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*Chapter VII*

# A Novel Cytodiagnostic Fluorescence Assay for the Diagnosis of Periodontitis

***Marco Giannelli*[*1], *Lucia Formigli*[2] *and Daniele Bani*[2•]**
1. Odontostomatologic Laser Therapy Center,
Florence, Italy
2. Dept. Anatomy, Histology and Forensic Medicine,
University of Florence, Florence, Italy

## Abstract

A topical issue in periodontology is to find objective diagnostic methods which may be combined with the classical clinical inspection parameters to yield a reliable grading of the severity and extent of periodontal disease. This study deals with a novel cytodiagnostic fluorescence test, performed on exfoliation samples taken from periodontal/oral tissues, useful to assess the severity of periodontal disease. Twenty-one patients with different degrees of periodontitis were subjected to clinical and histopathological grading and the results compared with those obtained from the cytodiagnostic fluorescence assay. We found that the amount of blood cells (polymorphonuclear and mononuclear leukocytes, erythrocytes), the occurrence of morphologically abnormal epithelial cells, and the number of spirochetes showed a statistically significant correlation with the clinical and histopathological diagnostic parameters, the latter being considered as the most reliable predictors of the severity of periodontal disease. On these grounds, we suggest that this cytodiagnostic method may greatly help dental practitioners to achieve a chair-side, reliable and objective evaluation of the degree and activity of periodontitis at first dental visit, and to perform a targeted treatment and an accurate follow up of the patients during supportive periodontal therapy.

[*] Author for correspondence: Marco Giannelli, Odontostomatologic Laser Therapy Center, Via dell'Olivuzzo 162/164, I-50143, Florence. Phone:(+39) 055 701665; Fax:(+39) 055 7131692; e-mail: dott.giannellimarco@dada.it.

[•]V.le G.B.Morgagni 85, I-50134 Florence, Italy.

# Introduction

Periodontal diseases comprise a group of inflammatory diseases of the gingival and supporting structures of the periodontium of high social impact, which, in the most severe cases, can result in tooth loss [Armitage et al. 1995; Papapanou et al. 1996]. Multiple infective agents are involved in the pathogenesis of this disease, including both Gram-negative anaerobic and facultative anaerobic bacteria [Loesche and Grossman, 2001]. In particular, it is generally assumed that Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Bacteroides forsythus, Campylobacter rectus, Prevotella intermedia, and the oral treponemes Treponema denticola, Treponema pectinovorum, Treponema vincentii, Selenomonas sputigena, and Eikenella corrodens are associated with the most aggressive and destructive forms of periodontitis [Socransky et al.1998; Holt et al. 1999]. Indeed, these bacteria release multiple virulence factors, such as lipopolysaccharide (LPS), that activate the host inflammatory response, characterized by migration into the periodontal tissues and gingival sulcus of polymorphonuclear leukocytes (PMN) in the acute phase and mononuclear (MN) leucocytes, such as monocytes and lymphocytes, in the chronic phase, which then initiate alveolar bone resorption [Jiang et al. 1999]. The frequent relapses and chronicization of periodontitis, may depend on complex host-parasite interactions, including the ability of the above pathogens to gain access into the cells of the sulcular and buccal epithelia [Rudney et al. 2005, Apsey et al. 2006; Colombo et al. 2007; Savage et al. 2009], thereby escaping the traditional antiseptic therapies [Rudney et al. 2001; Eick and Pfister, 2004; Rautemaa et al. 2004; Andrian et al. 2006].

Recently, chronic periodontitis has posed a significant public –health challenge, because it has been suggested to be a risk factor for cardiovascular disease and preterm births [Khader and Ta'ani, 2005; Friedewald et al. 2009]. In particular, increased carotid artery intimal/medial thickness, evaluated echographically, which is associated with increased risk for acute myocardial infarction and stroke often occurs in patients with periodontitis, suggesting that subclinical atherosclerosis is present in periodontopathic patients [Scannapieco et al. 2003; Bahekar et al. 2007]. The pathophysiology for such association may rely on multiple interwoven mechanisms, including systemic release of pro-inflammatory mediators [Linden et al. 2008], endothelial dysfunction [Amar et al. 2003; Tonetti et al. 2007], and dissemination of Gram-negative bacteria from the periodontal reservoir to atheromasic lesions [Haynes and Stanford 2003].

