

Application of Metagenomic Analyses in Dentistry as a Novel Strategy Enabling Complex Insight into Microbial Diversity of the Oral Cavity

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Abstract

The composition of the oral microbiome in healthy individuals is complex and dynamic, and depends on many factors, such as anatomical location in the oral cavity, diet, oral hygiene habits or host immune responses. It is estimated at present that worldwide about 2 billion people suffer from diseases of the oral cavity, mainly periodontal disease and dental caries. Importantly, the oral microflora involved in local infections may spread and cause systemic, even life-threatening infections. In search for etiological agents of infections in dentistry, traditional approaches are not sufficient, as about 50% of oral bacteria are not cultivable. Instead, metagenomic analyses are particularly useful for studies of the complex oral microbiome – both in healthy individuals, and in patients with oral and dental diseases. In this paper we review the current and future applications of metagenomic studies in evaluation of both the composition of the oral microbiome as well as its potential pathogenic role in infections in dentistry.

Key words: dental caries, endodontics, metagenomics, periapical abscess, periodontitis

Introduction

The oral microbiome plays a very important role both in health as well as in disease (Duran-Pinedo and Frias-Lopez, 2015; Struzycka, 2014; Xu and Gunsolley, 2014). It is now known – based on 16S rRNA gene sequence analyses – that the bacterial flora of the oral cavity exceeds 1000 taxa (Dewhirst *et al.*, 2010; Zehnder *et al.*, 2015). It consists of “protective” bacteria, transient invaders and opportunistic microflora of specific niches in the oral cavity (Zehnder *et al.*, 2015).

Even in healthy individuals the composition of the oral microbiome is complex and dynamic, depending on many factors, such as anatomical location in the oral cavity (supragingival or subgingival plaque, tongue, mucous membrane lining the oral cavity), diet, oral hygiene habits or host immune responses (Xu and Gunsolley, 2014). Disruption of a symbiotic relationship between the oral microbiome and the host results in dysbiosis, which may cause overgrowth of pathogenic microflora and diseases of the oral cavity (Beli-

basakis and Mylonakis, 2015). Furthermore, oral health may affect the health status of the host leading to systemic infections.

It is now estimated that about 2 billion people suffer from oral diseases, such as periodontal disease and dental caries. They are therefore some of the most prevalent infectious diseases of humans (Xu and Gunsolley, 2014; He *et al.*, 2015). Importantly, the oral microflora may cause not only local infections, but contributes also to the pathogenesis of systemic – even life-threatening infections – such as infective endocarditis, bacterial meningitis or brain abscess (Hsiao *et al.*, 2012; Maurer *et al.*, 2009; Mang-de la Rosa *et al.*, 2014). However, microbial composition in different conditions affecting the oral cavity remains unknown. The use of traditional microbiological methods (culturing and identification of microorganisms) is unsatisfactory, as it is known at present that at least 50% of the oral microflora cannot be cultured, as revealed by genetic studies, including metagenomic strategies. Furthermore, microbial populations may be studied with the use of modern “omics”

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techniques combined with thorough bioinformatics analyses, detecting not only metagenome (DNA-based analysis), but also metatranscriptome (RNA level), metaproteome (protein composition of the oral microbiome) and metabolome (functional activity of a studied microbial population) (Duran-Pinedo and Frias-Lopez, 2015).

Amongst the above mentioned analytical strategies, metagenomic analyses are particularly useful for studies of the oral microbiome. They allow not only evaluation of bacterial composition in different locations and conditions in the oral cavity, but also contribute to the detection of novel, not yet described, but potentially pathogenic species. Furthermore, they allow detection of even non-viable bacteria (Belda-Ferre *et al.*, 2012).

