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Microbiological Diagnosis for Periodontal Diseases

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1. Introduction

Periodontitis, an infectious disease caused by bacteria, brings about destructive changes leading to loss of bone and connective tissue attachment (Williams, 1990). Several oral bacteria are considered to be possible pathogens in periodontitis (Darveau et al., 1997). In particular, the black-pigmented, Gram-negative anaerobic rods *Porphyromonas gingivalis* and *Tannerella forsythia* have been implicated as major pathogens in the etiology of this disease. These two species are frequently isolated together, implying the existence of an ecological relationship between these organisms (Darveau et al., 1997). *Treponema denticola*, a helical oral spirochete, has also been considered as a major pathogen in periodontitis (Darveau et al., 1997). Mixed infection with these three bacteria in periodontal sites is correlated strongly with the severity of adult periodontitis (Socransky & Haffajee, 1998). Socransky named this combination the “red complex” and found that these bacteria were most crucial for the progression of this disease (Socransky & Haffajee, 1998). Thus, the detection of these organisms provides essential information about the severity of periodontitis. *Aggregatibacter actinomycetemcomitans* is suspected to be the most probable causal factor for aggressive periodontitis in adolescents (Darveau et al., 1997).

Although we cannot completely rule out the possibility of exogenous infection, periodontitis is thought to be primarily an endogenous infection caused by oral bacteria. Various systems for the detection of oral pathogens have been reported, but most are qualitative (Yoshida et al., 2005a; Yoshida et al., 2005b). Because periodontal pathogens exist not only in infected pockets but also in the healthy sulcus, qualitative detection is not suitable for the diagnosis of periodontitis. For this purpose, we have developed a quantitative detection system that uses real-time polymerase chain reaction (PCR) methodology (Yoshida et al., 2003a; Yoshida et al., 2003b).

The best time for the detection of oral bacteria remains unclear. When during the periodontal treatment process should a diagnostic system be used? Can a quantitative detection system be used for the initial diagnosis of periodontitis? Furthermore, periodontitis is influenced by multiple factors such as genetic, environmental, and lifestyle-related factors that complicate the determination of a microbial cut-off value for disease onset. The use of microbiological detection for the initial diagnosis of periodontitis is thus likely to be of limited value. Nevertheless, microbiological diagnosis is meaningful in evaluating the effects of periodontal therapy. During periodontal therapy, factors

associated with the etiology of periodontitis, other than microbiological factors, are relatively stable, whereas the number of bacteria is variable. Previously, we found a positive relationship between pocket depth and *P. gingivalis* and *T. denticola* counts and percentages, and the cell numbers were significantly lower after initial periodontal treatment compared with before treatment, which included scaling, tooth-brushing instruction, and professional mechanical tooth cleaning (Kawada et al., 2004; Yoshida et al., 2004).

A microbiological diagnosis involving bacterial detection can be useful for periodontal treatment. However, before considering these applications, the purpose of bacterial examinations in the course of treatment and the etiology of periodontitis must be understood. In this chapter, we describe the factors associated with the diagnosis of periodontitis and discuss the role of microbiological diagnosis in periodontal treatment.

2. Periodontal disease as an infectious disease

Previous investigations have revealed that periodontal disease is an infectious disease caused by oral bacteria and that it has complex associations with immunological, genetic, and environmental factors (Williams, 1990). It also is associated closely with dental plaque, which has been recognized as a biofilm contributing to representative oral diseases such as dental caries and periodontal disease (Keyes & Likins, 1946). The features of periodontitis as an infectious disease are listed in Table 1.

1.	Endogenous infection by normal oral microbiological flora
2.	Mixed infection by various normal oral microbiological flora
3.	<i>Porphyromonas gingivalis</i> , <i>Tannerella forsythia</i> , <i>Treponema denticola</i> , and <i>Aggregatibacter actinomycetemcomitans</i> as possible causative bacteria
4.	Biofilm-associated infectious disease caused by subgingival microflora

Table 1. Features of periodontitis as an infectious disease.

