) [S]	DNA–DNA hybridization 16S rRNA sequencing (Nakazawa *et al*, 2002)	Isolated from necrotic dental pulp
	Olsenella uli Olsenella profusa [G]	16S rRNA sequencing G+C content (Dewhirst *et al*, 2001)	Detected through metagenomic approach from acute apical abscesses (Siqueira and Rocas, 2009), chronic apical periodontitis (74%) (Rocas and Siqueira, 2008)
	Shuttleworthia satelles (DSM 14600T) [G]	Biochemical tests Analysis of cellular proteins 16S rRNA sequencing (Downes *et al*, 2002)	Detected in endodontic lesions (Jacinto *et al*, 2007)
	Treponema lecithinolyticum [S]	DNA-DNA hybridization Enzymatic activity (phospholipase A and C) 16S rRNA sequencing (Wyss *et al*, 1999)	Detected in 26% of primary root canal infections (Siqueira and Rocas, 2003)
	Treponema maltophilum (HO2A and PNA1) [S]	Protein profiles on SDS–PAGE Biochemical properties 16S rRNA sequencing (Wyss *et al*, 1996)	Inhabit 37% of endodontic infections (Sakamoto *et al*, 2009) and most prevalent in primary root canal infections (Siqueira and Rocas, 2003)
	Treponema parvum (OMZ 842) [S]	Cellular protein profiling Specific growth requirements 16S rRNA sequencing (Wyss *et al*, 2001)	Detected in 26% primary endodontic lesions
Odontologic infections	*Porphyromonas uenonis* [S]	16S rRNA sequence analysis DNA–DNA hybridization tests (Finegold *et al*, 2004)	Phenotypic characteristics of the novel organism are very similar to *P endodontalis* and *P asaccharolytica* indicating the possible role of pathogenesis of oral infections
	Neisseria bacilliformis (MDA2833) [S]	16S rRNA sequence analysis (Han *et al*, 2006)	Isolated from a submandibular wound infection

Table 2 (Continued)

Oral source	*Species (type strain) [G/S]*	*Method of identification (Reference)*	*Association with disease (Reference)*
Subgingival plaque	*Campylobacter showae* (SU A4) [S]	Morphological features like number of flagella DNA–DNA hybridization 16S rRNA sequencing (Etoh *et al*, 1993)	Higher prevalence in deep periodontal pockets, although it is lower than other *Campylobacter* strains the prevalence is the highest in gingivitis (Macuch and Tanner, 2000)
	Cryptobacterium curtum (12-3T) [S]	SDS–PAGE of whole cellular proteins DNA–DNA hybridization 16S rRNA sequencing (Nakazawa *et al*, 1999)	Prevalence is nearly twice higher in periodontitis as opposed to health (Kumar *et al*, 2003)
	Desulfomicrobium orale [S] *Desulfovibrio* strain NY682 [S]	16S rRNA sequencing Distinct sulfate reducing properties Cellular lipid composition profiles (Langendijk *et al*, 2001)	Isolated from periodontal pockets in periodontitis
	Prevotella multiformis (PPPA21) [S]	16S rRNA sequence analysis (Sakamoto *et al*, 2005a)	Isolated from the subgingival plaque in periodontitis
	Treponema putidum (OMZ 758T) [S]	SDS–PAGE analysis of the cell extracts Immuno staining for flagellin patterns Enzymatic tests 16S rRNA sequencing (Wyss *et al*, 2004)	Only been occasionally noted in apical periodontitis (2%) (Rocas and Siqueira, 2005)

Table 3. In essence, this method involves the extraction of genomic DNA from a clinical sample(s) followed by amplification of 16S rRNA genes. Complementary primers are designed to anneal the conserved regions of the approximately 1500 bases long gene. When the genomic DNA is amplified with these 'universal' primers, the amplicons consist of 16S rDNA of number of species. Subsequent analysis of these involves two major paths: first, and the most widely used approach thus far, is the cloning of purified PCR products into a bacterial vector followed analysis of the clones. Clones are analyzed by number of methods to reveal the identity of the species. These include denaturing gradient gel electrophoresis (DGGE) (Fujimoto *et al*, 2003; Rasiah *et al*, 2005; Ledder *et al*, 2007), restriction digestion and terminal restriction fragment length polymorphism (T-RFLP) (Sakamoto *et al*, 2004) and, sequencing in combination with the above methods or in isolation. Of these methods, sequencing is considered the most sensitive and specific in analyzing the bacterial diversity in a given niche.