Metagenomics strategies

Metagenomics – while being still a relatively novel science – has already helped to disclose many complicated, and often unexpected, relationships between the human microbiome and diseases it may cause (Padmanabhan and Wang, 2013; Alcaraz *et al.*, 2012; Xu and Gunsolley, 2014). Originally, metagenomics mostly found application in ecological analyses, and it was called environmental genomics or ecogenomics. However, currently it finds a broad application also in

medical sciences, as clinical metagenomics revealed to be useful in detection and analysis of non-cultivable microorganisms (both commensal and pathogenic) and their mutual relationships in the community which they form within the host or in the particular anatomical location.

Microbial metagenomics may involve one of two possible strategies (Fig. 1). The first one is a targeted strategy called deep amplicon sequencing (DAS). It employs a pre-sequencing PCR amplification step, during which a particular taxonomic marker (*e.g.* 16S rRNA, *recA* or *rpoB* gene) is amplified selectively. Afterwards, the thorough bioinformatic analysis and assignments of the assembled, individual reads into the appropriate operational taxonomic units (OTUs, clustering closely related individuals into one group), further taxonomic classification is possible. This leads to answering the question “Who is there?”, *i.e.* enables a deep insight into overall microbial diversity of the particular environment. Although, the DAS strategy is slightly biased, mainly due to the preliminary PCR step, the progress in sequencing technologies and *in silico* analytical methodologies currently allows efficient taxonomic community characterization with application of this strategy and makes DAS being commonly used in various environmental and clinical applications (Scholz *et al.*, 2012; Turaev and Rattei, 2016).

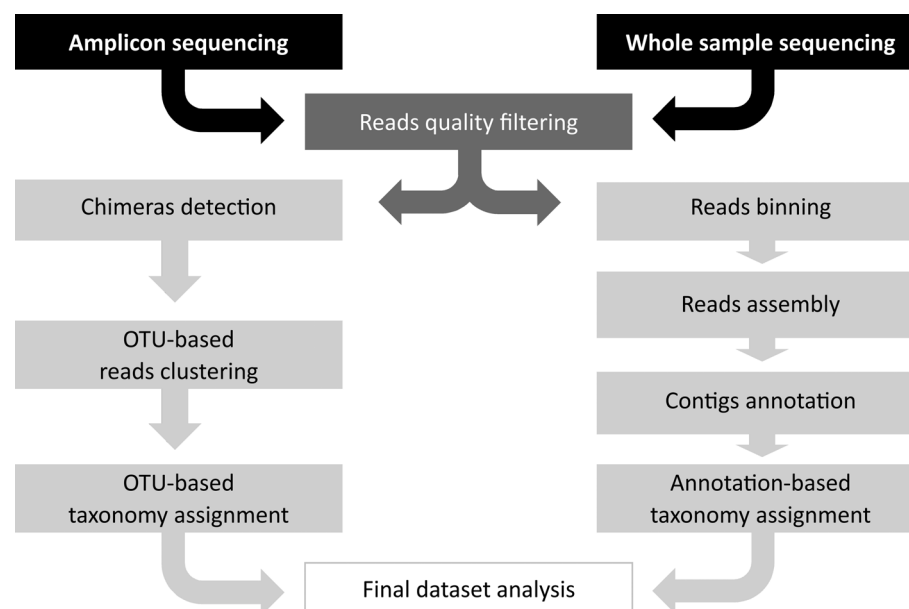


Fig. 1. Metagenome sequencing strategies.

Two types of metagenomic sequencing approaches, amplicon (on the left) and whole sample (on the right) sequencing, require specific library preparation and provide different sequencing data. The first step, common for both strategies, involves adaptor and quality-based reads trimming and length filtering. Amplicon sequencing, the strategy based on pre-PCR amplification of specific *loci* may result in point mutations and chimeras creation during that step. The later one should be filtered from the analyzed dataset as they could result in misleading biodiversity distribution. Then, the reads (or assembled read pairs) are clustered into operation taxonomy units (OTUs) based on their percentile identity between each other, *i.e.* 97% commonly used for 16S rRNA gene. Afterwards, representative sequences for each OTU are assigned to proper taxonomy group based on sequence similarity. The whole sample sequencing does not require previous pre-library DNA processing as the whole genomic DNA, including plasmid and phage DNAs, is sequenced. This kind of approach results in much bigger dataset. The reads are binned (sorted) into groups that might refer to individual genomes. After that, the reads may be assembled using either *de novo* (preferred for environmental samples) or reference-based assembly. Resulting contigs are then annotated and their taxonomy is assigned and used for biodiversity calculations.