2.1 Etiology of periodontal disease

Numerous bacterial products are released in the crevice fluid in the periodontal pockets. This fluid contains histiolytic enzymes, endo- and exotoxins, and nontoxic materials that interfere with cell function. Of these, collagenase and other proteases released by bacteria in the periodontal pockets are related to the features of periodontitis, such as the extensive destruction of collagen and the connective-tissue matrix (Kuramitsu, 1998). Bacterial lipopolysaccharide can also induce bone destruction (Miyata et al., 1997). Low-molecular-weight metabolites released by oral bacteria such as sulfides are considered to be cytotoxic molecules in the periodontium (Socransky, 1990). On the other hand, some bacteria can inactivate a specific antibody, which enables them to prevent their own death by phagocytosis. *A. actinomycetemcomitans* produces a leukotoxin that specifically kills human leukocytes (McArthur et al., 1981). Thus, some bacteria can inhibit the normal immune-defense system of the host. The bacterial etiological agent is pathogenic because of its capacity to induce response mechanisms that destroy periodontal tissue. Bacterial

substances can thus directly and indirectly destroy periodontal tissues, and it is difficult to distinguish “good” from “bad” bacteria because one bacterial species may behave both beneficially and destructively in humans. However, some bacteria are considered to be periodontopathic due to the production of etiological agents; the monitoring of these pathogens is important in periodontal treatment.

2.2 Infection mechanism of periodontal disease

Periodontal disease is characterized by inflammation caused by periodontopathic bacteria in the subgingival plaque. In general, periodontal infection is thought to be endogenous. In contrast to an exogenous infection, endogenous periodontal infection involves the internal proliferation of the normal bacterial flora in the oral environment. This significantly influences the potential use of microbiological examinations in the diagnosis of periodontal disease, as will be described later. Periodontopathic bacteria proliferate in periodontal environments such as the sulcus and induce inflammation around the periodontium. Both vertical transmission (e.g., between child and mother) and horizontal transmission (e.g., between husband and wife) of periodontopathic bacteria are commonly observed (Kobayashi et al., 2008).

Periodontitis usually involves infection with a combination of oral bacteria, and several specific bacterial species are suspected as contributors to this disease. *Porphyromonas gingivalis*, a Gram-negative anaerobic rod, is thought to be a major pathogen in adult and aggressive periodontitis. *Tannerella forsythia*, another Gram-negative anaerobic rod, and *Treponema denticola*, an oral spirochete, are associated with adult periodontitis, whereas *A. actinomycetemcomitans*, a Gram-negative anaerobic rod, is related to aggressive periodontitis. Socransky reported that a “red complex” of three bacteria, *P. gingivalis*, *T. forsythia*, *T. denticola*, is associated with the severity of periodontitis (Socransky & Haffajee, 1998). Oral bacterial examinations to monitor periodontal status generally focus on these three bacteria.

2.3 Bacterial examination of periodontal disease

To date, many detection methods for bacteria in periodontal disease have been reported (Suzuki et al., 2004a; Suzuki et al., 2004b; Yoshida et al., 2003a; Yoshida et al., 2003b). Representative methods for the microbiological examination of periodontal disease are shown in Table 2. The selection of a suitable examination method requires the definition of clear objectives for the results. For example, in order to confirm the horizontal or vertical transmission of a specific periodontal pathogen or to select appropriate antibiotics, the presence of target bacteria must be determined.

Bacterial examination methods that detect the presence of bacteria, but not the amount, are termed qualitative examinations. Owing to the endogenous nature of periodontal infection, periodontal bacteria often exist in both healthy gingival sulcus and diseased periodontal pockets, making qualitative methods unsuitable for the diagnosis of periodontal disease. We propose that the most important application of microbiological examination in periodontal disease is in monitoring changes in bacterial numbers after periodontal treatment compared with before treatment, providing an assessment of the effectiveness of periodontal treatment. For this purpose, quantitative bacterial examinations are required.

