Oral microbiology in the microbiome era – when, how and why to perform microbiological diagnostics

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ABSTRACT

Knowledge about the oral microbiota has increased greatly during the past decade after the introduction of high-throughput sequencing technologies. These culture-independent technologies have enabled the detection of the as yet uncultured bacterial species that make up about half of the 700 species identified in the oral microbiome. Similarly, the oral mycobiome has been shown to be much more diverse than previously expected. Currently, studies are underway to clarify the differences between the microbiome in health and disease with regard to both the species involved and the functional properties of the microbiome. The implications for disease management and diagnostics still remain undetermined.

Culture is still the preferred diagnostic method both for bacterial and fungal infections. The benefit of using culture is that it enables identification of multiple species and antimicrobial susceptibility testing. Nucleic acid detection methods have become increasingly available for detection of a number of suspected periodontal pathogens as well as for diagnostics of viral infections. Microbiological diagnostics are not routinely needed but may be helpful in complicated or refractory infections and in differential diagnostics.

THE ORAL MICROBIOME

Microbiome is the term used to refer to our resident microbiota (1). The oral microbiome consists of bacteria, fungi, Archaea, viruses, and protozoa (2). Throughout the 20th century improvements in cultivation and biochemical analyses revealed an increasingly diverse microbiota but the introduction of sequencing technology caused an explosion in microbial diversity because it enables detection of both cultivable and as yet uncultured species (3). In 2007, the Human Microbiome Project...
was launched (4) and in 2010 the Human Oral Microbiome Database was established (5). Bacteria have been predominantly identified by sequencing the 16S ribosomal RNA (16S rRNA) gene that contains regions conserved in all bacteria and regions that vary between species (3). This far, over 700 species of bacteria have been identified in the oral bacteriome and about half of them are as yet uncultured (6,7).

Microbiome studies have revealed that the microbiome may differ significantly between individuals and different oral niches. This has led to the definition of a ‘core’ oral microbiome consisting of the microorganisms found in all or the vast majority of individuals and a ‘variable’ part that has a lower prevalence (4). Three studies based on high-throughput sequencing of 16S rRNA genes from oral samples of up to 200 individuals have shown that the predominant oral taxa belong to the phyla Firmicutes (genus Streptococcus, Veillonella, Granulicatella), Proteobacteria (genus Neisseria, Haemophilus), Actinobacteria (genus Corynebacterium, Rothia, Actinomyces), Bacteroidetes (genus Prevotella, Capnocytophaga, Porphyromonas), Fusobacteria (genus Fusobacterium) and Spirochaetes (genus Treponema) (2,8-10). Frequently detected as yet uncultured phyla are GN02, SR1, and TM7 (2,6). Although we now have an understanding of the core oral microbiome, it is important to bear in mind that for example poor oral hygiene, wearing dentures, immunosuppression, use of antimicrobials, hospitalization and being bedridden all significantly alter the composition of the oral microbiome with for example opportunistic respiratory pathogens and staphylococcal species being introduced (11,12).

Over 75 fungal genera have been detected in the oral microbiome by high-throughput sequencing using fungal internal transcribed spacer (ITS) primers (13,14). In these studies, Candida species have been the most frequent finding (75%-100% of healthy individuals). Other common genera detected were Cladosporium, Aureobasidium, Aspergillus, Fusarium, Cryptococcus (13,14), and Malassezia (14).

The oral virome consists of both eukaryotic viruses and bacteriophages (15). Metagenomic studies on the oral virome are still rare. In a recent study, members of the virus families Herpesviridae and Papillomaviridae were found to be the most common of the human DNA viruses detected (16).

HOW HAS HIGH-THROUGHPUT SEQUENCING AFFECTED OUR UNDERSTANDING OF THE ORAL MICROBIOTA?
The main contribution of high-throughput sequencing is the revelation of the immense diversity of the oral microbiota (3). In addition, the microbiota of a specific oral site may differ between individuals (10). Thus, it is necessary to define the microbiota associated with health and to follow the transition from health to disease in longitudinal studies. This can disclose microbial changes associated with disease. A cross-sectional study of a limited number of individuals most likely will reflect differences in the microbiota between health and disease but due to the large inter-individual differences it is far from certain that this indicates a significance when the microorganisms are found only in the diseased subjects.

The Human Genome Project revealed that our genome does not contain all genes necessary for the functions of the human body (4). The resident microbiota of man provides far more genes necessary for the well-being of man than hitherto anticipated. Through evolution man has coevolved with the members of the resident microbiota and together they form a ‘superorganism’ (3). Because of the coevolution, the immune system has developed immune tolerance towards the resident microbiota. There is a beneficial relationship between the resident microbiota and man which should be carefully valued for example by avoiding unnecessary use of antimicrobials.