The second metagenomics strategy is much broader, as it leads to answering not only the question “Who is there?”, but also “What are they doing?”. This strategy is known as shotgun metagenomics. According to this approach after the isolation of the total DNA from a particular sample, the total nucleic acid content of a sample is sequenced either directly or after applying an enrichment step, which might be a capture-based approach or subtraction prior to sequencing. The application of the high-throughput sequencing leads to generation of enormous number of short reads, which in the first step of bioinformatic analysis have to be assembled into contigs. Then, their taxonomic classification and functional assignments may be performed (Scholz *et al.*, 2012).

Both above mentioned strategies find application in analyses of the oral cavity microbiomes. However, many researchers underline the need for standardized sampling methods for metagenomic studies of the oral microbiome. This would ensure reliable results, which will make it possible to compare the microbiome in different intraoral locations and in diverse clinical conditions of health and disease. Bacterial flora composition may be influenced by many factors, such as anatomical location (*e.g.* soft palate, hard palate, tongue, tooth surface, supra- or subgingival sample) or other factors (*e.g.* diet, smoking and oral hygiene habits) (Xu and Gunsolley, 2014; Wu *et al.*, 2016). Xu and Gunsolley (2014) also indicate that sampling methods differ significantly. In dental caries specimens should be taken precisely from the affected tooth, avoiding contamination from subgingival sites. On the other hand, there is a risk of human DNA contamination of the samples taken from periodontitis lesions (Xu and Gunsolley, 2014). Even different tools for supragingival sample collection (*e.g.* cotton swabs *vs* loop-like devices) may influence the results of metagenomic studies (Xu and Gunsolley, 2014).

Bacterial metagenome in dental caries

Dental caries is one of the most common diseases in many parts of the world despite a decline in its rate in some regions due to prevention programmes (Belibasakis and Mylonakis, 2015; Gross *et al.*, 2012; Gooch *et al.*, 2009; Edelstein, 2006; Petersen *et al.*, 2005). It may affect even very young children shortly after the eruption of their milk teeth. It is estimated by the WHO, that worldwide 60–90% of school children and nearly 100% of adults have dental caries (WHO, 2012). As indicated above, clinically it can have a severe course, which may even require hospitalization, with some fatal cases (Gross *et al.*, 2012; Colak *et al.*, 2013). Similarly, in adults dental caries may cause severe, even life-threatening complications. Most authors believe that dental caries constitutes an infectious, transmissible and

polymicrobial disease, which results from a shift within the biofilm community of the oral cavity, however its etiology and pathogenesis remain unsolved (Gross *et al.*, 2012; Simon-Sorro *et al.*, 2014; Belibasakis and Mylonakis, 2015).

At present there are three major hypotheses of the etiology of dental caries: the specific, the non-specific, and the ecological plaque hypothesis (Aas *et al.*, 2008; Xu and Gunsolley, 2014). Therefore, verification of these hypotheses is urgently needed and possible with the use of modern molecular techniques comprising metagenomic analyses of the oral microbiome. This would contribute to an improvement in diagnosis, treatment and prevention of caries and its complications, such as dental pulp necrosis and periapical abscess (Alcaraz *et al.*, 2012; Belda-Ferre *et al.*, 2012).