Method	Principle	Advantages	Disadvantages	Comments
Culturing	Culturing of oral specimens on a medium	Detection of viable bacteria. Antibiotic sensitivity.	Unculturable bacteria. Requires bacteriology skill.	Important for antibiotic selection.
Enzymatic	Measurement of enzymatic activities produced by oral bacteria	Rapid and low-cost method.	Cannot identify bacterial species.	Commercial kits are available.
Immunological	Detection of specific bacteria using antibodies	Available for specific bacteria.	Cannot discriminate between living and dead cells.	Requires special techniques.
Conventional PCR	Detection of bacteria by DNA amplification	High sensitivity, qualitative analysis.	Same as above. Quantitative detection is not available.	Requires a thermal cycler.
Real-time PCR	Detection of bacteria by DNA amplification	High sensitivity, quantification.	Cannot discriminate between living and dead cells.	Requires a thermal cycler.
Loop-mediated isothermal amplification (LAMP)	Isothermal DNA amplification	High sensitivity, isothermal amplification, visual detection.	Same as conventional PCR.	Developed by Eiken Chemical Co., Ltd.

Table 2. Representative microbiological examination methods in dental practice.

3. Microbiological examination methods for periodontal disease

In selecting the appropriate microbiological examination method, the objectives and purposes of the analysis must be defined, as specimen collection procedures vary according to the goals of the assessment. Clinical specimens to be analyzed for a patient’s periodontopathic bacterial levels should be collected from the saliva or tongue coat. Saliva samples should be diluted with phosphate-buffered saline (PBS), and salivary components and debris must be removed by centrifugation before the sample is analyzed. Tongue-coat samples are collected from the tongue dorsum and suspended in PBS, and debris is then removed by centrifugation. For the analysis of bacteria in specific periodontal pockets, subgingival plaque or crevicular fluid samples are suitable. To collect subgingival plaque, a paper point is inserted into the periodontal pocket and then transferred to a tube containing PBS; the subgingival plaque is suspended, and debris is removed by centrifugation (Fig. 1). Properly prepared samples can then be analyzed by qualitative and quantitative methods.

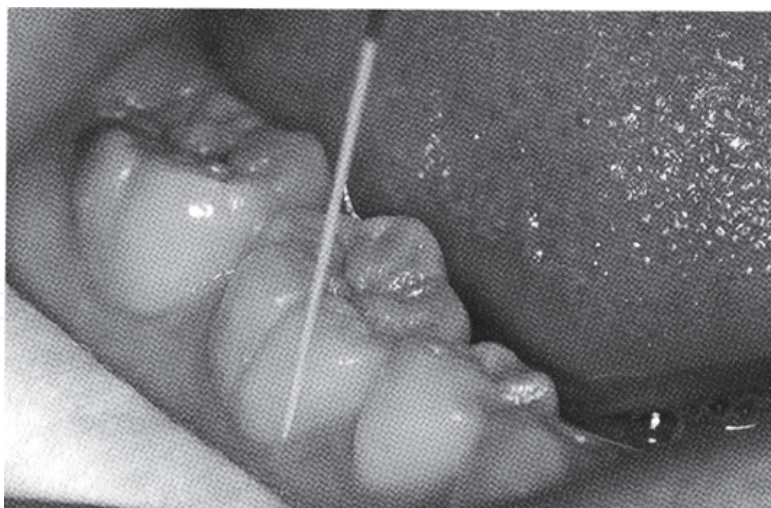
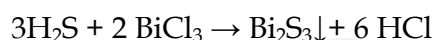


Fig. 1. Sampling of the subgingival plaque using by paperpoint.

3.1 Qualitative examination of periodontal disease

Both enzymatic and PCR-based methods are often used for the qualitative examination of periodontal bacteria. Enzymatic methods do not require special technology or equipment, are relatively inexpensive, and are commercially available as kits (Schmidt et al., 1988). However, because enzymatic methods identify only a group of bacteria associated with periodontitis and not specific bacteria, these analyses are not helpful in the selection of antibiotics.