Clinical attachment level (CAL), probing depth (PD), gingival recession and radiographic assessments of inflammation are the most widely accepted and used tools to diagnose periodontitis [Savage et al. 2009], although they may not closely reflect the actual disease activity at given sites [Caton et al. 1981; Apsey et al. 2006; Schatzle et al. 2009]. A major limitation of periodontal probing is its inability to distinguish previous tissue loss from current disease activity and is not a safe criterium for evaluating gingival health [de Souza et al. 2003]. Since periodontal tissue damage accumulates over time, the disease may appear more severe in elderly patients than in young ones, although in terms of disease progression, the contrary may be the case [Hujoel et al. 2005]. The evaluation of the outcome of therapy and detection of periodontal disease recurrence also suffers from the low sensitivity of the clinical diagnostic tests, often leading to false conclusions concerning the efficacy of the methods and treatments applied [Kaldahl et al. 1996; Mombelli, 2005]. To overcome these

limitations, additional diagnostic tools have being developed to diagnose and assess therapeutic efficacy, including microbiological and histopathogical tests. On the other hand, both these methods have several intrinsic limitations that hinder their widespread use in current dental practice. In fact, microbiological criteria fraught with technical problems related to the culturing of plaque samples for anaerobesand results can take weeks to be obtained [Apsey et al. 2006]. Likewise, histopathology on periodontal biopsies, which in principle may be the objective method of choice, finds limited application because is it invasive and need several days and specifically trained examiners to be carried out properly [Gillet et al. 1990]. Therefore, a topical issue in periodontology is to find objective diagnostic methods for the "chairside" determination of the oral disease which, in combination with the clinical inspection, may allow a rapid and reliable evaluation of the severity, extent and progression of periodontal disease, required for the set up of appropriate therapeutic protocols and follow up. Indeed, new diagnostic tools have been proposed, based on the use of highly sensitive immunoassays, enzymatic (BANA test) and real time PCR, which allow the identification of specific inflammatory and bacterial-derived biomarkers from the whole saliva and plaque bio-film of periodontal patients [Loeshe et al. 1990; Ramseier et al. 2009]. The present report provides evidence for the possible usefulness of a novel cytodiagnostic fluorescence test performed on exfoliation samples taken from periodontal/oral tissues to predict the severity of periodontal disease at individual sites. This approach is more sensitive compared with the clinical judgement and shows a strong positive correlation with the histopathological parameters indicative of disease activity.

# Materials and Methods

## Patients and Sampling

The study was designed in compliance with the guidelines of the Declaration of Helsinki, as amended in Edinburgh, 2003. It received a favourable ethical opinion from the Ethical Committee of the Faculty of Medicine, University of Florence, Italy. Twenty-one volunteer patients (14 males, 7 females , aged 35-65 years; mean, 58.4 years) with clinical diagnosis of periodontitis were enrolled. They attended a screening visit at which written informed consent was taken. One week before entering the study, the patients were subjected to supra-gingival tooth cleaning. The exclusion criteria were: systemic diseases (diabetes mellitus, cancer, HIV, metabolic and endocrine diseases), pregnancy and lactation, chronic high-dose steroid therapy, radiation or immunosuppressive therapy, smoking (more than 10 cigarettes/day), orthodontic treatments, extensive carious lesions, and antibiotic medication during or within the 6 months preceding the study. Periodontal clinical measurements were performed by an experienced dentist and included clinical attachment level (CAL), pocket depth (PD) and gingival index and bleeding on probing (GI/BOP). Measurements were carried out using a conventional manual periodontal probe (Hu-Friedy, Chigago, IL, USA) at 6 sites per tooth. Twenty-one randomly chosen sites (1 from each patient) were selected for histological and cytofluorescence monitoring . To delineate the area for histological analysis, a reference small incision was made on the facial surface on the gingiva which corresponded to the depth and mesio-distal extent of the area probed and evaluated for visual signs of inflammation.All sites

were isolated with cotton rolls and the supra-gingival biofilm was carefully removed with a sterile gauze. Samples of exfoliated cells were taken with a sterile micro-curette in the proximity of the free gingival margin, i.e. in the same location where histological sampling was scheduled. The samples were processed for fluorescent staining as described below. Small gingival biopsies, about 2 $mm^3$, were taken under local anesthesia (Articain HCl, Ultracain,, Frankfurt, Germany) from each cytological sites using a biopsy punch, 2-mm diameter, taking care not to expose the marginal alveolar bone and periostium, and routinely processed as described below.

## Histological Analysis

The periodontal biopsies were immediately fixed by immersion in 4% (w/v formaldehyde in 0.2 M phosphate-buffered saline, pH 7.4, dehydrated in graded ethanol and embedded in paraffin. Five μm-thick cross sections were stained with hematoxylin and eosin, viewed and photographed under a light microscope (Nikon, Tokyo, Japan).

