The Prospects of Advanced Molecular Methods in the Detection of Periodontal Pathogens: Hybridization in Situ

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Abstract: The paper summarizes the present state in the diagnostics of periodontal pathogens. Both the main advantages and drawbacks of the classic cultivation methods and those of the new DNA techniques are discussed. From the emerging methods of molecular diagnostics, the method of in situ hybridization is presented in more details. Its principle, various modifications of performance and possibilities of use are explained, including examples of its application in the detection of periodontal pathogens.

Microorganisms of importance to the etiopathogenesis of diseases of the periodontium

The complexity of the microbial ecosystem of the mucous membrane of the oral cavity and the intricate interactions of the microorganisms with the periodontal habitat of the host considerably hamper the apparent description of the role of the microorganisms in the etiopathogenesis of periodontal diseases. Neither a single species nor the whole group of microorganisms which could be found exclusively in the locality with pathological alterations, i.e. an unambiguous etiological agent, has yet been described. In various individuals more than 300 species of microorganisms (up to 40 species from a single location) have been isolated from the environment of the gingival sulcus or the periodontal pocket; however, only several of them are related to periodontal lesions [1]. Most authors, as well as the conclusions of the American Academy of Periodontology World Workshop [2] agree with the definition of three periodontal pathogens for which strong evidence about their role in etiopathogenesis of the disease exists. These microorganisms are Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis (the latter was formerly called Bacteroides forsythus). These bacterial species, besides exhibiting statistically significant association with destructive periodontitis, secrete a broad spectrum of potential virulent agents, which increase their pathogenicity [3].

A. actinomycetemcomitans produces leukotoxin, P. gingivalis arginine and lysine proteases (so called gingipains) connected with the destruction of the periodontal tissues, and T. forsythensis secretes sialidase, a protease similar to trypsin, and BspA protein which facilitates the adhesion to fibronectin and fibrinogen. Other microorganisms from the mucous membrane of the oral cavity probably also take part in the development of the disease. However, they are in most cases commonly found in healthy persons. The most common ones are Prevotella intermedia and Prevotella nigrescens, Campylobacter rectus, Eikenella corrodens, Capnocytophaga ochracea, Eubacterium nodatum, Peptostreptococcus micros, Fusobacterium nucleatum and various spirochetes, e.g. Treponema denticola [4, 5].

The search for new detection possibilities of periodontal pathogens

Although cultivation methods are still considered as a reference standard in periodontal microbiology, they cannot be regarded as optimal ones. However, none of the present new DNA methods complies fully with all requirements. In contrast with the cultivation method these methods do not detect unusual and new species.

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Cultivation methods are usually considered to be easy to perform and inexpensive, but this does not hold for the diagnostics of periodontal pathogens. The cultivation of the species spectrum of periodontal pathogens is not a simple process, as all of them are anaerobic microorganisms. Therefore a specialized laboratory with a specially trained staff, costly equipment and relatively high operating costs is needed. Even so, the scope of detection attained by cultivation methods is in some cases problematic. The main reason of its failure is the very principle inherent to this method, namely the necessity of cultivation. The form of a visible colony is attained only by viable cells, i.e. those with the ability of replication. As known from practice incorrect sampling, storage and transport of samples may render the subsequent cultivation and detection of periodontal pathogens completely impossible. Thus the method makes high demands not only on the specialized diagnostic laboratory but also on the staff carrying out the sampling. These demands represent an insurmountable obstacle for a routine application in practice.

That is why the above mentioned consent concerning the most important periodontal pathogens has been achieved only after the appearance of detection methods based on molecular genetics. Until then, some of the most important species, the presence of which, when determined by DNA techniques, exhibits the best correlation with the affliction, were not ranged among those pathogens. The most important example is T. forsythensis [6, 7, 8]. Most of the spirochetes of the genus Treponema [9] cannot be cultivated and the results based on those tests strongly distort the actual percentage and thus also the importance of the individual species for the origin of the disease.

From these facts it is clear that for the periodontal pathogens the ideal diagnostic method obviating the cultivation step ha to be found. That method should detect not only living cells but also "dead" cells. This requirement is generally fulfilled by molecular methods aimed at the nonliving and chemically much more resistant DNA. These methods must have a high sensitivity. Cell replication must be substituted by the amplification of a specific target structure (a specific segment of DNA in case of the PCR methods) or by the possibility of amplifying the signal. This is the case of hybridization methods, especially of the method of in situ hybridization, which gives the possibility of visualizing species or group specification of individual cells of the selected microorganisms (even of several species simultaneously) directly in the tissue samples.

In situ hybridization and its applicability to periodontal pathogens
A non-specific labelling with fluorescent dyes (which in most cases exhibit the affinity for nucleic acids) can be generally used for the visualization of bacterial cells. The dyes SYTO BC and SYBR Green I can be mentioned as examples of fluorochromes applicable in this labelling technique. An example of cells of a culture of Capnocytophaga ochracea visualized in this way is given in Fig. 1. This method is very effective, but, it does not allow specific detection and visualization of a single

species or of several selected species of bacteria in mixed cultures as well as in infectious or contaminated material.

On the other hand, in situ hybridization is a highly selective technique which is able to detect specific sequences of nucleic acids in the morphologically preserved tissue, in tissue sections or in whole cells by means of labelled DNA or RNA probes. The infectious agent can thus be detected as bacterial or viral nucleic acids. With a multiple labelling of the specific probes this method gives the possibility of detecting even several bacterial species in a single step. If suitable markers are used as target structures for the labelled probe, even the individual carrying genes with virulent factors or genes for resistance to antibiotics may be detected in the population. Such suitable target structures specific for the three main periodontal pathogens are, e.g. their potential virulence factors mentioned in the first paragraph of this paper. They have been already used with another advanced DNA technique, the "real-time PCR" [3].