We previously developed a detection system for hydrogen sulfide (H_2S), a causative agent for oral malodor produced by bacteria, especially periodontopathic bacteria (Yoshida et al., 2009). This type of detection system can be used to evaluate treatment efficiency even when specific bacteria cannot be identified, providing that the treatment objectives and detection targets are the same. Hydrogen sulfide produced by oral bacteria reacts with bismuth chloride to form bismuth sulfide as a black precipitate, as described by the following reaction:



Hydrogen sulfide-producing bacteria can be detected by measuring the absorbance of the black precipitate. As shown in Fig. 2, these precipitates are detectable in small subgingival plaque samples from periodontal pockets, obtained using paper points. This system for the comprehensive detection of hydrogen sulfide-producing bacteria can be used to evaluate the elimination of these organisms.

On the other hand, PCR techniques are relatively sensitive and can be used with species-specific primers to identify specific bacteria (Yoshida et al., 2005b). A major disadvantage of PCR techniques is that they cannot discriminate between viable and dead bacteria, because PCR methods use chromosomal DNA as a template. This makes PCR techniques unsuitable for sensitivity tests guiding the selection of antibiotics. A modification of the PCR method, loop-mediated isothermal amplification (LAMP), was developed by Eiken Chemical Co., Ltd. (Japan). LAMP reactions are performed under isothermal conditions, in contrast to the thermal cycling necessary for PCR. In addition to this advantage, LAMP technology has a rapid analysis time of about 1 h and requires no special detection equipment, as the results

can be observed by the naked eye (Fig. 3). Using this technology, we have developed a method for the rapid detection of the “red complex” of *P. gingivalis*, *T. forsythia*, and *T. denticola*, which is closely related to the severity of periodontitis (Yoshida et al., 2005a). Osawa et al. have developed a LAMP-based detection system for *A. actinomycetemcomitans*, one of the causative bacteria for aggressive periodontitis (Osawa et al., 2007). LAMP technology is currently one of the most rapid bacterial diagnostic methods (Kato et al., 2007; Nagashima et al., 2007).

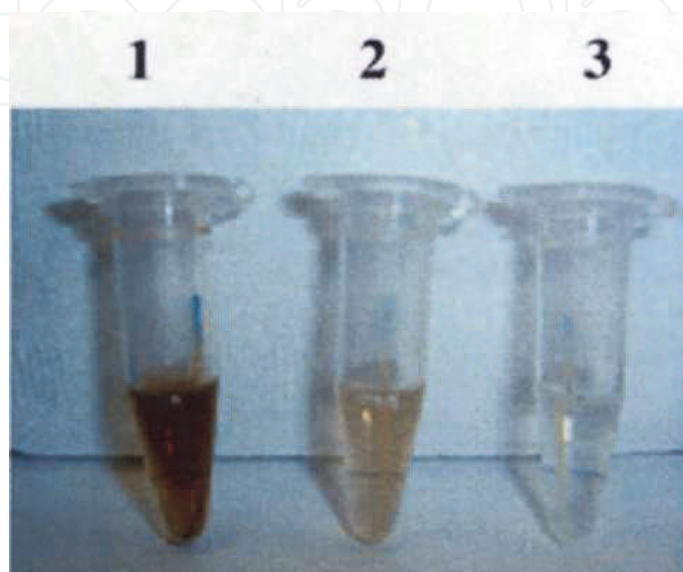


Fig. 2. Visualization of hydrogen sulfide production by precipitation of bismuth trichloride. 1. *Poryphyromonas gingivalis* ATCC 33277 culture; 2. Subgingival fluid sample (*P. gingivalis* positive); 3. Subgingival fluid sample (*P. gingivalis* negative)

