

# Bacterial Signatures in Thrombus Aspirates of Patients With Myocardial Infarction

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**Background**—Infectious agents, especially bacteria and their components originating from the oral cavity or respiratory tract, have been suggested to contribute to inflammation in the coronary plaque, leading to rupture and the subsequent development of coronary thrombus. We aimed to measure bacterial DNA in thrombus aspirates of patients with ST-segment–elevation myocardial infarction and to check for a possible association between bacteria findings and oral pathology in the same cohort.

**Methods and Results**—Thrombus aspirates and arterial blood from patients with ST-segment–elevation myocardial infarction undergoing primary percutaneous coronary intervention (n=101; 76% male; mean age, 63.3 years) were analyzed with real-time quantitative polymerase chain reaction with specific primers and probes to detect bacterial DNA from several oral species and *Chlamydia pneumoniae*. The median value for the total amount of bacterial DNA in thrombi was 16 times higher than that found in their blood samples. Bacterial DNA typical for endodontic infection, mainly oral viridans streptococci, was measured in 78.2% of thrombi, and periodontal pathogens were measured in 34.7%. Bacteria-like structures were detected by transmission electron microscopy in all 9 thrombus samples analyzed; whole bacteria were detected in 3 of 9 cases. Monocyte/macrophage markers for bacteria recognition (CD14) and inflammation (CD68) were detected in thrombi (8 of 8) by immunohistochemistry. Among the subgroup of 30 patients with myocardial infarction examined by panoramic tomography, a significant association between the presence of periapical abscesses and oral viridans streptococci DNA–positive thrombi was found (odds ratio, 13.2; 95% confidence interval, 2.11–82.5;  $P=0.004$ ).

**Conclusions**—Dental infection and oral bacteria, especially viridans streptococci, may be associated with the development of acute coronary thrombosis. (*Circulation*. 2013;127:1219–1228.)

**Key Words:** bacteria ■ inflammation ■ myocardial infarction ■ thrombosis ■ viridans streptococci

Bacterial infections have been suggested to have a role in the origin of atherosclerosis. Of the infective agents, *Chlamydia pneumoniae* has been the most common suspect (reviewed by Vainas et al<sup>1</sup>), but antimicrobial treatment against *C pneumoniae* failed to show any benefit in the secondary prevention of coronary events.<sup>2</sup> Oral pathogens such as viridans group streptococci and the periodontal pathogen *Porphyromonas gingivalis* have been detected by immunohistochemistry in human carotid atherosclerotic plaques.<sup>3</sup> With the use of various polymerase chain reaction (PCR) techniques, periodontal bacterial DNA from *P gingivalis*, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Treponema denticola*, and *Campylobacter rectus* have been detected in the atherosclerotic lesions of

aortic tissues.<sup>4–7</sup> We have previously reported the presence of ribosomal DNA from oral streptococcal bacteria, including viridans *Streptococcus mitis* group,<sup>8</sup> in coronary atherosclerotic plaques. Subsequently, other groups have confirmed this finding and found >50 different bacteria, including other *Streptococcus* species, existing within the same atherosclerotic lesion.<sup>9,10</sup> Koren et al<sup>11</sup> identified *Chryseomonas* sp. in all and *Veillonella* sp. and *Streptococcus* sp. in the majority of atherosclerotic plaque samples, and their abundance in plaques correlated with their abundance in the oral cavity. At present, however, there is no consensus on the most pathogenically relevant bacteria.