The second, more recent method of analyzing the products of a broad range or universal primer PCR is pyrosequencing. This, especially the 'next generation'

Table 3 Key developments in the discovery of oral microbiome using metagenomic approach

Study scope	*Main findings and remarks*	*References*
Bacterial flora of the subgingival and other oral mucosal surfaces in healthy and subjects with varying degrees of periodontal disease, endodontic infections and other oral mucosal lesions	Bacteria from at least 9 phyla inhabit the mouth of which nearly 40–50% are yet uncultivable Some universal primers used to amplify 16S rDNA preferentially amplify some species Uncultivable bacterial phyla such as obsidian pool and TM7 could be identified through microscopic cell sorting	(Dymock *et al*, 1996; Kroes *et al*, 1999; Paster *et al*, 2001; Munson *et al*, 2004; Aas *et al*, 2005; Hooper *et al*, 2006; de Lillo *et al*, 2006; Marcy *et al*, 2007)
Detection of archaeal species in oral niches and possible associations with disease conditions	*Methanobrevibacter oralis*-like phylotypes are found in oral niches but their pathological associations are yet to be determined	(Lepp *et al*, 2004; Vickerman *et al*, 2007)
DNA microarrays to study the diversity of subgingival plaque microflora	Enables the large scale use in comparing bacterial communities in different oral conditions	(Huyghe *et al*, 2008)
Pyrosequencing to study the oral bacterial microbiome (saliva and subgingival plaque)	High throughput analysis of bacterial diversity, surpassing the need for cloning and cycle sequencing 99% of sequences were in one of seven phyla (Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, Spirochaetes and TM7) 318 distinct species identified from 71 individuals Prediction analysis showed there may be >19 000 more species in the mouth	(Keijser *et al*, 2008)

pyrosequencing technology, is a nanotechnology based method where individual DNA segments are compartmentalized into nano-wells and sequenced. This technique obviates the need for cloning and, the miniaturization permits the sequencing of much larger 'whole genome' scale analyses in a relatively shorter time.

The first study in the indexed literature using metagenomics in oral microbiology was performed over a decade ago and examined the cultivable and uncultivable bacterial species in dentoalveolar abscesses (Dymock *et al*, 1996). Abscesses from three individuals were studied and although the study was not a true metagenomic analysis *sensu stricto*, it was seen that the number and the diversity of the bacterial species isolated through molecular methods outnumbered the species isolated by cultivating the samples on traditional non-selective bacteriological media. It was later postulated that the diversity of the organisms isolated from dentoalveolar abscesses could be much higher than that detected from cultivation alone (Wade *et al*, 1997). These early studies paved the way for molecular microbiologists to study other oral niches.

Subgingival plaque and oral mucosal surfaces

In a landmark study, 59 different phylotypes or species were identified through amplification of rDNA from a sample of subgingival plaque, as opposed to 28 species recovered through cultivation (Kroes *et al*, 1999). These species were from five taxonomical divisions; Low GC Gram Positives, *Fusobacteria*, *Actinobacteria*, *Proteobacteria* and *Cytophagales*. Furthermore, it was found that 48% of the total phylotypes isolated were less than 99% identical to the prevailing sequences in the databases, and hence were considered sequences belonging to novel bacterial species. However, due to the remarkable expansion of sequence databases in the past few years, these 'novel' sequences could now be assigned into definitive species.