The method of in situ hybridization was first described by Pardue and Gall [10]. Thanks to the progress in molecular biology, immunology and modern technologies a broad range of applicable labels, signal detection methods, techniques of probe preparation and working procedures is currently available. This gives the possibility of using in situ hybridization for detection and visualization of specific sequences of nucleic acids and for signal amplification not only in laboratories but also for routine diagnostic purposes.

The in situ hybridization includes the following steps:
a) preparation of a labelled DNA or RNA probe;
b) preparation of the target structure: tissue, tissue section, cell suspension;
c) pre-treatment of the target in order to facilitate the penetration of the probe;
d) hybridization of the probe with the target nucleic acid;
e) rinsing – removal of the unbound probe;
f) detection of the hybridized probe depending on the type of the applied label.

The method can be combined also with PCR: it brings further amplification of the signal, improvement of sensitivity and detection possibilities.

There are two main alternatives of the probe labelling: radioactive labelling (detectable by autoradiography) and non-radioactive one (detectable directly or indirectly immunocytochemically). The advantages of the application of radioactive isotopes are the possibility of subsequent quantification of the signal and high sensitivity. Non-radioactive (haptens) labels have a great advantage in the safety of manipulation, stability and high rate of signal processing. In principle, there are two types of hybridization methods using non-radioactively labelled probes: a direct and an indirect one. The direct method consists in the visualization of the detectable marker (mostly fluorochrome, which has a direct covalent bond to the probe) immediately after the hybridization reaction. If antibodies against this molecule are available, the direct method can be converted into the indirect one [11]. The indirect method requires a

probe with a bonded molecule which is subsequently detectable by an affinity cytochemical reaction. Very often it is the case of haptene detection by a specific antibody conjugated with fluorochrome or with an enzyme producing a stable coloured or fluorescent substrate.

Preparations hybridized with probes labelled directly with fluorochromes can be immediately visualized in a fluorescence microscope. The fluorochromes most often used for in situ hybridization are for example: AMCA (aminomethylcoumarin-acetic acid), fluorescein, rhodamine, Texas Red, Cy3, Cy5 or Alexa Fluor dyes. An example of a species-specific visualization of the cells of P. gingivalis is given in Figure 2.

The cells selected for in situ hybridization must be first fixed. The fixation of the sample is one of the most important steps necessary for successful hybridization. It gives the possibility of preserving a correct morphology of the sample, shapes and sizes, the fixing of nucleic acids in the cells and the facilitation of probe penetration, The most efficient cross-linking agents are paraformaldehyde, formaldehyde or glutaraldehyde. The sample can be further treated with organic solvents or detergents in order to remove the lipidic bilayers; with proteases to split partly cell proteins and thus to facilitate cell disruption and penetration of the probe or with nucleases to remove unwanted DNA or RNA. The blocking of unspecific bonding sites for the probe (e.g. BSA) can be also very important. The hybridization reaction itself must take place under optimum reassociation conditions, above all temperature, concentration of monovalent cations and pH. The removal of the unbound or unspecifically bound probe after the terminated hybridization is also absolutely essential for obtaining meaningful results.

The method of in situ hybridization was used in the detection of periodontal pathogens A. actinomycetemcomitans and P. gingivalis directly inside human epithelial cells of the buccal mucosa which form a "protective coat" against these sensitive
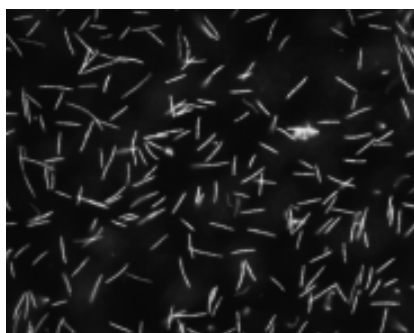

Figure 1 – Bacterial cells of Capnocytophaga ochracea visualized in a fluorescence microscope, unspecifically labelled with a fluorescent dye (SYTO BC) with affinity to DNA. Magnification 1250 times.
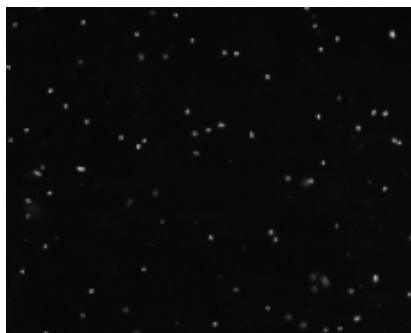

Figure 2 – Specific detection of the suspension of the Porphyromonas gingivalis cells by means of the DNA probe labelled with Alexa Fluor 488, visualized in the fluorescence microscope. Magnification 1250 times.

anaerobes and enhance their penetration into the otherwise unfavourable aerobic milieu and enables their proliferation [12]. A further recent example of application of this method is the visualization and detection of periodontal pathogens in situ in the periapical foci at endodontally treated teeth [13].

Conclusion

Hybridization in situ is one of the prospective methods of the molecular diagnostics potentially applicable in the detection of periodontal pathogens in both the basic research and in the clinical diagnostics. It offers a unique possibility to detect target microorganisms directly within the tissues, and permits the quantification and simultaneous investigation of several bacterial species and groups.

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