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Poor dental health has been linked to an increased risk of cardiovascular diseases.<sup>12</sup> Chronic periodontal infection especially has been associated with the risk of acute myocardial infarction (MI)<sup>13</sup> and coronary heart disease.<sup>14</sup> In addition, Mendez et al<sup>15</sup> found that periodontal disease was a significant independent risk factor for peripheral vascular disease. Almost all the patients in their study (51 of 53 [96%]) had moderate or severe periodontitis or were edentulous. The most recent meta-analysis showed an increased risk of the simultaneous presence of cardiovascular disease in patients with periodontitis.<sup>16</sup> According to a recent large population study, angiographic coronary stenosis is linked to alveolar bone loss, pathological periodontal pockets, and the number of missing teeth.<sup>17</sup>

In a recent study by Ohki et al,<sup>18</sup> periodontal pathogens were detected in the thrombus aspirates of MI patients. Most studies<sup>13–18</sup> have focused on the role of periodontal pathogens or periodontal infections as a risk factor for atherosclerosis. We hypothesized that not only typical periodontal bacterial DNA but also other oral bacterial DNA can be detected in the thrombus aspirates of MI patients and that dental procedures are also linked to bacterial findings in the atherosclerotic tissues. We collected a series of thrombus aspirates from ST-segment–elevation MI patients from 2 heart centers and measured candidate bacterial DNA in these using real-time quantitative PCR. We also aimed to verify the link between periapical abscesses and MI by analyzing the association between bacterial findings and dental pathology in a subset of our patients. To evaluate the pathological significance of our bacterial findings, monocyte/macrophage markers for bacteria recognition (CD14) and inflammation (CD68) were immunohistochemically stained in available thrombus aspirates.

## Methods

### Subjects and Sampling

#### *MI Patients Treated With Primary Percutaneous Coronary Intervention and Clinical Parameters*

One hundred one consecutive patients with acute MI treated with primary percutaneous coronary intervention and successful thrombus aspirations were recruited from Satakunta Central Hospital, Pori, Finland, and Turku University Hospital, Turku, Finland, between December 2009 and March 2011. Aspiration of thrombi from the culprit artery was performed as part of the routine treatment of MI in a standardized fashion with sterile equipment (Export Catheter, Medtronic; or Quickcat Catheter, Spectranetics). After aspiration, the contents of the aspiration syringe were emptied into a pyrogen-free Eppendorf tube. A control blood sample was obtained for each patient from the arterial sheath before the procedure and stored in a similar tube for use as a reference. The percutaneous coronary intervention procedure was otherwise carried out according to local standards, and the sampling of aspirates did not affect the patients' treatment. For clinical laboratory measurements, peripheral venous blood samples were collected (nonfasting). Triglycerides, low-density lipoprotein, high-density lipoprotein, and total cholesterol were determined in serum with the use of standard laboratory techniques.

### Scoring of Dental Status

Of the 101 patients, 30 (29.5%) were subjected to dental panoramic tomography. Dental pathology was performed in 1 heart center and included 50 patients. Of those 50 patients, the first 30 subjects were included. With this technique, an image of the entire dentition is obtained on a single film or on a digital phosphor plate. The tube and the cassette holder of the panoramic tomography apparatus rotate in a scanning manner from one side of the patient's head around his or her back to the other side. During this motion, the film and plate cassette move synchronically, producing a 2-dimensional image of the upper and lower jaws with the orthogonal projections of the teeth (Figure 1). Panoramic x-rays were assessed by a board-certified dentist without knowledge of the clinical patient data. For every x-ray picture, 9 parameters of dental findings were scored. Altogether, 270 values were assessed for the 30 panoramic radiographs.

We assessed periodontal health as described in detail earlier.<sup>19</sup> The number of dental osseous lesions (periapical lesions at the root tips Ø1–4 mm) and signs of dental caries (caries lesions Black classes I–VI and residual roots, eg, totally decayed teeth) were calculated, and pericoronal lesions (infected areas around eighth molars) were recorded. Moreover, signs of dental treatments, for example, the number of fillings and root canal treatments, were recorded. Periodontal health was assessed by scoring vertical bony pockets (depth >3 mm) and furcation lesions (grade III; eg, no jaw bone left at the base of the root trunk of a tooth where ≥2 roots meet).<sup>20</sup> Scoring was done on a personal computer workstation (CliniView 7.1, Instrumentarium Dental) with the use of the digital panoramic x-rays, and distance and diameter evaluations were performed with panoramic tomography software tools (Carestream, Carestream Health).