**Table 1. Severity scoring criteria**

| SCORE | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| CAL | - | < 2 mm | 2-5 mm | > 5 mm |
| PD | - | < 3 mm | 3-6 mm | > 6 mm |
| GI | normal periodontum | slight erythema<br>slight oedema<br>no bleeding on probing | mild erythema<br>mild oedema with smooth gingival surface<br>bleeding on probing | severe erythema<br>severe oedema and ulcers<br>spontaneous bleeding |
| II | absent | slight, perivascular and scattered intraepithelial | mild, continuous in the lamina propria and intraepithelial | severe, continuous in the lamina propria and dense intra-epithelial |
| DE | normal epithelium | outer layer shedding | outer layer shedding<br>cell swelling | as in # 2, plus ulceration |
| PMN, MN, RBC | absent | < 5 | 5-10 | > 10 |
| DEC | normal | aberrant shape | plasma membrane rupture<br>vacuolation | conglutination, vacuolation<br>cell debris |
| cocci, bacilli, spirochetes | absent | < 10 | 10-30 | > 30 |

CAL, clinical attachment level; PD, probing depth; GI, gingival index; II, inflammatory infiltrate; DE, damaged epithelium; PMN, polymorphonuclear leukocytes; MN, mononuclear leukocytes; RBC, erythrocytes; DEC, damaged epithelial cells.

**Table 2. Severity scoring of periodontitis**

| Case No. | Clinical score | | | | Bioptic score | | | Cytodiagnostic fluorescence score | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CAL | PD | GI | Total score | II | DE | Total score | PMN | MN | RBC | DEC | Cocci | Bacilli | Spiro-chete |
| 1 | 1 | 2 | 1 | 4 | 2 | 1 | 3 | 2 | 1 | 0 | 0 | 1 | 1 | 0 |
| 2 | 1 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| 3 | 3 | 3 | 2 | 8 | 3 | 1 | 4 | 1 | 3 | 0 | 1 | 1 | 0 | 0 |
| 4 | 2 | 2 | 2 | 6 | 2 | 2 | 4 | 2 | 2 | 3 | 0 | 1 | 1 | 0 |
| 5 | 3 | 3 | 3 | 9 | 3 | 3 | 6 | 3 | 3 | 1 | 3 | 1 | 2 | 3 |
| 6 | 3 | 3 | 3 | 9 | 3 | 3 | 6 | 3 | 3 | 2 | 3 | 1 | 2 | 1 |
| 7 | 3 | 3 | 3 | 9 | 3 | 3 | 6 | 3 | 3 | 1 | 1 | 1 | 1 | 1 |
| 8 | 3 | 3 | 3 | 9 | 3 | 3 | 6 | 3 | 3 | 2 | 3 | 3 | 2 | 1 |
| 9 | 3 | 2 | 0 | 5 | 2 | 1 | 3 | 1 | 2 | 0 | 1 | 2 | 2 | 0 |
| 10 | 2 | 1 | 0 | 3 | 2 | 1 | 3 | 1 | 2 | 0 | 1 | 2 | 1 | 0 |
| 11 | 2 | 2 | 3 | 7 | 3 | 3 | 6 | 3 | 1 | 3 | 2 | 3 | 3 | 3 |
| 12 | 1 | 1 | 0 | 2 | 2 | 0 | 2 | 0 | 2 | 0 | 0 | 2 | 0 | 0 |
| 13 | 2 | 2 | 0 | 4 | 2 | 1 | 3 | 1 | 2 | 0 | 1 | 2 | 0 | 0 |
| 14 | 1 | 2 | 0 | 3 | 1 | 1 | 2 | 1 | 1 | 0 | 1 | 2 | 1 | 0 |
| 15 | 2 | 3 | 3 | 8 | 3 | 3 | 6 | 3 | 1 | 2 | 3 | 1 | 3 | 1 |

**Table 2. (Continued)**

| Case No. | Clinical score | | | | Bioptic score | | | Cytodiagnostic fluorescence score | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CAL | PD | GI | Total score | II | DE | Total score | PMN | MN | RBC | DEC | Cocci | Bacilli | Spiro-chete |
| 16 | 2 | 2 | 3 | 7 | 3 | 3 | 6 | 3 | 1 | 3 | 3 | 1 | 1 | 3 |
| 17 | 3 | 3 | 0 | 6 | 3 | 1 | 4 | 1 | 3 | 0 | 0 | 2 | 2 | 1 |
| 18 | 3 | 2 | 1 | 6 | 2 | 1 | 4 | 1 | 2 | 0 | 1 | 2 | 2 | 0 |
| 19 | 3 | 1 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 |
| 20 | 2 | 2 | 3 | 7 | 3 | 3 | 6 | 3 | 1 | 2 | 3 | 1 | 3 | 2 |
| 21 | 2 | 2 | 2 | 6 | 3 | 3 | 6 | 3 | 3 | 1 | 3 | 3 | 0 | 0 |

CAL, clinical attachment level; PD, probing depth; GI, gingival index; II, inflammatory infiltrate; DE, damaged epithelium; PMN, polymorphonuclear leukocytes; MN, mononuclear leukocytes; RBC, erythrocytes; DEC, damaged epithelial cells.