3.2 Quantitative examination of periodontal disease

Recently, real-time PCR has become a popular method for the quantitative detection of periodontal bacteria (Suzuki et al., 2004a; Suzuki et al., 2005). Originally used for the measurement of DNA copy numbers, this technique has also been applied to the quantification of bacteria (Yoshida et al., 2003a; Yoshida et al., 2003b). One advantage of this technique is its wide dynamic range of bacterial detection, making it suitable for the determination of oral bacteria, which occur in various and variable amounts. We have developed a detection system based on real-time PCR for the quantification of periodontopathic bacteria, including *P. gingivalis*, *A. actinomycetemcomitans*, *T. denticola*, *T. forsythia*, and *Prevotella* species, in oral specimens such as saliva and subgingival plaque (Kato et al., 2005; Suzuki et al., 2004a; Nagashima et al., 2005; Yoshida et al., 2003a). Using this system to quantify *P. gingivalis* and *T. denticola* in subgingival plaque samples taken from periodontitis patients, we demonstrated a correlation between the numbers of these organisms and periodontal pocket depth (Kawada et al., 2004; Yoshida et al., 2004). The number of *P. gingivalis* bacteria increased ten-fold with every millimeter increase of pocket depth (Fig. 4). Furthermore, the number of this organism decreased significantly after scaling and root planning (Kawada et al., 2004). Thus, this method can be used to quantitatively evaluate the number of periodontopathic bacteria at periodontal sites, making it applicable for the evaluation of therapeutic efficacy. Although the specific equipment and chemical

requirements of real-time PCR technology may limit its use, several laboratories have recently begun to offer real-time PCR analytical services for the quantification of periodontopathic pathogens, expanding access to this type of analysis.