### Detection of Bacteria

#### *Real-time Quantitative PCR*

Oligonucleotide primers and probes for real-time quantitative PCR are listed in Table I in the online-only Data Supplement. The primers and probes were designed and confirmed using BLAST with the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov>) or the Ribosomal Database Project (<http://rdp.cme.msu.edu/probematch/search.jsp>). Amplification primers and probes designed by other laboratories were synthesized according to the sequences published by the authors and examined in the same manner. Detailed information is given in the online-only Data Supplement. Briefly, the presence and amount of human DNA, total bacterial DNA, candidate bacterial DNA for endodontic bacteria (*Streptococcus* sp. mainly *Str mitis* group, *Str mitis*, *Str oralis*, *Str sanguinis*, *Str gordonii*, *Streptococcus anginosus* group, *Staphylococcus aureus*, *Sepidermidis*, *Parvimonas micra*, and *Prevotella intermedia*) and periodontal bacteria (*P gingivalis*, *Aggregatibacter* [née *Actinobacillus*] *actinomycetemcomitans*, *Fusobacterium nucleatum*, *Dialister pneumosintes*, and *T denticola*), as well as *Chlamydia pneumoniae*, were determined in thrombus aspirates and control arterial blood samples with real-time quantitative PCR and the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA). The relative amounts of bacterial DNA in thrombus were calculated in comparison to that in the inner control (blood) with the comparative Ct



**Figure 1.** Dental panoramic tomography of the upper and lower jaws. Arrows indicate periapical abscesses.

method ( $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{inner control}}$ )<sup>21</sup> with a simplification. The method gives n-fold difference in the amounts of candidate bacteria between the sample and control in relation to a reference gene. Thrombi results were classified into candidate bacteria positive or bacteria negative. The samples were marked to be positive for candidate bacteria if  $2^{-\Delta\Delta Ct} \geq 2$  or if there was amplified bacterial DNA in the thrombi but not in the control (blood) sample. The bacterial results from the first 11 aspirates were also verified with the broad-range PCR as described earlier.<sup>8</sup>

### Transmission Electron Microscopy

Randomly selected frozen thrombus aspirates (n=9) for electron microscopy were allowed to melt overnight in a glutaraldehyde-formaldehyde mixture (1% glutaraldehyde and 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.4), and were postfixed with buffered 1% osmium tetroxide and embedded LX 112 (Ladd). Thin sections were cut with an Ultracut E microtome (Reichert-Jung) and post-stained with uranyl acetate and lead citrate (Carlsberg system, LKB, Sweden). The electron micrographs were taken with a JEOL 1200EX transmission electron microscope operating at 60 kV.

### Immunohistochemistry

The activity of bacteria recognizing receptors in frozen formalin-postfixed histological sections from 8 thrombus aspirates was studied with CD14, clone 7, 1:70 (Novocastra, Leica Biosystems Newcastle Ltd), and CD68, clone KP1, 1:1000 (Nordic Biosite, AIB-30047, Nordic Biosite AB), antibodies diluted in normal antibody diluent (BD09-125) supplied by Immunologic. The diluted antibodies were pipetted onto slides for 40 minutes and washed twice in TBS-Tween for 5 minutes. Secondary staining was performed with BrightVision+ detection system (DPVB110-HRP), and visualization was done with diaminobenzidine (Bright DAB, BS04-110) according to the manufacturer's protocol. Confirmatory staining was prepared with primary antibody replaced with dilute and with DAB only to exclude the possibility of any erroneous staining result caused by endogenous peroxidase activity or necrotic cells.

### Ethical Issues

The studies were approved by the ethics committees of Satakunta Central Hospital and Turku University Hospital. All patients gave informed consent.