**Table 3. Spearman's non-parametric correlation test**

| | vs. | *r* | *r squared* | *p* value |
|---|---|---|---|---|
| Clinical score | II (biopsy) | 0.7670 | 0.5882 | <0.0001 |
| | PMN | 0.8327 | Gaussian approx. | <0.0001 |
| | MN | 0.5248 | 0.2754 | 0.0146 |
| | RBC | 0.6131 | 0.3759 | 0.0031 |
| | EDC | 0.6993 | 0.489 | 0.0004 |
| | cocci | -0.07588 | 0.005758 | 0.7437 (n.s.) |
| | bacilli | 0.4263 | 0.1817 | 0.0540 (n.s.) |
| | spirochetes | 0.6012 | 0.3615 | 0.0039 |

A.

| | vs. | *r* | *r squared* | *p* value |
|---|---|---|---|---|
| Inflammatory infiltrate (biopsy) | PMN | 0,8076 | Gaussian approx. | <0.0001 |
| | MNC | 0,6629 | 0,4395 | 0.0011 |
| | RBC | 0,5084 | 0,2584 | 0.0186 |
| | EDC | 0,6465 | 0,418 | 0.0015 |
| | cocci | 0 | 0 | 1 (n.s.) |
| | bacilli | 0,2919 | 0,08523 | 0.1991 (n.s.) |
| | spirochetes | 0,5477 | 0,3 | 0.0102 |

B.

II, inflammatory infiltrate; PMN, polymorphonuclear leukocytes; MN, mononuclear leukocytes; RBC, erythrocytes; EDC epithelial damage, cytological.

## Cytodiagnostic Fluorescence

The LIVE/DEAD BacLight™ bacterial viability kit (Invitrogen Molecular Probes, Milan, Italy) is originally developed as an easy-to-use method for monitoring the viability of micro-organisms as a function of the membrane integrity of bacteria [Boulos et al. 1999, Berney et al. 2007, Tomàs et al. 2009]. It uses a mixture of SYTO 9 green-fluorescent and propidium iodide red-fluorescent nucleic acid stains. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, SYTO 9 labels all bacteria in a population — those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in SYTO 9 fluorescence when both dyes are present. Therefore, cells with a compromised membrane, considered dead or dying, stain red, whereas cells with an intact membrane stain green. In addition, this staining method can offer a broader range of diagnostic information, such as the occurrence of inflammatory PMN and MN leukocytes, erythrocytes, and the morphological alteration of the exfoliated epithelial cells. Briefly, the collected material was smeared on a histological slide, fixed in 90% ethanol, air-dried and stained with 1 ml of the fluorescent dye solution for 2 min at 37° C. After thorough rinsing in distilled water, the samples were mounted in oil and immediately observed under a Leica 4000B fluorescent microscope (Leica Microsystems, Milan, Italy).