According to the specific plaque hypothesis, only a few bacterial species, such as *Streptococcus mutans* and *Streptococcus sobrinus*, are actively involved in the initiation of dental caries (Alcaraz *et al.*, 2012; Xu and Gunsolley, 2014; Karpinski and Szkaradkiewicz, 2013; Kuramitsu and Wang, 2011). Apart from mutans streptococci, also lactobacilli and *Actinomyces* spp. may contribute to the development of dental caries (Beighton, 2005). There is, however, a conflicting opinion for and against this hypothesis as some authors claim that 10% of subjects with rampant caries in permanent teeth do not have detectable levels of *S. mutans* (Aas *et al.*, 2008). It is even postulated by some researchers that the association of mutans streptococci and caries is weak and no greater than for other bacteria, and that the mere presence of *S. mutans* and *S. sobrinus* in dental plaque does not account alone for the cariogenic potential of such biofilms, as caries occurs in the absence of these species and their presence does not necessarily indicate caries activity (Gross *et al.*, 2012; Beighton, 2005; Belda-Ferre *et al.*, 2012; Kuramitsu and Wang, 2011; Simon-Sorro *et al.*, 2014).

The non-specific plaque hypothesis maintains that caries is caused by a heterogenous mixture of many bacterial species and results from the overall activity of the total plaque microflora (Aas *et al.*, 2008). Other dental plaque bacteria – apart from mutans streptococci, lactobacilli and *Actinomyces* spp. – also possess some characteristics thought to be important in cariogenicity. It seems likely that interaction of different bacteria may cause initiation of caries, and therefore the plaque flora may be non-specific in nature.

The ecological plaque hypothesis suggests that cariogenic flora of the oral microbiome constitutes only a minority of the total community and caries results from an imbalance of the metabolic activity of the resident microflora in the dental biofilm, due to changes in local environmental conditions (Astorga *et al.*, 2015). A diet rich in carbohydrates causes prolonged pH

change, which promotes tooth demineralisation and the growth of acid-tolerant and acid-producing bacteria (*e.g.* mutans streptococci and lactobacilli), while eliminating acid-labile species (Astorga *et al.*, 2015).

In a recent study Zhou *et al.* (2016) applying high-throughput metagenomic sequencing reported that in dental caries a synergistic effect may influence microbial community assembly and the co-prevalence of the pathogenic genera. In contrast to these findings, in caries-free individuals the authors found that the function of clustered genera was more random and competition appears to play a more significant role in the oral microbiome. They also found, that the following genera were more abundant in the caries group in comparison to healthy subjects: *Veillonella*, *Bifidobacterium*, *Selenomonas*, *Olsenella*, *Parascardovia*, *Scardovia*, *Chryseobacterium*, *Terrimonas*, *Burkholderia* and *Sporobacter*.

Metagenomic studies help to elucidate the potential role of oral bacteria in the initiation and establishment of a dental plaque. Many authors report that *S. mutans* is not present in all patients with dental caries (Gross *et al.*, 2012). Instead, in these individuals other streptococci are predominant (*e.g.* *Streptococcus salivarius*, *S. sobrinus*, and *Streptococcus parasanguinis*) as well as strains of *Veillonella* spp. Detailed metagenomic analysis may therefore contribute to modification of current treatment of this disease and establishment of effective prophylactic measures.

Bacterial diversity in endodontics and purulent complications of severe dental caries

Progression of dental caries may cause pulpitis, infection of the root canal and tooth necrosis (Belibasakis and Mylonakis, 2015; Zehnder *et al.*, 2015). Further expansion of the infection may lead to periapical abscess and apical periodontitis (Narayanan and Vaishnavi, 2010). The course of disease appears to depend on the interaction between the microbial flora and the host's immune system (Zehnder *et al.*, 2015).