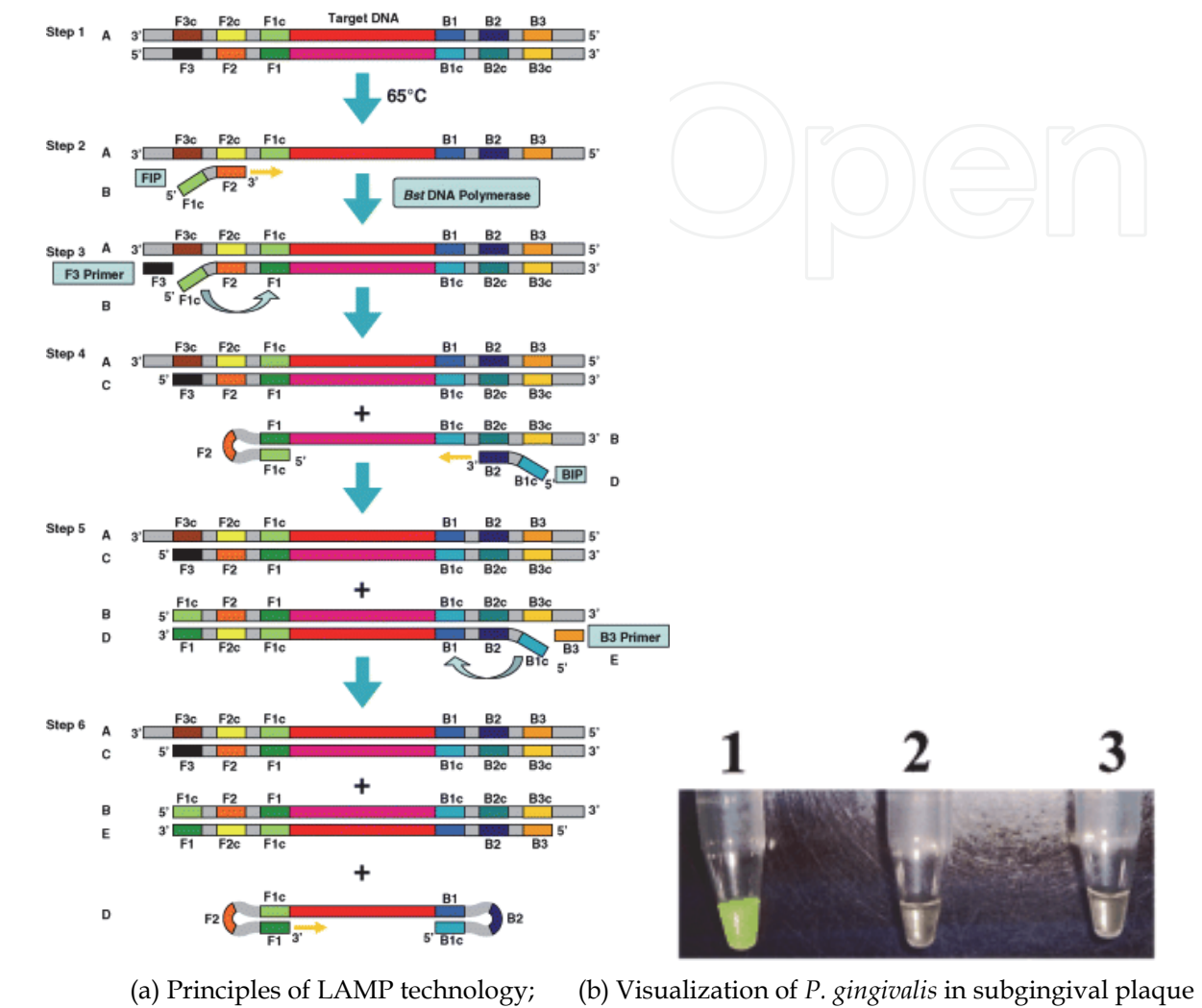


Fig. 3. Principles of LAMP technology (a) and visualization of *Porphyromonas gingivalis* in subgingival plaque (b). 1. *P. gingivalis* positive subgingival plaque; 2. *P. gingivalis* negative subgingival plaque; 3. Negative control (without DNA)

We also have provided technical support for GC Corporation Co., Ltd. (Japan), which provides services for the quantitative analysis for periodontopathic bacteria (Fig. 5). One disadvantage of this technology is that because PCR uses DNA as a template, it quantifies both viable and dead bacteria, which usually results in overestimated cell numbers. To discriminate between living and dead bacteria, we have used propidium monoazide, which selectively penetrates the membranes of dead cells and combines with the DNA, thereby inhibiting its amplification by PCR. Masakiyo et al. evaluated the LED-based fluorescence microscopy which distinguishes between live and dead bacteria for oral bacteria (Masakiyo et al., 2010). Future investigations of the relationship between bacterial cell viability and the severity of periodontitis would further clarify the etiology of periodontitis.

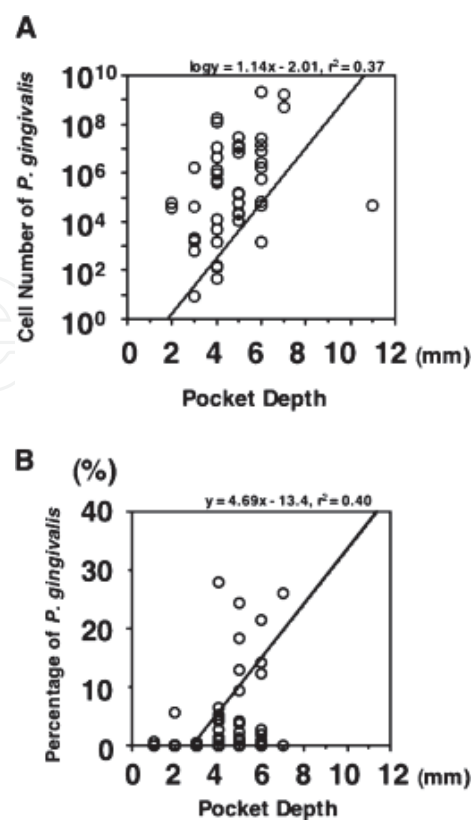


Fig. 4. The correlation between the amount of *P. gingivalis* and pocket depth. A. The correlation between the cell number and pocket depth. B. The correlation between the percentages and pocket depth.



Fig. 5. The commercial kit of real-time PCR assay for periodontopathic bacteria.

4. Microbiological examinations for the purpose of diagnosis

Although quantitative detection methods may be necessary for evaluating therapeutic efficacy, as described above, qualitative methods may be sufficient and even preferable for diagnostic purposes. For example, qualitative culturing methods are more practical than molecular methods for evaluating antibiotic sensitivity. After antibiotic sensitivity has been

established, quantitative methods, ideal one that incorporates a way of discriminating between viable and dead cells, can be used to evaluate the therapeutic efficiency of the antibiotics.