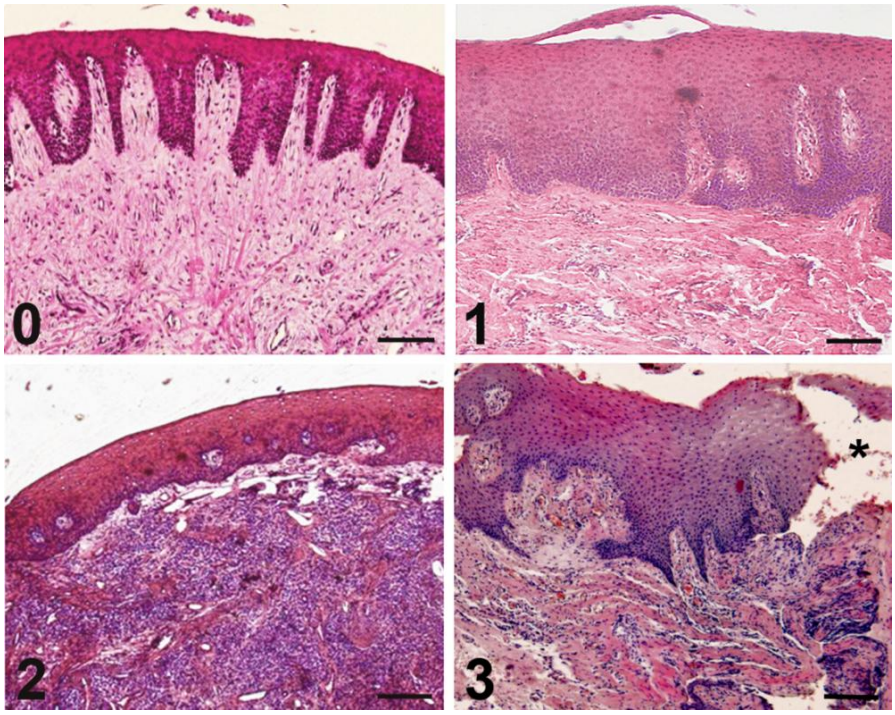


Figure 1. Histopathological scoring. Representative images of periodontal biopsies scored as indicated in Table 1: 0, normal mucosa; 1, moderate perivascular inflammatory infiltrate; 2, dense inflammatory infiltrate and reduction of epithelial cristae; 3, diffuse inflammatory infiltrate, dilated blood vessels, epithelial ulceration (asterisk). Bars = 200 μm.

### Data Analysis and Statistical Evaluation

A semi-quantitative scoring was used to evaluate the severity of periodontitis based on current clinical and histopathological criteria. A similar scoring was used for the cytodiagnostic fluorescence parameters. For evaluation of bacterial score, only viable, green fluorescent stained bacteria were considered. Details are reported in Table 1. Individual patientswere assigned a total clinical and bioptic score, calculated as the sum of the scores of each clinical and histopathological parameters, respectively. The complete semi-quantitative data are reported in Table 2.

Statistical analysis of the relationships between the assayed scoring criteria was performed by the Spearman's non-parametric correlation test, assuming $p<0.05$ as statistically significant. Calculations were carried out using Prism 4.0 statistical software (Graph Pad Software, San Diego, CA).

## Results

The patients under study were divided into 4 groups based on the assigned score for the clinical, histopathological and cytodiagnostic parameters (Table 2). Using the Spearman's non-parametric statistical correlation analysis, we first found a significant, positive correlation

between the clinical severity and the histopathological parameters (Table 3A). As expected, the most severe clinical signs, e.g. epithelial ulceration, spontaneous bleeding and wide dental root exposure, were associated with surface epithelial thinning and shedding, as well as with abundant inflammatory infiltrate invading the lamina propria and the epithelium in the matched bioptic samples (Figure 1)

Most of the assayed cytodiagnostic fluorescence parameters also positively correlated with the clinical severity of the periodontal disease (Table 3A), the number of PMN leukocytes and the occurrence of damaged exfoliated epithelial cells (i.e. showing spiked profile, cytoplasmic shrinkage and plasma membrane rupture) being those with the highest significance values (Figures 2, 3).

Regarding the presence of micro-organisms in the exfoliative samples (Figure 4), no significant correlation was detected between the clinical score and the number of viable cocci and bacilli within the exfoliative material - both extracellular or internalized into the epithelial cells - suggesting that their presence cannot be taken as an indicator of the active disease stage. By contrast, the presence of viable spirochetes (Figure 4) was consistently associated with severe periodontitis.