Above all, the immense diversity of the oral microbiota has finally buried the specific disease concept that prevailed for a prolonged period for both caries and periodontal disease, and which initiated the era of antimicrobial treatment especially in periodontal disease. The ecological plaque hypothesis introduced by Marsh (17) (1994) framed the contribution of more members of the oral microbiota for the development of caries and periodontitis by acknowledging the significance of plaque formation for the development of microbiota whose concerted action surpasses the level for a balanced relationship of microbiota with the host (today referred to as dysbiosis).

Further studies should now be done aiming at understanding the functional properties of the microbiome as well as interactions between bacteria and other members of the oral microbiome.

ORAL INFECTIONS
Ooral bacterial and fungal infections are usually endogenous in nature and caused by the commensal microbiota. Clinical viral infections, on the other hand, are either acute exogenous infections or chronic (e.g. HIV) or result from endogenous reactivation of viruses (e.g. herpesviruses).

The polymicrobial nature and biofilm formation are typical for dental infections (18). Bacteria living in a biofilm show calcification towards antimicrobials (19). This is a result of the biofilm growth pattern, antimicrobial resistance genes in bacteria, and microbial tolerance towards antimicrobials. A tolerant bacterium does not grow in the presence of an antimicrobial but survives and continues to grow after withdrawal. Expression of antimicrobial resistance genes (e.g. beta-lactamase) may inactivate an antimicrobial to the extent that also neighbouring susceptible bacteria are protected. Mobile resistance genes can be transmitted between the bacteria within biofilm. Finally, dormant or resting bacteria in a biofilm are less susceptible to antimicrobials due to lack of metabolic activity.

Consequently, mechanical dental treatment is the primary choice for dental biofilm diseases and if needed, antimicrobials can be used in addition but they should never be used alone.

INDICATIONS AND METHODS OF SAMPLING AND DIAGNOSTICS
Dental abscesses
In uncomplicated cases mechanical treatment of the infection focus alone may be enough for complete cure and the benefit of using antimicrobials is questionable (20,21). If antimicrobi-
Diagnostic methods

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Table 1. Recommended diagnostic methods for bacterial, fungal and viral infections according to infection type.

Als are used, these can be chosen following local antimicrobial guidelines for empiric treatment (21,22).

Sampling for culture is, however, recommended in complicated infections with risk of local spreading or signs of systemic infection, to specify microbes and their antimicrobial susceptibility within the abscess to ensure optimal treatment (Table 1). Other indications for sampling are persistent or recurrent infections, infections of the immunocompromised and patients with recent history of hospitalization or antimicrobial treatment, as in these situations unexpected bacterial species or antimicrobial sensitivities may be discovered (11,23).

The sample should be taken aseptically, with great care to avoid contamination by mucosal microbes outside of the infection focus (18,24,25):

1) Disinfect the area with chlorhexidine mouthwash or careful chlorhexidine swabbing.
2) Use a sterile syringe to aspirate pus from the abscess or the root canal. Transfer the sample aseptically into transport medium which supports survival of both aerobe and anaerobe bacteria.
3) Transport the sample to the laboratory as quickly as possible to facilitate the yield of anaerobic bacteria.

Swabs should not be used for sampling, because it is often impossible to avoid contamination by mucosal bacteria outside the infection focus, and the number of species recovered are often lower (24,26).

Infections are dominated by strict anaerobic bacteria together with facultatively anaerobic species from the commensal...
microbiota (18, 24). Phyla Firmicutes and Bacteroidetes constitute over 70% of the findings both by culture and molecular methods (18). At the species level, common findings are viridans streptococci (Streptococcus anginosus, Streptococcus mitis), anaerobic Gram-negative rods such as Prevotella, Porphyromonas and Fusobacterium spp., anaerobic Gram-positive cocci belonging to the genera Parvimonas or Peptostreptococcus, and Eikenella corrodens (18, 24). Infrequently, beta-hemolytic streptococci, enterococci, staphylococci, enteric rods and Pseudomonas and Candida species may be found (24, 25, 27). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has greatly facilitated identification of strains isolated on solid culture media (28).

It is debated whether certain species in the polymicrobial abscess are especially pathogenic, and should be selectively targeted by antimicrobials, or whether all the bacteria are of equal significance (18). Nevertheless, it is important to remember that in addition to the predominant cultivable flora, the samples contain slow-growing, fastidious, and less abundant species that may go undetected as well as the as yet uncultured species.

**Other severe cervicofacial infections**

In suspicion of osteomyelitis, actinomycosis, Mycobacterium infection, or invasive fungal infection, an aseptically acquired tissue sample (biopsy/needle aspirate) or pus aspirate in an empty sterile tube should be taken for culture. In addition, a tissue sample should always be taken for histopathological analysis to help set the diagnosis and for differential diagnosis.