### Data Analysis

SPSS version 18.0 was used for the statistical analysis (SPSS Inc, Chicago, IL). The associations between the presence of bacterial DNA (positivity/negativity) in the sample and clinical parameters and between the presence of bacterial DNA and dental findings were calculated with the Fisher exact test. To calculate adjusted *P* values, binary and multinomial logistic regression analyses were used in which age and sex were covariables. Odds ratios and confidence intervals were calculated by CIA software version 1.1.<sup>22</sup> *P* values were not corrected for multiple comparisons because of the hypothesis-oriented study approach.<sup>23</sup> A value of *P*<0.05 was considered statistically significant.

## Results

### Patient Characteristics

The study included 101 MI patients. Detailed clinical characteristics are presented in Table 1. When we compared the clinical parameters of patients between the 2 heart centers, no significant differences (*P*<0.05) were seen, except that dyslipidemia was more common in subjects in center 1 (76.0% versus 37.3%; *P*<0.001,  $\chi^2$ ). All patients received aspirin and clopidogrel or prasugrel before the intervention. Bivalirudin was used in 55.4% and glycoprotein IIb/IIIa inhibitors in 18.8% of the patients.

**Table 1. Characteristics of Patients With Myocardial Infarction**

	MI Patients (n=101)
Male/female sex, %	76.2/23.8
Age, mean±SD, y	63.3±12.02
BMI, kg/m <sup>2</sup> , mean±SD	28.3±4.90 (n=71)
Hypertension, %	44.6
Diabetes mellitus, %	17.8
Dyslipidemia, %	56.4
Smoking (0/1/2), %*	59.4/36.6/4.0
CRP (<5 mg/L/>5 mg/L/no data), %	65.3/31.7/3.0
Total cholesterol, mean±SD, mmol/L	4.8±1.24 (n=85)
HDL, mean±SD, mmol/L	1.3±0.49 (n=86)
LDL, mean±SD, mmol/L	2.8±1.07 (n=84)
Triglycerides, median±IQR, mmol/L	1.4±1.10 (n=86)
STEMI, %	93.1
No. of stenotic arteries, %†	
1	50.5
2	31.7
3	17.8
Culprit lesion, %	
LAD	42.6
LCx	11.9
RCA	39.6
SVG	3.0
DG/IM	3.0
TIMI before, %	
0	83.2
1	3.0
2	5.9
3	7.9
TIMI after, %	
2	6.9
3	93.1
Killip class, %	
1	83.2
2	11.9
3	4.0

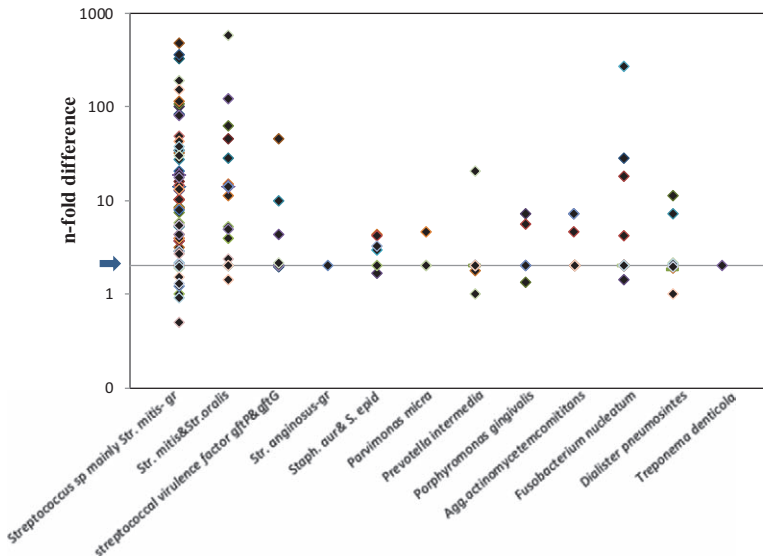
BMI indicates body mass index; CRP, C-reactive protein; DG/IM, diagonal/intermediate; HDL, high-density lipoprotein cholesterol; IQR, interquartile range; LAD, left anterior descending; LCx, left circumflex artery; LDL, low-density lipoprotein cholesterol; MI, myocardial infarction; RCA, right coronary artery; STEMI, ST-segment-elevation myocardial infarction; SVG, saphenous vein graft; and TIMI, Thrombolysis in Myocardial Infarction.