In a separate study on subgingival plaque, on a relatively larger cohort of subjects, analyzing 2522 clones from healthy as well as HIV-infected, periodontitis, refractory periodontitis and acute necrotizing ulcerative gingivitis, 347 different species or phylotypes were identified (Paster *et al*, 2001). These represented nine bacterial divisions or phyla and *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Deferribacteres* that are known to have both cultivable and uncultivable species. On the other hand, no cultivable species have been identified yet in the Obsidian pool and TM7 phyla. These two divisions are considered as environmental isolates and most of the members have been located from marine environments and peat bogs. Through comparison of sequence profiles obtained across different subject categories, it was found that there could be putative bacterial pathogens in all except 'obsidian pool' of organisms. Although the sample sizes of each category was relatively too small to arrive at a definitive conclusion, these data point to the fact that much more diverse consortia of organisms could be associated with diseased periodontium. Another aspect of this pioneering study is the fact that the sequence data were obtained from the near complete length of the rDNA. In the earlier studies, bacterial identification was based on the sequence data of relatively shorter segments of 300–400 bases. As the 16S rRNA gene is approximately 1550 bases in length, it could be surmised that more sensitive and specific information on taxonomical lineage and bacterial diversity might be obtained through analyzing longer lengths of sequences.

In an attempt to determine the normal bacterial flora of the healthy mouth, a metagenomic analysis of samples collected from oral mucosal surfaces, tooth surfaces, supra- and sub-gingival plaque, 141 predominant bacterial species were found to inhabit these niches, out of which 60% were uncultivable species (Aas *et al*, 2005). A collection of 20–30 predominant species were detected from each oral niche studied. These bacterial species were members of six different phyla, but species of the genus *Streptococcus* (Phylum: *Firmicutes*) were the predominant species found in nearly all niches. The greatest number of bacterial species was detected from samples obtained from brushings of tonsillar surfaces. It is important to note in the aforementioned study, the cohort of five healthy individuals (i.e. free of periodontal disease), no known periodontal pathogens such as *P. gingivalis*, *T. denticola* and *Tannerella forsythia* were detected in any of the niches sampled.

The metagenomic approach has been used to determine the subgingival bacterial diversity profiles under different circumstances. For example, analysis of terminal RFLP (T-RFLP) patterns of PCR amplicons of 16S rDNA followed by sequencing and quantitative PCR has shown a community shift in bacterial profiles 3 months after periodontal treatment with marked reduction of *P. gingivalis*, *T. forsythia, T. denticola* and *T. socranskii* (Sakamoto *et al*, 2004). In addition, 16S rDNA amplicons from 47 subgingival plaque samples subjected to denaturing gradient gel electrophoresis (DGGE) and sequencing of each unique band have shown that *Actinobacillus actinomycetemcomitans* (new term: *Aggregatibacter actinomycetemcomitans*) was higher among individuals with periodontitis (Ledder *et al*, 2007). However, the discriminatory power of DGGE, when used alone, was only limited to the detection of *T. denticola* from clinical samples.

A study of the tongue microflora in halitosis have shown that *Streptococcus salivarius* (absent in halitosis), *Rothia mucilaginosa* and *Eubacterium* strain FTB41 are the most prevalent organisms found on the healthy tongue dorsa. In contrast *Atopobium parvulum*, *Cryptobacterium curtum*, *Eubacterium sulci*, *Fusobacterium periodonticum*, *Solobacterium moorei*, *Dialister* clone BS095, *Streptococcus* clones BW009 and TM 7 clone DR034 were found in halitosis. By comparing the previously published sequence data from other oral niches, it has been found that there could be more than 25 bacterial phylotypes unique to the tongue dorsum (Kazor *et al*, 2003).