Mycobacteria do not grow on standard bacterial growth media and cannot be stained by regular gram stain. Therefore, a sample should be examined by Mycobacterium culture, acid-fast staining, and PCR using mycobacteria-specific DNA probes.

Suspicion of Actinomycetes must be stated in the referral so that the laboratory can use selective culture media and increase the culture duration.

PCR with species-specific probes, staining, and culture is used for microbiological diagnosis of invasive fungal infections, such as mucormycosis, aspergillosis, cryptococcosis and histoplasmosis. Certain fungi, for example Aspergillus species, are common environmental contaminants. Therefore, interpretation of a positive finding always requires consideration.

**Periodontitis**

The main indication for sampling is severe periodontitis, which has not responded to standard treatment including good oral hygiene. Samples are taken with paperpoint technique. Analysis is performed by culture or DNA-probes for a panel of species known to associate with severe periodontitis, such as Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans (29). Samples for testing with species-specific DNA probes by PCR or checkerboard method are easily transported in an empty vial. Bacterial culture is needed when sensitivity testing is required. For this purpose, paper points are submerged in anaerobic transport medium. It is noteworthy, that as spirochetes are unculturable by standard methods, Treponema denticola is never reported from culture samples, but instead can be found by molecular methods.

**Clinical relevance**

High-throughput sequencing studies have expanded our knowledge about the diversity of the oral microbiome by detecting a great number of as yet uncultured species both in health and disease. However, the clinical significance of these findings remains undetermined. In clinical practice, culture methods are still preferred for diagnostics of bacterial and fungal infections because culture allows susceptibility testing. In addition, nucleic acid detection methods have become available for detection of periodontitis-associated pathogens and viral infections.

**Mucosal infections**

Diagnosis of oral candidiasis is fundamentally clinical. Local or systemic immunosuppressive conditions and disturbances of the commensal bacterial microbiome favours Candida overgrowth and establishment of clinical infection (30). C. albicans is the predominant species detected both by culture and molecular methods in oral samples (31, 32).

Sampling is helpful in refractory symptoms, in cases of poor response to treatment, and if there is risk for systemic fungal infection in an immunocompromised host. A swap scrap, or imprint sample of the diseased mucosa or the biofilm on adjacent non-renewing surface, or an oral rinse sample can be taken for fungal culture and, if needed, sensitivity testing. Candida are generally susceptible to chlorhexidine, and polyene and azole antifungals. Importantly, however species such as, C. glabrata and C. krusei are commonly resistant to azoles (30, 31).

A biopsy is required for histopathological diagnosis of hyperplastic candidiasis as well as for differential diagnosis, or diagnosis of a coexisting mucosal disease. Candidal hyphae can be visualized from tissue samples with special stains such as Periodic acid-Schiff (PAS).

As Candida species are part of the commensal oral microbiota, detecting Candida in culture does not prove infection. Routine diagnostic culture methods only give a robust estimate of the amount of Candida but cannot reliably differentiate colonization from infection. Various mucosal diseases such as lichen planus or epidermoid cancer can clinically mimic candidiasis and lesions may be colonized with Candida. Therefore, the interpretation of the culture finding always has to be done by the clinician with careful consideration for differential diagnosis.

The role of bacteria is probably underestimated in mucosal infections. Especially Staphylococcus aureus and beta-hemolytic streptococci have been reported in patients with mucosal erythema, erosive lesions and burning sensation (33, 34). They are also common findings along with Candida species in angular cheilitis. The diagnosis of angular cheilitis is clinical. Both fungal and bacterial cultures can be made from swab samples to help direct the local antimicrobial treatment if needed.
Heavy growth of S. aureus and beta-hemolytic streptococci in a symptomatic patient should primarily be treated with local disinfectants, for example, chlorhexidine. Coliforms (e.g. Escherichia coli and Klebsiella spp) or Pseudomonas species are usually transient colonizers without any disease association and need not be targeted with antimicrobial agents at all. (33)

**Viral infections**

Herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus and enterovirus infections manifest as blisters or ulcers on the oral mucosa (35). Usually clinical diagnosis is enough, but for differential diagnosis, a swab sample from ulceration can be taken for viral culture, species specific PCR/RT-PCR or antigen detection. When using the very sensitive nucleic acid detection methods for diagnostics of herpesvirus infections, one should be careful to only sample patients on proper clinical suspicion. Otherwise there is risk for misinterpreting asymptomatic shedding of herpesviruses for infection.

If Kaposi’s sarcoma or oral hairy leukoplakia is suspected, a biopsy is required for histopathological diagnosis that can be complemented with immunohistochemistry with specific antibodies against viral proteins or in situ hybridization with virus species specific probes.