\*0 corresponds to nonsmoker; 1, smoker; and 2, ex-smoker.

†At least 50% stenosis in the RCA, LAD, or LCx.

### Bacterial Findings in Thrombus Aspirates

With the use of real-time quantitative PCR, the median value for the total amount of bacterial DNA in thrombi was 16 times higher than that found in their peripheral arterial blood samples (median, 16.2; 25th–75th percentile, 2.10–64.39). Figure 2 shows the relative amounts of candidate bacteria measured in their thrombus aspirates compared with that in their arterial blood. The greatest amounts were found in the measurements



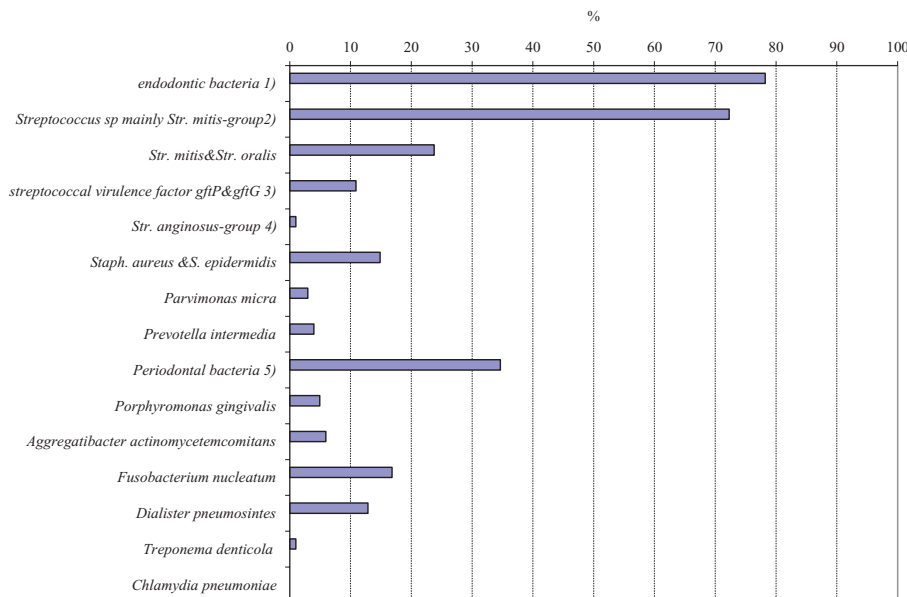
**Figure 2.** Relative amounts of bacterial DNA in patients thrombus aspirates. N-fold difference compared with the subject's own peripheral arterial blood. Individual values (♦). The arrow shows the bacteria positivity limit (2-fold difference). gr Indicates group.

of *Streptococcus* sp. mainly *Str mitis* group and *Str mitis*, and *Str oralis*. None of the samples contained DNA from *C pneumonia*.

Thrombus aspirates were categorized into candidate bacteria positive and negative. Figure 3 shows the frequencies of bacterial DNA–positive findings in thrombus aspirates. The most frequently found bacterial DNA was from *Streptococcus* sp. mainly *Str mitis* group in 73 of 101 thrombus aspirates (72.3%). Bacterial DNA from *A actinomycetemcomitans* was found in 6 aspirates (5.9%), and *P gingivalis* was found in 5 aspirates (5.0%). Positive results from ≥1 measurements of typical endodontic bacteria (*Streptococcus* sp. mainly *Str mitis* group, *Str mitis*, *Str oralis*, glucosyltransferase [*gtf*]

P&G streptococcal virulence factor, *Str anginosus* group, *S aureus*, *S epidermidis*, *Parvimonas micra*, or *Pr intermedia*) were detected in 78.2% of thrombus aspirates, and periodontal pathogens were detected (*P gingivalis*, *A actinomycetemcomitans*, *F nucleatum*, *D pneumosintes*, or *T denticola*) in 34.7%.