Dental caries

In a comparative study using a culture and metagenomic approach, a higher degree of precision in species discrimination was observed from dental caries samples using molecular methods (Munson *et al*, 2004), although some bacterial species with a high G+C content were detected better by culture. This could be attributed to a problem in DNA extraction or a PCR bias of universal primers rather than a shortcoming of the metagenomic method *per se*. In another study, it was shown that 4 of ten carious lesions analyzed had a predominance of *Lactobacillus* spp. and the other lesions showed species such as *Prevotellaceae*, *Lachnospiraceae* and *Olsanella* (Chhour *et al*, 2005). These points to confounding data that species other than *Lactobacillus* spp. could be implicated as aetiological agents of dental caries. It has also been shown that the bacterial diversity in severe early childhood caries is less than that of caries-free tooth surfaces (Li *et al*, 2007). Upon analysis of 1285 bacterial 16S rDNA sequences from 42 individuals, Aas *et al* (2008) showed that nearly half of the bacteria from the caries lesions are yet to be identified. It has also been argued that, apart from *Streptococcus mutans*, other species such as *Veillonella*, *Bifidobacterium*, *Propionibacterium*, *Actinomyces* and *Atopobium* species could play a role in initiating caries. A study in older subjects has also shown that species other than *Streptococcus mutans*, *Lactobacillus* spp. and *Actinomyces* spp., belonging to *Atopobium*, *Olsenella*, *Propionibacterium* and *Pseudoramibacter* genera may also be associated with caries (Preza *et al*, 2008).

Endodontic infections

Infected root canals are colonized by a consortium of bacterial species (Chu *et al*, 2005, 2006). 16S rDNA sequencing has shown that the infected canals contain a more diverse flora than previously recognized (Vickerman *et al*, 2007), but surprisingly symptomatic root canal lesions harbor a less diverse flora than do asymptomatic lesions. Certain *Streptococcus* spp. persists in treated root canals (Sakamoto *et al*, 2007). Diversity of bacterial species across geographical locations has confirmed that there are shared, location specific and exclusive bacterial species (Machado de Oliveira *et al*, 2007).

Other lesions

A common lesion found in oral mucosa, recurrent aphthous ulcers has been investigated through culture independent methods and analysis of 535 clones has yielded 57 bacterial species (Marchini *et al*, 2007). It has been noted that several species of the genus *Prevotella* were found to be associated with 16% of the lesions. However, these data are insufficient to prove any causal relationship.

Clonal analysis and sequencing have been used to study the bacterial diversity of oral squamous cell carcinoma (Hooper *et al*, 2006). A cohort of 20 lesions has been shown to harbor both indigenous oral organisms and environmental species deep within the carcinomatous tissues, but a larger cohort of subjects needs to be investigated to arrive at any definitive conclusions.

Detection of archaea and eucarya from oral niches

The metagenomic approach in studying oral microflora is not only restricted to the detection of bacterial species. The same technique has been utilized to study the organisms from the domain archaea in oral niches exhibiting different pathological conditions. In a cohort of 48 subjects with periodontitis, 77% of the subgingival plaque samples were shown to contain archaeal rDNA (Kulik *et al*, 2001), but the presence of archaea was not correlated with the clinical parameters of periodontal disease. Two of the three distinct phylotypes have been shown cluster with either *Methanobrevibacter oralis* or *M. smithii* on phylogenetic positioning. These two organisms have previously been shown to inhabit the oral cavity (Belay *et al*, 1988). The association of periodontal Diseases and presence of archaeal species have been demonstrated using quantitative PCR using archaeal 16S rRNA specific primers, where quantity of archaeal load was shown to correlate with the disease severity of the periodontal sites (Lepp *et al*, 2004). The identified archaea were predominantly (81%) of a single phylotypes closely related to *M. oralis*. As most archaeal species have long incubation periods and exacting nutrient requirements, a metagenomic approach could shorten the time taken for their detection.

The quest for archaeal species and the disease association have extended to niches beyond periodontal pockets, with considerable attempts made to study them in endodontic lesions. In a series of 20 subjects with root canal infections, archaeal species were detected in five subjects using an archaeal specific gene, *mrcA* (Vianna *et al*, 2006). Depending on the *mrcA* and 16S rDNA sequences, these phylotypes were clustered in the phylum Euryarchaeota and were more closely related to *M. oralis* than to *M. smithii*. It has also been found that in root canals, *M. oralis*-like phylotypes were associated with *T. denticola* (Vickerman *et al*, 2007). This finding, however, needs to be confounded by much larger cohort studies, as the cited study reported this in only two subjects.