It is estimated that periapical abscesses and accompanying pain (which can be excruciating) constitute about 56% of all non-traumatic dental emergencies (Hsiao *et al.*, 2012). Infection may complicate the outcome of endodontic treatment and the survival of the tooth (Hsiao *et al.*, 2012). Furthermore, pathogens involved in purulent complications may spread to the circulation, causing systemic diseases and infections in anatomically distant organs and sites (Pappa and Jones, 2005; Sequeira and Rocas, 2013; Robertson, 2015). However, despite major progress in endodontic techniques and many chemicals being available for root canal treatment, periapical abscesses remain the main cause of tooth loss and severe, even life-threatening complications.

Periapical abscesses constitute an enclosed environment, separated from the oral cavity. Indeed, recent metagenomic analyses revealed that bacterial composition in the root canal and abscess samples differs significantly from the microflora present in the oral cavity (Hsiao *et al.*, 2012; Tavares *et al.*, 2010). Metagenomic studies are therefore needed to characterize bacterial flora present in the endodontic system and in periapical abscesses in order to establish their etiology and proper treatment (Ribeiro *et al.*, 2011). This can be done by 16S rDNA sequence analysis. Using this approach Ribeiro *et al.* (2011) were able to detect in samples from root canals of 12 untreated asymptomatic teeth, on average 10 different bacterial taxa per root canal (range: 3–21), out of which as many as almost 66% represented non-cultivable bacteria. Earlier, Siqueira *et al.*, (2000) identified up to 17 taxa in a single root canal.

Molecular studies made it possible to detect uncultivable bacteria present in root canals of teeth with apical periodontitis, such as *Spirochaetes*, *Synergistetes* and *Dialister* (Munson *et al.*, 2002; Zehnder *et al.*, 2015). Recent approaches using 16S rRNA gene pyrosequencing revealed high diversity of bacteria in the apical portion of infected root canals (Siqueira and Rocas, 2009; Siqueira *et al.*, 2011). It now appears that bacterial flora present in different types of endodontic infections comprises as many as >460 bacterial taxa, classified in 100 genera and 9 phyla (Siqueira and Rocas, 2009). Most of them represent *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. Tavares *et al.* (2011) evaluated the microbiota of 32 samples obtained from the root canal system of deciduous teeth with pulp necrosis. In their study the mean number of species detected was 19 per sample, with a range from 3 to 66.

Santos *et al.* (2011) examined the root canal content of 8 teeth with chronic apical periodontitis and compared it with the aspirate from 9 abscesses of endodontic origin. They found, using a high-throughput multiplexed 16S rRNA gene pyrosequencing analysis that bacteria from the genus *Peptostreptococcus*, but also *Fusobacterium*, *Atopobium*, *Parvimonas*, *Dialister*, *Porphyromonas* and *Prevotella* were much more common in abscesses as compared to chronic root canal infections. It stands in agreement with a study by Sequeira and Rocas (2009), who found that the most prevalent species in apical abscess aspirates from 42 patients were *Fusobacterium nucleatum*, *Parvimonas micra* and *Porphyromonas endodontalis*. Other common taxa were *Olsenella uli*, streptococci and *Eikenella corrodens*. In another study Sequeira and Rocas (2013) confirmed that the most common genera found in acute apical abscesses are *Fusobacterium*, *Parvimonas*, *Prevotella*, *Porphyromonas*, *Dialister*, *Streptococcus*, and *Treponema*. On the other hand, Hsiao *et al.* (2012) found in 8 patients that although strains of *Prevotella* spp. and