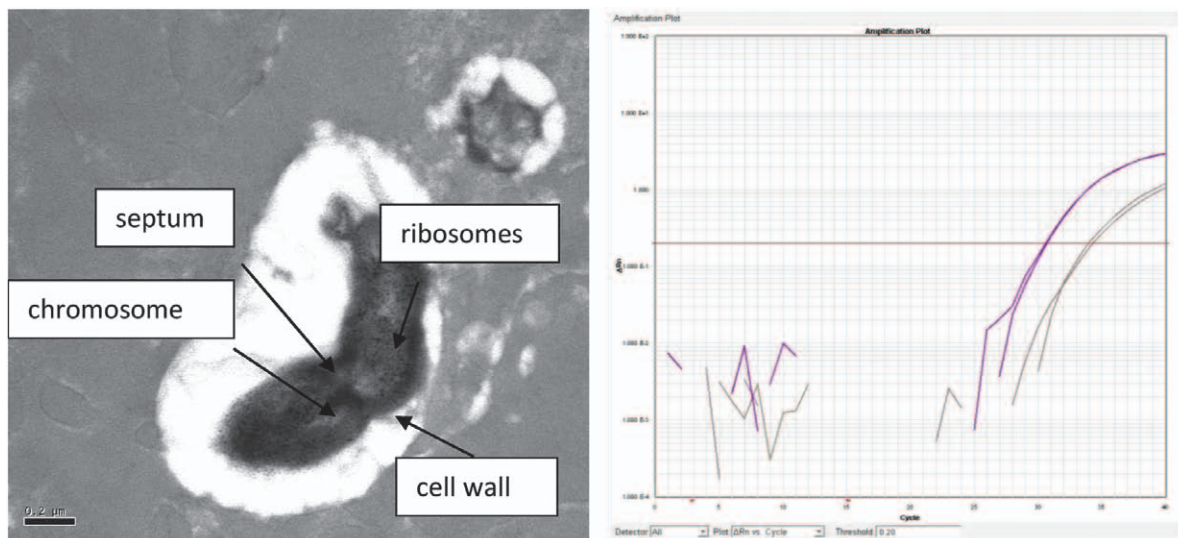
In 16 cases, the n-fold difference was between 0 and 2, that is, under the bacterial positivity limit for thrombi, suggesting possible bacterial DNA in arterial blood but not in thrombus aspirates. Ten arterial blood samples were positive for *Streptococcus* sp. mainly *Str mitis* group, 1 for *Str mitis* and *Str oralis*, 1 for *S epiderm* and *S aureus*, 1 for *P gingivalis*, 1 for *Pr intermedia*, 1 for *F nucleatum*, and 1 for *D pneumonistes*.



**Figure 3.** Frequencies of bacterial DNA-positive findings in thrombus aspirates of patients with ST-segment–elevation myocardial infarction using specific primers and probes in real-time quantitative polymerase chain reaction. Blood sample obtained from the arterial sheath before the procedure was used as reference.

1) positive result from one or more measurements of *Streptococcus* sp. (mainly *Str. mitis*-group), *Str. mitis*, *Str. oralis*, *gtfP&gtfG*-streptococcal virulence factor, *Str. anginosus*-group, *Staphylococcus aureus*, *S. epidermidis*, *Parvimonas micra*, *Prevotella intermedia*  
 2) recognition of *Str. mitis*, *Str. oralis*, *Str. gordonii*, *Str. sanguinis*, *Str. pneumoniae*, *Str. salivarius*, *Str. thermophilus*, uncultured streptococci, *Lactobacillus lactis*  
 3) recognition of *Str. sanguinis*(*gtfP*) and *Str. gordonii*(*gtfG*)  
 4) recognition of *Str. anginosus*, *Str. intermedius*, and *Str. constellatus*  
 5) positive result from one or more measurements of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Dialister pneumosintes*, *Treponema denticola*





**Figure 4.** Transmission electron micrographs (left) and bacteria quantitative polymerase chain reaction results (right) from the same thrombus aspirates. Arrows indicate bacterial structures. Total amount of bacterial DNA (purple curve) and amount of *Streptococcus* sp. mainly *Str mitis* group bacteria (green curve).

