Case Report

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Molecular detection of *Treponema denticola* and *Porphyromonas gingivalis* in carotid and aortic atheromatous plaques by FISH: report of two cases

Francesca Cavrini, Vittorio Sambri, Annette Moter, Dora Servidio, Antonella Marangoni, Lucio Montebagnoli, Federico Foschi, Carlo Prati, Roberto Di Bartolomeo and Roberto Cevenini

1 Section of Microbiology, DMCSS, University of Bologna, Bologna, Italy
2 Institut für Mikrobiologie und Hygiene, Charité Universitätsmedizin, Berlin, Germany
3 Department of Oral Sciences, University of Bologna, Bologna, Italy
4 Department of Surgical Sciences, Anaesthesiology and Transplants, University of Bologna, Bologna, Italy
5 Centro Regionale di Riferimento per le Emergenze Microbiologiche, Bologna, Italy

*Treponema denticola* and *Porphyromonas gingivalis* have been identified in atheromatous plaques of two patients suffering from atherosclerosis by PCR and fluorescence *in situ* hybridization (FISH). The use of the FISH technique suggested that these periodontopathic micro-organisms might be metabolically active within the wall of arteries, under the atherosclerotic lesion.

Case reports

**Case 1.** The patient was a 73-year-old caucasian Italian male with a 12 year history of atherosclerosis and systemic hypertension. The patient also had a previous history of smoking and dislipidemia. He underwent percutaneous transluminal coronary angioplasty (PTCA) of the left coronary artery in 1991, and 2 years later he underwent coronary artery by-pass grafting (CABG). In 2003 an ecotomodoppler study of his carotid arteries showed a bilateral stenosis (70 % in the right vessel and 50 % in the left vessel). Consequently a computerized tomography (CT) scan was performed, which revealed the presence of partially calcified atheromatous plaques located at the carotid bulbs. The patient was then scheduled to undergo an endarterectomy to remove the plaque in the right bulb followed by the application of a dacron patch. Clinical and radiological examinations of the patient immediately before surgery showed a very poor periodontal situation, as indicated by a high value (103) of CPSS (clinical periodontal sum score: the sum of the number of sites with probing pocket depths of 4 mm or greater, the number of gingival sites with bleeding after probing or visible suppuration on probing, and the number of furcation lesions exceeding grade 1), which was the system used to evaluate the periodontal situation (Mattila *et al.*, 2000).

The crevicular fluid was sampled by using a paper cone inserted into the periodontal pockets and stored at −80 °C until it was processed for DNA extraction. During surgery, a section of the arterial wall including the atheromatous plaque was removed and longitudinally cut into two sections that were used for PCR and FISH testing.

The PCR protocol has previously been reported (Donati *et al.*, 1997) and was performed as described by Mättö *et al.* (1998) and by Siqueira *et al.* (2000) for the detection of *Porphyromonas gingivalis* and *Treponema denticola*, respectively. DNA extracted from *in vitro* grown *P. gingivalis* (ATCC 33277) and *T. denticola* (ATCC 35405) was used as a positive control; DNA extracted from *Treponema pallidum* (Nichols strain) (Sambri *et al.*, 2001) was used as a negative control in each PCR reaction set. DNA was extracted from the vessel biopsy as follows: the tissue was treated with a mechanical homogenizer and then 700 mg was incubated with buffer K [10 mM Tris (pH 8.3), 50 mM KCl, 1.75 mM MgCl₂, 0.01 % (w/v) bovine serum albumin, 0.45 % (v/v) Tween 20, 0.45 % (v/v) Nonidet P-40 and 100 µg ml⁻¹ Proteinase K] at 56 °C for 7 h. After incubation the sample was extracted with phenol/chloroform, precipitated with 0.3 M sodium acetate and 2-propanol, and resuspended in 200 µl of TE buffer.

The PCR analysis of the DNA extracted from the crevicular fluid showed the presence of *T. denticola* 16S rRNA gene sequence in the sample studied, but showed a negative result for *P. gingivalis*. The *T. denticola* 16S rRNA gene sequence

Abbreviations: CABG, coronary artery by-pass grafting; FISH, fluorescence *in situ* hybridization; PTCA, percutaneous transluminal coronary angioplasty.
lining the atherosclerotic lesion, as shown in Fig 1a. The atheromatous plaque was also evaluated for the presence of \( P. \) gingivalis-related oral treponemes using FISH performed with the bacterial probe TRE II, specific for treponemes of phylogenetic group II, including \( T. \) denticola, according to Choi et al. (1994), and eubacterial-specific probe EUB 338. Probe TRE II was 100 % homologous to the target site of the sequenced PCR product from the patient’s sample. Details of the molecular probes used are reported in Table 1; the sequences are deposited in ProbeBase (http://www.microbial-ecology.de/probebase/index.html) and were all targeted at the small subunit of the ribosome. These oligonucleotides were commercially synthesized and labelled at the 5' end either with fluorescein isothiocyanate (FITC), to give a green fluorescence, or with the Cy3 fluorochrome (indocarbocyanine, Thermo Hybaid Interactiva), giving a bright orange signal. Slides with selected strains of oral treponemes \( [T. \) denticola ATCC 35405, Treponema vincentii ATCC 33580 (member of group I), Treponema maltophilum ATCC 51939 (group IV)] were used as positive or negative controls. The FISH technique was performed as reported above with minor modifications. Control slides prepared with the following bacteria were used: \( T. \) denticola ATCC 35405, \( P. \) gingivalis ATCC 33577, and showed no higher homology with any published DNA sequence. However, the target sequence of the \( P. \) gingivalis probe (POGI) used for FISH was not included in the sequenced 16S rRNA gene fragment.