The diversity of eukaryotes in oral niches, although not as extensive as bacteria and archaea, has been the subject of recent interest. A diversity study of fungal species using fungal SSU DNA (18S rDNA) primers, showed that only *Saccharomyces cerevisiae* and *Candida albicans* were found in periodontal niches from 306 clones analyzed from 20 patients (Aas *et al*, 2007). This low abundance could be attributed to a methodological bias. *Trichomonas tenax*, a protozoan parasite found in the oral cavity has been successfully detected using genus-specific 18S rDNA primers (Kikuta *et al*, 1997). However, their role in periodontal or other oral infections has yet to be defined.

In summary, the metagenomic approach provides a powerful tool to detect uncultivable and fastidious bacterial communities in oral niches and to identify novel bacterial phylotypes. It could be regarded as the most important contributory factor for the expansion of the *status quo* of the oral microbial diversity. In addition, it allows the determination of the genetic homogeneity of species/phylotypes isolated from two different niches. However, this method is not infallible, for the whole methodology operates under the premise that enough genomic DNA is recovered from the clinical sample, the amplification process (PCR) is unbiased and that the database sequences against which the nascent sequences are compared are accurate. In fact it has been shown that, in some databases, as much as 25% of the deposited sequences are of chimeric origin. The choice of using 16S rRNA genes as bacterial profile signatures is also questionable in some instances. For example, discrimination of some enteric bacterial species is not possible with 16S rDNA. The usefulness and limitations of this method have been addressed elsewhere (Woo *et al*, 2008).

Nevertheless, novel approaches have been developed to circumvent some of the problems associated with the amplification-cloning-sequencing approach. For example, DNA microarray chips have been designed to identify 20–100% of the 32 phyla described in the ribosomal database project (RDP). Armed with nearly 9500 probes, this is claimed to be able to identify 78.3% of the RDP sequences (Huyghe *et al*, 2008). Although the time spent on oral microbe identification is shortened with the use amplification-based clonal analysis, this could be shortened even further by using pyrosequencing. Furthermore, because of the high throughput nature of this technique, tens of thousands of samples could be analyzed in one experimental setting. Following pyrosequencing analysis of 197600 16S PCR amplicons, 318 different bacterial species (185 from saliva and 267 from plaque) were identified in a cohort of 71 subjects (Keijser *et al*, 2008). Furthermore, based on these findings and prediction analysis, it has been surmised that the estimated number of phylotypes could be as high as 19000. However, one of the limitations in this method is the relatively short sequence read length. Whether a 300–400 bases long segment has sufficient sensitivity and specificity to determine the phylogenetic position is questionable (Wommack *et al*, 2008).

Conclusions

Despite the large bacterial flora that inhabits the oral cavity, microbiological diagnosis of oral disorders remains largely confined to research laboratories. However, advances in technology indicate that some of the traditional as well as novel techniques could readily be employed to aid clinicians at point of care. Comprehensive and miniaturized kit based methods of traditional microbiological testing methods, could help identify pathogen(s) even by novices with a basic knowledge in microbiology. It has to be emphasized that despite the lack of sensitivity and the time-consuming nature in detecting the fastidious and slow-growing organisms, culture methods are still the method of choice for determining the antimicrobial sensitivity and the determination of virulence factors.

The metagenomic approach has revolutionized the *status quo* of the oral microbiome by its ability to identify increasing numbers of novel species. However, caution needs to be exercised when interpreting the voluminous data. For example, some of the universal primers tend to bias the PCR towards certain bacterial genera leading to over or under-representing the true magnitude of bacterial diversity (Kroes *et al*, 1999). Furthermore, interpretation and generalization of findings of metagenomic studies are open to dispute as a result of relatively fewer numbers of subjects studied. Apart from detecting novel pathogens, genetic homogeneity of bacterial species in different oral niches and those isolated from non-oral sites could be clarified by such technology (Parahitiyawa *et al*, unpublished data). Although the 'traditional' metagenomic approach of amplification-cloning-sequencing routine may not be ideal for expediting a diagnosis at point of care, it is likely that the newer 16S rDNA microarray technology will pave the way for rapid diagnostics in oral microbiology.

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