To demonstrate the possible identifiable bacteria structures in the thrombus aspirates, 9 frozen aspirates were also analyzed by transmission electron microscopy. Bacteria-like components were seen in all 9 cases, and whole bacteria were detected in 3 of 9 cases (Figure 4).

Through the use of immunohistochemistry, intensive staining of CD14 and CD68 was observed in all 8 thrombus aspirates (Figure 5). The presence of bacterial DNA was detected in all those thrombi.

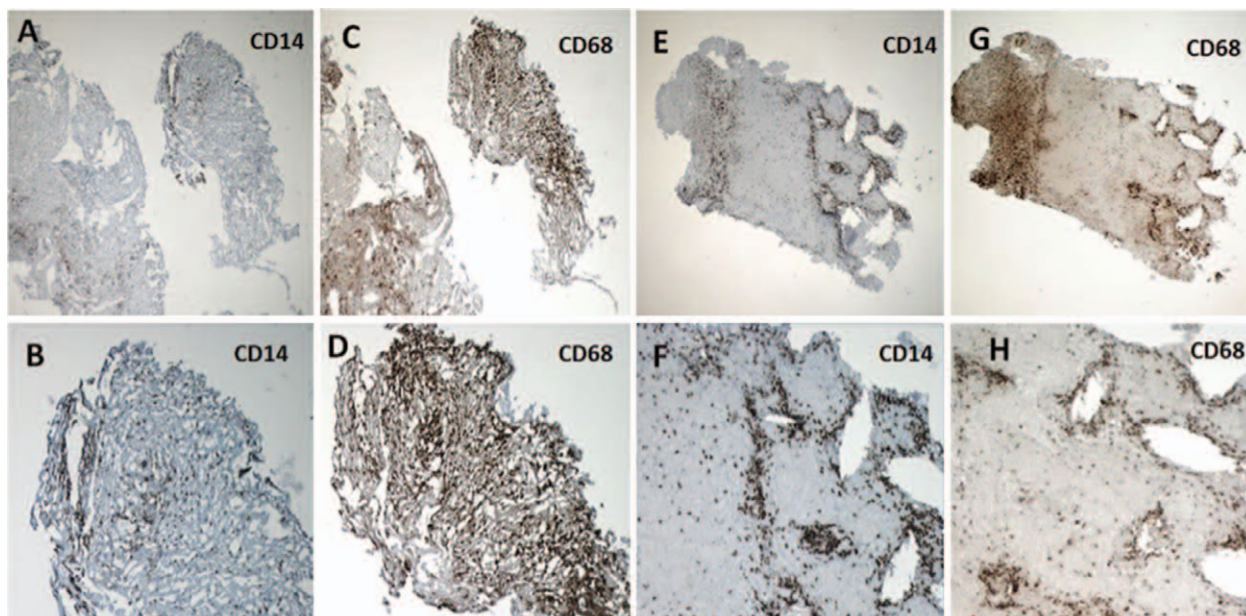
**The Presence of Bacterial DNA and Clinical Findings**

There was a trend of an inverse association ( $P=0.070$ , Fisher exact test; Table 2) between the presence of bacterial DNA

from *Streptococcus* sp. mainly *Str mitis* group and the number of stenotic arteries (narrowing by quantitative coronary angiography of >50%). The percentage of bacterial positivity was lowest (50.0%) in subjects with 3-vessel disease compared with patients with 1-vessel (74.5%) or 2-vessel (81.3%) disease. After adjustment for age and sex, the association remained ( $P=0.065$ , logistic regression). No other associations were found between different bacteria findings and clinical parameters.