The atheromatous plaque was also evaluated for the presence of \( P. \) gingivalis with the probes POGI and EUB 338 using FISH. This technique was performed as reported above with minor modifications. Control slides prepared with the following bacteria were used: \( T. \) denticola ATCC 43037, Prevotella intermedia (ATCC 25611), Actinobacillus actinomycetemcomitans (ATCC 43718). The FISH showed the presence of bright yellow-green fluorescent microcolonies compatible with \( P. \) gingivalis within the aortic wall underlying the atherosclerotic plaque as shown in Fig. 1b.

**Discussion**

The relationship between periodontopathogenic bacteria and the development of atherosclerosis has been under investigation for many years providing increasing evidence that the chronic inflammation in periodontal disease may act as an additional factor for atherogenesis (Mattila et al., 2000). In the case of the patient described in the first report we confirmed by PCR the simultaneous presence of \( T. \) denticola in the crevicular fluid obtained from periodontal pockets and in the wall of the artery. In the case of the second patient this was not possible since this subject was completely edentulous. It should nevertheless be underlined that periodontal disease is among the major causes of tooth loss and that this chronic infection leads to edentulism within some decades after its appearance (Papapanou, 1993).

Several authors have described the identification, by PCR, immunofluorescence assay and electron microscopy, of \( T. \) denticola and \( P. \) gingivalis in dental lesions (Asai et al., 2002; Ishihara et al., 2004; Mättö et al., 1998) and in

**Table 1. Oligonucleotide probes used for the FISH technique**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')</th>
<th>Target species</th>
<th>Reference</th>
<th>Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRE II</td>
<td>GCTCCTTTCCTATTACCTTTAT</td>
<td>Group II treponemes (including ( T. ) denticola)</td>
<td>Moter et al. (1998)</td>
<td>Cy3</td>
</tr>
<tr>
<td>POGI</td>
<td>CAATACCTGATCCCGTATTCC</td>
<td>( P. ) gingivalis</td>
<td>Sunde et al. (2003)</td>
<td>Cy3</td>
</tr>
<tr>
<td>EUB 338</td>
<td>GCTGCTCCGGTAGAGT</td>
<td>Eubacteria</td>
<td>Amann et al. (1990)</td>
<td>FITC</td>
</tr>
</tbody>
</table>
atherosclerotic plaques (Haraszthy et al., 2000; Okuda et al., 2001) or in artery cells (Deshpande et al., 1998; Dorn et al., 1999), but these data did not support the presence of living bacteria in the vascular tissues. The use of the FISH technique allowed the detection of metabolically active bacteria (Moter & Göbel, 2000): in particular this method identified the presence of 16S rRNA, which is present only in cells that are actively synthesizing proteins. Although positive FISH results have been reported from starving cells (Oda et al., 2000), this fact allows us to assume that the bacteria identified within the wall of atherosclerotic vessel were living microorganisms with the typical spirochaetes morphology in case 1 and with the shape of microcolonies in case 2. The specificity of the FISH technique is very high, especially when performed with two different probes contemporarily, as in this study. The first oligonucleotide probe (EUB 338) detected the 16S rRNAs from most eubacteria (Amann et al., 1990) and was labelled with FITC, the second one was specific for the individual species (TRE II for T. denticola-related organisms and POGI for P. gingivalis) and was labelled with the Cy3 fluorochrome. In this way, the microscopic observation of tissue sections, with narrow band filter sets (HQ-F41-001; AHF, Analysentechnik) to visualize the FITC and Cy3 signals, gave an orange-yellow fluorescence in contrast with the green autofluorescence of the artery wall in the image overlays.

The use of the FISH technique supports the hypothesis that actively metabolizing periodontal pathogens might be located within the atherosclerotic artery wall and this is the first report describing the presence of living T. denticola and P. gingivalis in the intimate layer underlying the atheromatous plaque. However, the small number of cases studied do not allow any conclusion about the correlation between periodontal disease and the appearance of atherosclerosis. Further work is in progress to extend the study of the arterial biopsies from periodontopathic or edentulous patients suffering from atherosclerosis, by PCR and FISH, to investigate the presence of T. denticola and P. gingivalis.

References
Papapanou, P. N. (1993). Epidemiology and natural history of perio-

Fig. 1. FISH on tissue sections of the carotid wall from patient described in case 1 (a) and of the aneurysmatic aortic wall from case 2 (b). The hybridization was performed simultaneously with the eubacteria-specific probe EUB 338 labelled with FITC and with species-specific Cy3-labelled probes TRE II for T. denticola (a) and POGI for P. gingivalis (b). Observation was made by using the narrow band filter set HQ-F41-001. The presence at several sites of T. denticola within the wall of the aorta is indicated by the black arrows in (a), showing an orange-yellow fluorescence. Microcolonies of P. gingivalis are indicated by the black arrow in (b), showing a yellow-green fluorescence.