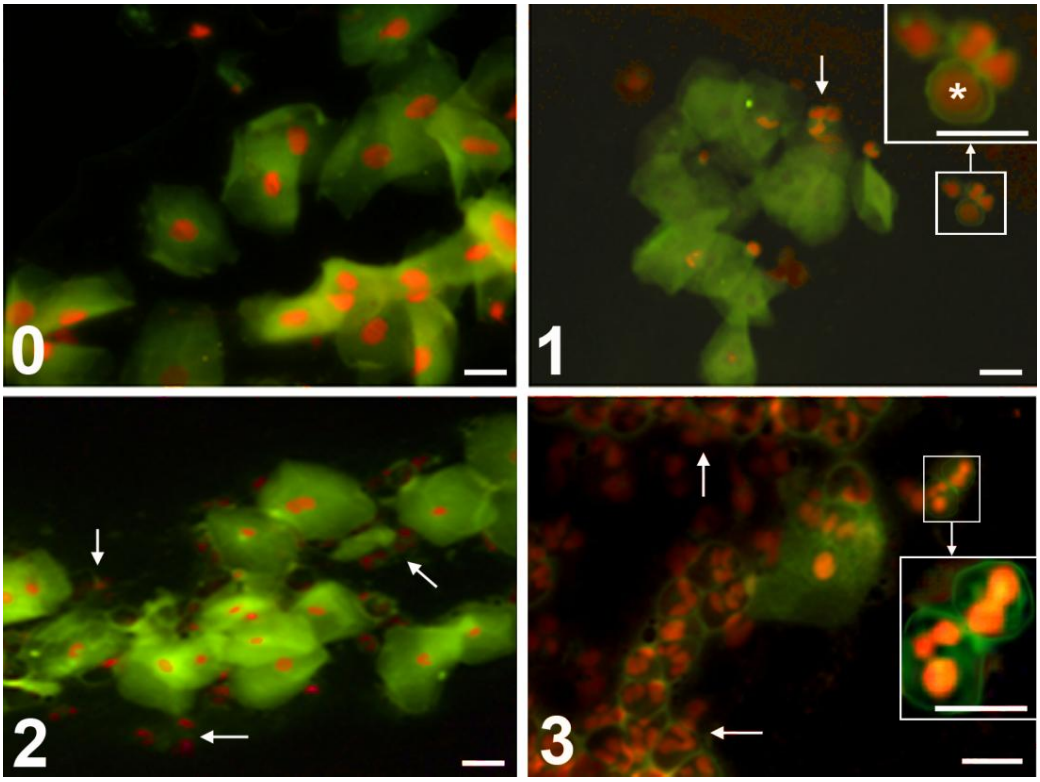


Figure 2. Cytodiagnostic fluorescence scoring. Representative images of cytological smears stained with LIVE/DEAD BacLightTM, scored as indicated in Table 1: 0, normal epithelial cells; 1, clustered epithelial cells with scattered PMN and a MN (asterisk); 2, clustered epithelial cells with several PMN; 3, a vacuolated epithelial cell with very numerous PMN. The insets show details of the leukocytes. Some PMN are indicated with arrows. Bars = 20 μm.

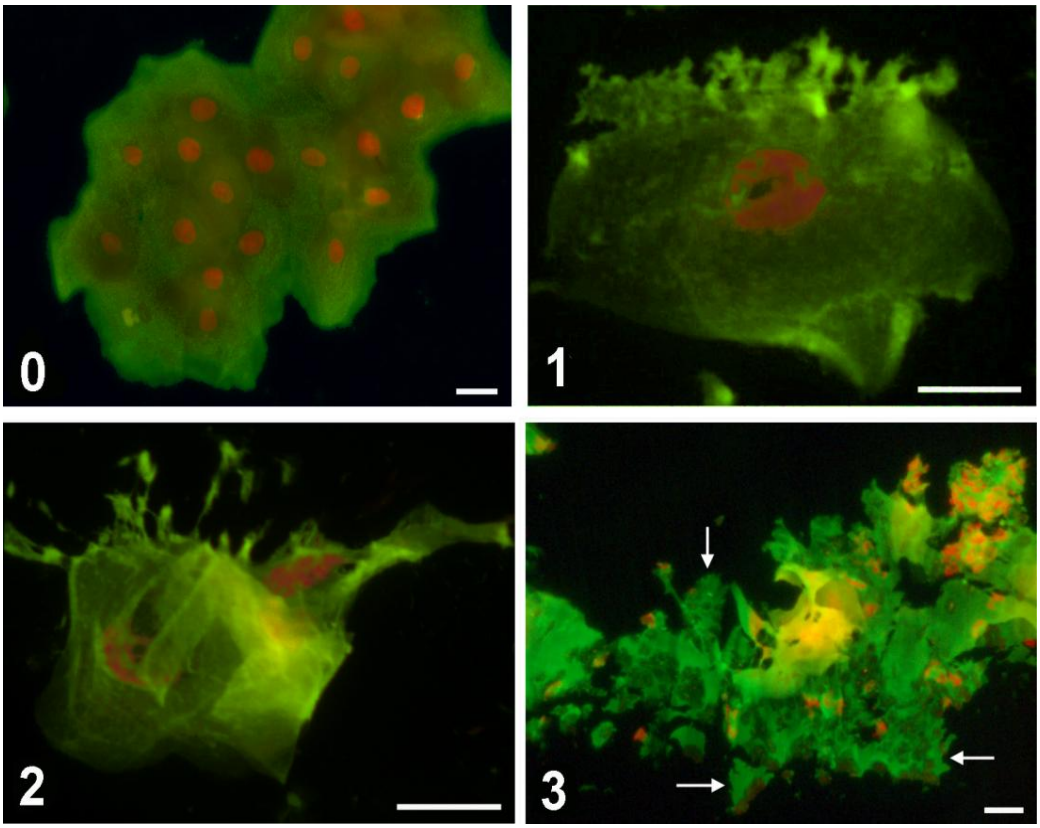


Figure 3. Cytodiagnostic fluorescence scoring. Representative images of cytological smears stained with LIVE/DEAD BacLight™, scored as indicated in Table 1: 0, normal epithelial cells; 1, an epithelial cell with spiked profile; 2, an epithelial cell with cytoplasmic shrinkage and vacuolization; 3, clustered epithelial cells with severe cytoplasmic shrinkage and vacuolization surrounded by cell debris (arrows). Bars = 10 μm.