Currently, over 200 human papillomavirus (HPV) genotypes have been identified. HPV is a known cause of oral warts and condylomas (35), focal epithelial hyperplasia (HPV genotypes 13 and 32) (36), and for its association with a subset of squamous cell carcinomas of the oral cavity and oropharynx (oncogenic HPV genotypes, in particular HPV 16) (37). Molecular methods applied to tissue and brush samples are available for determination of HPV genotype. Currently this is not done routinely but is an emerging analysis as part of further characterization of head and neck squamous cell carcinomas in clinical setting as HPV status may have implications for disease management and prognosis.

Immunocompromised hosts are susceptible to Kaposi’s sarcoma-associated herpesvirus infections, oral hairy leukoplakia associated with Epstein-Barr virus, and cytomegalovirus induced mucosal ulcerations. Similarly, an unusually severe or wide-spread HSV, VZV or HPV infection may be suggestive of defective immune defence. Finding these conditions in a previously healthy patient should therefore prompt further investigations to rule out conditions like HIV or other causes for immunodeficiency.

**WRITING THE REFERRAL**

Oral bacterial and fungal samples pose a diagnostic challenge. The samples are practically always polymicrobial and composed of commensal oral microbiota, both in health and disease. Therefore, it is extremely important to convey all necessary information for the laboratory for optimising both culture conditions and reporting of findings.

Vital information includes sample type (mucosal sample, biopsy or pus aspirate), clinical suspicion of certain bacteria or fungi, and clinical diagnosis. Based on the referral, the laboratory makes the decision about growth conditions. Pus samples and deep samples are grown both under anaerobic and aerobic conditions whereas superficial samples from oral mucosa are only cultured aerobically. Knowing the clinical condition and sample type, the laboratory can use selective media in order to increase the recovery of certain bacteria, including Prevotella, Fusobacterium, Tannerella and Actinomyces species, or in case of periodontitis Aggregatibacter actinomycetemcomitans.

The referral has major influence on reporting of the findings. Oral commensal bacteria found in a superficial mucosal sample signifies absence of pathogenic microbes, but the same finding from a normally sterile compartment, such as bone or dental pulp, means that the sterile tissue has been colonized or infected with commensal oral microbes. In the former case the laboratory normally reports finding as normal flora whereas in the latter case, the prominent bacterial species or groups of bacteria will be reported with a susceptibility report. From mucosal samples, only bacteria that differ from commensal microbes and heavy growth of Candida will be reported as separate findings with antimicrobial sensitivities. If culture in periodontitis is needed, the sample should be sent to a laboratory specialized in oral microbiology.

**SUMMARY**

The immense diversity of the oral microbiota and the biofilm formation has implications for the treatment of oral infections. Microbiological sampling is indicated in severe and refractory cases. At present, the routine diagnostic method for oral bacterial and fungal infections is still culture whereas nucleic acid detection methods are widely used in virological diagnostics. The major advantage of culture over molecular methods is the possibility of performing antimicrobial susceptibility testing for bacteria and fungi. Although the laboratory report will not be available at the initiation of antimicrobial treatment of acute infections, the sample taken will help to redirect the treatment if the response is poor. Overall, culture samples allow surveillance of local antimicrobial susceptibility patterns. Therefore, the laboratories should continuously gather susceptibility data on the predominant bacteria discovered in dental infections. This is critically important for making appropriate treatment guidelines on the use of antimicrobials.

With the recent advances in high-throughput sequencing it might be possible to detect a larger proportion of the microbiota and their associated resistance genes. In the future this could lead to cost-effective diagnostic molecular methods for clinical microbiological laboratories. How the results should then be interpreted and applied to treatment decisions needs to be clarified in further studies on the oral microbiome in health and disease.
**ABSTRACT (DANSK)**

**ORAL MIKROBIOLOGI I MIKROBIOMÆRAEN – HVORNÅR, HVORDAN OGYHOFOR UDFØRE MIKROBIOLOGISK DIAGNOSTIK**

Forbedrede sekventeringsteknikker har ført til en markant stigning i kendskabet til den orale mikrobiota i løbet af det sidste år. Disse dyrkningsufhøngige teknologier har gjort det muligt at detektere bakterier, der endnu ikke kan dyrkes, og som udgør op til halvdelen af de omkring 700 arter, der til nu er identificeret i det orale mikrobiom. Tilsynden har det orale mykobiom vist sig at være meget mere forskelligartet end tidligere antaget. For øjeblikket foregår der sammenlignende undersøgelser af mikrobiomet ved sundhed og sygdom, hvor både mikrobielle arter og deres funktionelle egenskaber afdækkes. Implikationerne for diagnostik og behandling er endnu uafklarede.


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