Fusobacterium spp. were most prevalent in samples taken from the diseased endodontic sites, *Streptococcus* spp. were not common in these specimens. In this study, using next-generation sequencing of 16S rDNA amplicons, they found that the most common microbial species present in the samples from endodontic lesions were *Granulicatella adiacens*, *Eubacterium yurii*, *Prevotella melaninogenica*, *Prevotella salivae*, *Streptococcus mitis*, and *Atopobium rimae* (Hsiao *et al.*, 2012). In a study by Ribeiro *et al.* (2011) in 12 samples from root canals the most prevalent bacterial species identified by metagenomic investigations were *Atopobium rimae*, *Dialister invisus*, *Prevotella oris*, *Pseudoramibacter alactolyticus*, and *Tannerella forsythia*. In a study of 32 root canal samples from deciduous teeth with pulp necrosis, the most prevalent taxa were *Prevotella intermedia* (96.9%), *Neisseria mucosa* (65.6%), *Prevotella nigrescens* (56.2%) and *Tannerella forsythia* (56.2%), while *Aggregatibacter (Haemophilus) aphrophilus* and *Helicobacter pylori* were not detected (Tavares *et al.*, 2011).

It remains to be evaluated on a larger group of patients what is the etiological role of particular bacteria – or their specific compositions – in endodontic infections. Multiple species present in the root canals and/or periapical abscesses may result in network of interactions, which may affect their pathogenicity (Siqueira and Rocas, 2013). Understanding of the microflora associated with different forms of endodontic infections is necessary for improvement of the success of endodontic treatment (Narayanan and Vaishnavi, 2010).

Metagenomic studies in periodontal diseases

Periodontal disease may be defined as a cluster of infectious inflammatory conditions (gingivitis and different forms of periodontitis) which in a severe form may affect even 10–15% of the global population and is the major cause of tooth loss in adults (Belibasakis and Mylonakis, 2015). Apart from genetic factors, its development is strongly related to the polymicrobial biofilm formed by oral bacteria on the tooth surface, which stimulates pro-inflammatory responses in the surrounding tissues and their destruction (Liu *et al.*, 2012; Belibasakis and Mylonakis, 2015). It can often resolve by good oral hygiene which eliminates the biofilm formed by bacteria (Xu and Gunsolley, 2014). On the contrary, built-up of bacteria may contribute to development of severe periodontitis, which may lead to systemic complications, such as infective endocarditis as well as diabetes, pneumonia, low birth weight in infants, inflammatory bowel disease, systemic autoimmune disease and colon cancer, to name a few (Xu and Gunsolley, 2014; Han and Wang, 2013; Moodley *et al.*, 2013; Zarco *et al.*, 2012; He *et al.*, 2015; Liu *et al.*, 2012). Apart from insufficient

oral hygiene, other modifiable risk factors have been identified, such as tobacco use, excessive alcohol consumption, poor diet and nutrition, obesity and psychological stress, which contribute to periodontal disease prevalence (Petersen and Baehni, 2012).

Several metagenomic studies have indicated that samples of subgingival plaque from periodontitis patients contained different flora than in healthy subjects (Liu *et al.*, 2012; Xu and Gunsolley, 2014; Griffen *et al.*, 2012). Furthermore, a shift has been demonstrated in the oral microbiome from Gram-positive bacteria predominant in healthy individuals to Gram-negative microflora in patients suffering from periodontal disease. Indeed, Wang *et al.* (2013) observed that strains of *Streptococcus* spp. (13.7–41.3%), *Haemophilus* spp. (2.0–25.8%), *Rothia* spp. (0.9–16.7%), and *Capnocytophaga* spp. (3.1–13.0%) predominated in samples from individuals without periodontitis, while other genera comprised less than 10.0% of the microflora. In contrast, in specimens from individuals with periodontal disease the most prevalent were strains of *Prevotella* spp., which amounted to 14.4–44.7% of the bacterial communities.