The specific periodontal characteristics of a patient should also be considered when choosing a microbiological method for diagnosis. In patients with a specific periodontal locus, subgingival plaque samples would provide the most relevant information. To identify the population of periodontopathic bacteria present in the oral cavity of a patient, saliva or tongue-coat samples would be appropriate.

5. Microbiological examinations for the purpose of antibiotic selection

Periodontal tissue debridement and root planing are the initial therapeutic approaches for periodontal disease. However, mechanical periodontal debridement can have poor therapeutic efficacy in some cases, owing to the invasion of periodontopathic bacteria into the periodontal tissue. In such cases, antibiotic therapy is often effective (Slots et al., 2004). Antibiotics can be chosen based on the specific pathogens identified by microbiological examination. *Porphyromonas gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, and *T. denticola* are common target bacteria. Table 3 shows the recommended antibiotics according to bacterial type.

	Red complex: <i>Porphyromonas gingivalis</i> , <i>Tannerella forsythia</i> , <i>Treponema denticola</i>	<i>Aggregatibacter</i> <i>actinomycetemcomitans</i>	Orange complex: <i>Prevotella intermedia</i> , <i>Fusobacterium nucleatum</i>
Pathogenicity	High	High	Moderately high
Amoxicillin	-	+	-
Clindamycin	+	-	+
Doxycycline	+	+	+
Minocycline	+	+	+
Azithromycin	-	+	-
Ciprofloxacin	-	+	-
Metronidazole	+	-	+
Amoxicillin + Metronidazole	+	+	+

Table 3. Periodontopathic bacteria and recommended antibiotics (Shaddox & Waller, 2009).

However, this table presents only theoretical or *in vitro* data, and antibiotics selected based on these data may not be effective. Bacteria present in biofilm often obtain antibiotic-resistance genes through horizontal gene transfer, and periodontopathic bacteria may thus acquire novel antibiotic-resistance genes in addition to those they naturally possess, nullifying theoretical antibiotic data. To assess the effectiveness of antibiotics for an individual case of periodontitis, bacterial culturing and the construction of an antibiogram are useful methods for obtaining patient-specific antibiotic data.

6. Conclusions

In this chapter, the concept, selection, and procedure of microbiological examination have been described. Although not required in all cases, the importance of microbiological examinations in the diagnosis and treatment of periodontal disease cannot be ignored in some cases. When a patient's treatment history, present periodontal condition, and information required for diagnosis and treatment (e.g., bacterial species identification, antibiotic selection) are considered, a suitable microbiological examination method and timing can be determined.

Although many of the examination methods described in this chapter are difficult to perform in a private clinical setting, most can be performed in cooperation with commercial laboratories. We are currently focusing on the research and development of a periodontal microbiological examination that satisfies the accuracy, ease of handling, speed, and cost requirements of private clinics.

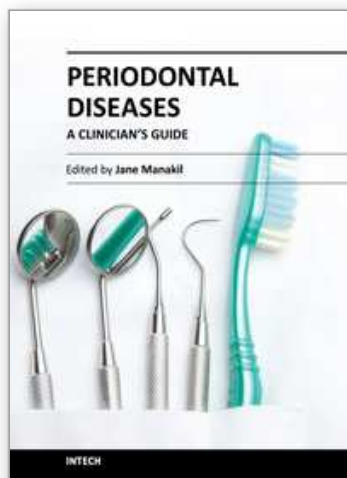
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"Periodontal diseases" is a web-based resource intended to reach the contemporary practitioners as well as educators and students in the field of periodontology. It is fully searchable and designed to enhance the learning experience. Within the book a description is presented of the current concepts presenting the complex interactions of microbial fingerprint, multiple genotypes, and host modulations. In addition, an overview is given of the clinical outcome of the disease's progression, as influenced by the epigenetic factors. Emerging concepts on periodontitis as a risk factor for various systemic diseases and as a bilateral modulating factor have been elucidated in detail as well.

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