We then compared the bioptic score, assumed as an objective measurement of the degree of periodontitis, with the cytodiagnostic parameters (Table 3B). Remarkably, the results showed that all the assayed parameters, except for the number of cocci and bacilli, correlated closely with the bioptic score. These findings indicated that the presence and amount of PMN, MN, RBC, DEC, and spirochetes in the cytodiagnostic samples had a diagnostic value comparable to that of conventional tissue biopsy.

Of note, some patients (for instances 10,12, 13, 19) diagnosed as having mild periodontal disease based upon the high CAL and PD scores, resulted either negative or positive for substantial inflammatory response and pathogenic bacterial colonization at both the biopsy and cytodiagnostic analyses. These findings, suggested that the mere clinical inspection was insufficient to correctly diagnose the periodontal disease and that its combination with the cytodiagnostic test, which displayed the same sensitivity as the histopathological one, could be essential to properly and rapidly recognize the periodontal process and address the therapeutic needs.

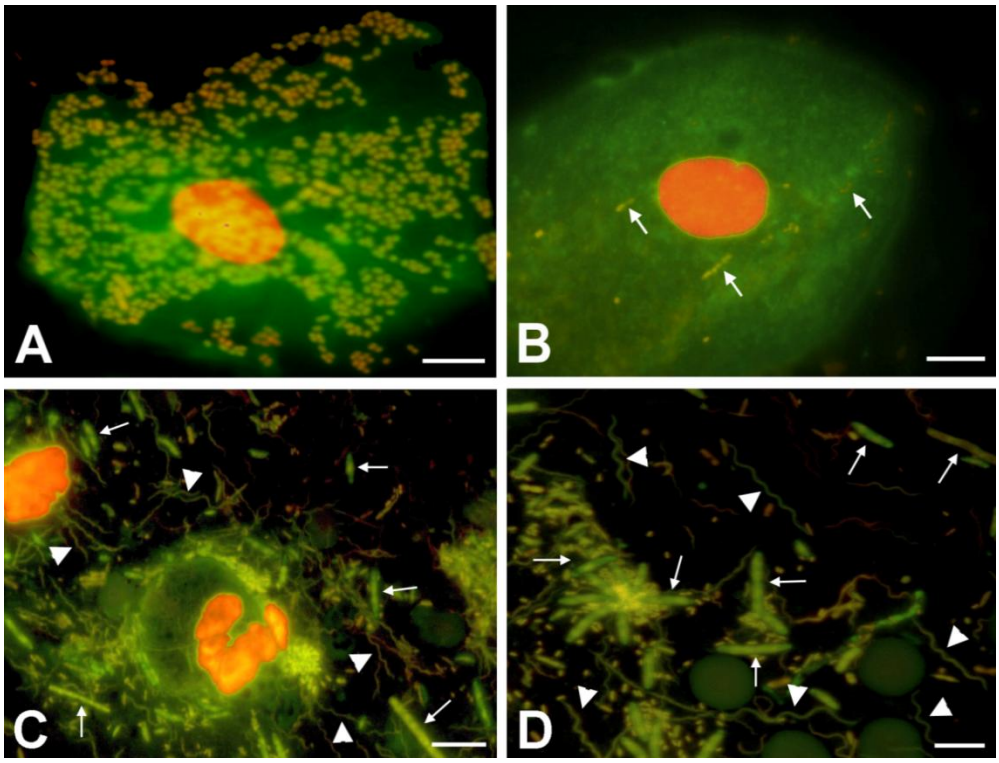


Figure 4. Cytodiagnostic fluorescence scoring. Representative images of typical oral bacteria in the cytological smears, as they appear upon staining with LIVE/DEAD BacLight™: A, cocci; adherent to and inside an epithelial cell; B, bacilli within an epithelial cell (arrows); C,D, bacilli and spirochetes within the exfoliative material. Arrows: cocci; arrowheads: spirochetes. Green and orange/red indicate viable and dead bacteria, respectively. Bars = 10 μm.