The predominant species isolated from dental plaques from patients with periodontal disease are *Porphyromonas gingivalis* (which has many virulence factors and an ability to evade the host's immune response), *Aggregatibacter actinomycetemcomitans* (causing aggressive periodontitis), *Treponema denticola*, and *Tannerella forsythia* (which may have invasive properties). Similarly, Wang *et al.* (2013) showed a higher proportion of anaerobic Gram-negative bacteria classified in the genera *Prevotella*, *Leptotrichia*, *Veillonella*, *Porphyromonas*, and *Treponema*, in samples from periodontitis patients in comparison to microflora in samples from healthy individuals. Therefore, these species were considered by many authors as specific pathogens of periodontal disease. It should be noted, that further microbiological studies revealed a strong correlation between periodontal disease and the proportions of some bacteria cultured from dental plaques, *e.g.* *Prevotella intermedia*, *Fusobacterium nucleatum*, *Selenomonas noxia*, *Aggregatibacter actinomycetemcomitans*, and *Eubacterium nodatum* (Slots and Genco, 1984; Tanner, 2015). However, the use of culture-independent molecular techniques allowed to identify other groups of bacteria prevalent in samples from patients with periodontal disease, including the following genera: *Megasphaera*, *Parvimonas*, *Desulfobulbus*, and *Filifactor* (Kumar *et al.*, 2005; Colombo *et al.*, 2009).

Elucidation of pathogenesis of periodontitis and an association between its progression and specific pathogens – or their composition – require further studies, particularly metagenomic analyses (Wang *et al.*, 2013; Jorth *et al.*, 2014). Wang *et al.* (2013) used metagenomic sequencing of 16 samples from patients with 4 different

clinical forms of periodontal disease to evaluate functional potential of detected bacterial microflora. They observed a strong correlation between the composition of identified microflora and periodontal disease status. They also claim that they were successful in identifying an essential (“core”) disease-associated set of bacterial taxa. Jorth *et al.* (2014) confirmed a high diversity of microbial flora composition in patients with periodontitis, however they found that disease-associated communities exhibit conserved changes in metabolic profiles and virulence gene expression.

Surprisingly, recent studies suggest that bacterial species present in low quantities in oral samples cannot be ignored as they may play a significant role in the oral microbiota, including inflammatory processes observed in periodontitis (Kawamura and Kamiya, 2012; Hajishengallis *et al.*, 2011; Wang *et al.*, 2013). Using a metagenomic approach, Wang *et al.* (2013) identified low-abundance genera, which were associated with periodontitis, including *Alistipes*, *Bulleidia*, *Butyrivibrio*, and *Parabacteroides*. They also claim that several functional genes and metabolic pathways (*e.g.* bacterial chemotaxis, flagellar assembly, and toxin biosynthesis) were over-represented in the microbiomes in periodontal disease, in comparison to the oral microflora in healthy individuals (Wang *et al.*, 2013). Furthermore, they found a large number of phages in samples from both healthy individuals and patients with periodontal disease. They postulate that phages may modify the oral microflora and therefore may also play an indirect role in the pathogenesis of oral diseases.

The future of metagenomics in dentistry

Metagenomics has the potential to revolutionize clinical diagnostics (Miller *et al.*, 2013). It enables simultaneous detection of all microorganisms in a clinical sample, including uncultivable, rare and novel pathogens. Furthermore, metagenomic techniques may help explain the role of variability in microbiome composition and function in relation to pathogenesis of infectious diseases.

Metagenomic analyses have been done so far on a relatively small number of dental patients and healthy individuals. Further studies are therefore urgently needed to establish true composition of the oral microbiome in health and disease. Metagenomic and related molecular techniques also offer perspectives for evaluation of pathomechanism of different dental infections and subsequently proper management of them. Function-based metagenomic analyses have already helped to discover new resistance mechanisms and potential targets for antimicrobial therapy, therefore evaluation of the oral resistome is very important (Sukumar *et al.*,

2016; Tansirichaiya *et al.*, 2016). As Zarco *et al.* (2012) stated, metagenomics may contribute not only to more effective diagnostic and therapeutic techniques, but also to personalized dental medicine. This may help to develop effective prophylaxis of oral diseases, such as dental caries and periodontitis, which according to the recent estimates affect as much as a quarter of the world’s human population.

Conflict of interest

The authors declare that there are no conflicts of interest.

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