## Conclusion

A serious concern in periodontology is the lack of reliable criteria to objectively distinguish between sites with active and inactive disease and differentiate between changes associated with aging and changes associated with disease. The traditional clinical parameters, such as CAL, PD and GI/BOP, as well as the assessment of dental plaque and calculus, namely tooth mobility, and radiological appearance of alveolar bone, are considered inappropriate *per se* to provide accurate diagnostic aids [Savage et al. 2009]. The present study introduces a novel cytodiagnostic method that shows a strong positive correlation with the histopathological grading. Since the histological evaluation of surgical biopsies from affected tissues is considered the best diagnostic tool to recognize the phases of periodontal disease and to differentiate the periods of destructive inflammatory response from those of effective host defense [Gillett et al. 1990], we suggest that this newly identified method, in a similar manner as histopathology but in a shorter time, may objectively reflect the activity of periodontal disease and contribute to reduce the misclassification of subjects based on the clinical evidence.

We found that the amount of blood cells, especially PMN and MN, within the exfoliated samples could be considered as reliable indicators of the degree of the periodontal inflammatory reaction, in full agreement with previous studies showing that the presence of white blood cells in the dental plaque is diagnostic for periodontal disease [Apsey et al. 2006; Vitkov et al. 2009]. This method also allowed the examiner to easily discriminate between PMN and MC, which are characteristic of the acute and chronic inflammatory phases, respectively, thus potentially providing information on the evolution of periodontitis and the efficacy of the therapy. The presence of RBC also had diagnostic value, as it was conceivably related to clinical or sub-clinical gingival bleeding.

It is worth noting that the occurrence of exfoliated epithelial cells with morphological alterations (DEC) was also correlated with the clinical and histopathological grading of periodontitis, suggesting that DEC may be a valuable diagnostic parameter of periodontitis. This assumption is in line with the well accepted role for periodontal epithelium in health and disease, providing a physical barrier to infection and participating actively in the innate host defense [Holt et al. 1999; Andrian et al. 2006; Dale 2002]. In particular, it has been demonstrated that periodontal epithelial cells respond to bacteria in an interactive manner; they secrete interleukin-8 and other chemokines and cytokines and produce natural antimicrobial peptides in response to bacterial plaque [Weinberg et al. 1998; Dale 2002; Laurina et al. 2009; Ren et al. 2009]. These cells may even respond to bacteria by changes in cell proliferation, differentiation and death, thus altering epithelial tissue homeostasis. All these data, combined with the present findings concerning the occurrence of damaged epithelial cells in the exfoliated samples of patients with periodontitis, invite dentists and researchers to pay more attention to the modifications of the periodontal epithelium and their connection with the initiation and progression of periodontal disease.

We have also demonstrated that this cytodiagnostic method can easily detect the presence of micro-organisms within the exfoliated material and, most importantly, it is able to quantify bacterial viability in real-time. Recent compelling evidence indicates that periodontal bacteria can establish symbiotic or parasitic relationships with the host depending on their ability to settle in appropriate ecological niches, such as gingival epithelial cells or fibroblasts, and produce virulence factors, affording them a competitive advantage over the commensal microbiota and resistance to host immunologic defenses [Holt et al. 1999]. Notably, this method was also able to distinguish between commensal and parodontopathic bacteria. Given that spirochetes, such as *Treponema denticola,* are considered the most aggressive periodontal pathogens [Holt et al. 1999; Colombo et al. 2007], their presence in subgingival plaque samples being associated with clinical parameters of periodontitis, such as PD and GI/BOP [Byrne et al. 2009], the cytodiagnostic fluorescence method reported in this study may help identifying sites at risk for progression and assist in the targeted treatment of periodontitis.Moreover, compared with conventional phase contrast microscopy, the present method has the advantage of distinguishing between dead and viable bacteria, thus allowing to discriminate inactive and active pathogens. At the opposite with spirochetes, the presence of cocci and bacilli within the exfoliated epithelial cells did not show any significant correlation with the degree of periodontitis: this paradox may be explained by the fact that these morphologically identified classes of micro-organisms include both active and quiescent parodontopathogens, such as for instance *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans,* as well as normal components of the oral microbial flora.

In conclusion, the current cytodiagnostic fluorescent method allows the identification of key morpho-pathological features in the exfoliation periodontal samples that strongly correlate with periodontitis. We suggest that this approach may have significant potential in assisting dental practitioners to achieve a chairside reliable and objective evaluation of the degree of the periodontal disease at the first observation and to correctly classify the patients as needing or not needing treatment. It may also represent an useful tool to perform an accurate follow up of the patients during supportive periodontal therapy. Studies are ongoing to apply this approach to the longitudinal predictions of disease activity. The development of a simple and rapid diagnostic method for periodontitis may also yield obvious benefits to reduce the related long-term cardiovascular risks and health care costs.

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