**Dental Pathology and Its Association With Bacterial DNA Findings**

The panoramic tomographies of the 30 MI patients showed that the most common dental findings were signs of dental



**Figure 5.** Immunohistochemical stainings with CD14 and CD68 antibodies of 2 thrombus aspirates. **A** through **D**, Thrombus aspirate comprising tissue fragments from ruptured fibrous cap. **E** through **H**, Thrombus aspirate comprising mainly thrombotic material.

**Table 2. Association of Clinical Parameters With Oral Bacteria Found in Thrombus Aspirates of Patients With ST-Segment–Elevation Myocardial Infarction Undergoing Primary Percutaneous Coronary Intervention**

	n	Endodontic Bacteria, n (%) <sup>*</sup>	P†	<i>Streptococcus</i> sp. Mainly <i>Str mitis</i> Group, n (%)‡	P†	Periodontal Bacteria, n (%)§	P†
All	101	79 (78.2)		73 (72.3)		35 (34.6)	
No. of stenotic arteries							
1	51	40 (78.4)	0.096	38 (74.5)	0.070	19 (37.3)	0.623
2	32	28 (87.5)		26 (81.3)		10 (31.3)	
3	18	11 (61.1)		9 (50.0)		6 (33.3)	
Culprit lesion							
LAD	43	33 (76.7)	0.588	30 (69.8)	0.577	15 (34.9)	0.526
LCx	12	8 (66.7)		7 (58.3)		4 (33.3)	
RCA	40	33 (82.5)		31 (77.5)		12 (30.0)	
DG/IM	3	3 (100)		3 (100)		2 (66.7)	
SVG	3	2 (66.7)		2 (66.7)		2 (66.7)	
TIMI before							
0	84	62 (73.8)	0.192	57 (67.9)	0.181	25 (29.8)	0.171
1	3	3 (100)		3 (100)		3 (100)	
2	6	6 (100)		5 (83.3)		4 (66.7)	
3	8	8 (100)		8 (100)		3 (37.5)	
TIMI after							
2	7	5 (71.4)	0.662	4 (57.1)	0.393	1 (14.3)	0.417
3	94	74 (78.7)		69 (73.4)		34 (36.2)	
Killip							
1	84	64 (76.2)	0.571	58 (69.0)	0.255	27 (32.1)	0.589
2	12	11 (91.7)		11 (91.7)		5 (41.7)	
3	4	3 (75.0)		3 (75.0)		2 (50.0)	

DG/IM indicates diagonal/intermediate; LAD, left anterior descending; LCx, left circumflex artery; RCA, right coronary artery; SVG, saphenous vein graft; and TIMI, Thrombolysis in Myocardial Infarction.

<sup>\*</sup>*Streptococcus* sp. mainly *Str mitis* group, *Str anginosus* group, *Staphylococcus aureus*, *S epidermidis*, *Parvimonas micra*, and *Prevotella intermedia*.

†Crude *P* values (Fisher exact test).

‡*Str mitis*, *Str oralis*, *Str gordonii*, *Str sanguinis*, *Str pneumoniae*, *Str salivarius*, *Str thermophilus*, uncultured streptococci, and *Lactobacillus lactis*.

§*Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Dialister pneumosintes*, and *Treponema denticola*.

||At least 50% stenosis in the RCA, LAD, or LCx.

treatment—fillings (1 or more) in 86.7% and previous root canal treatments in 66.7%—and further pathological findings: furcating lesions in 63.3%, vertical bone defects in 50.0%, and periapical abscesses in 46.6% (Table 3). Of the periapical abscesses, 33.3% coincided with previous root canal treatment. There was a significant association between the presence of periapical abscess and *Streptococcus* sp. mainly *Str mitis* group (odds ratio, 13.2; 95% confidence interval, 2.11–82.5;  $P=0.004$ , Fisher exact test) in the thrombus aspirates. Of 14 patients with periapical abscess, 12 subjects (85.7%) were positive for this bacteria group, whereas in subjects without periapical lesions, these bacteria were detected in 5 of 16 cases (31.3%). The relative amount of these bacteria was also higher (median 12.7; 25th–75th percentile, 2.0–27.55) in patients with periapical abscesses than in those without periapical abscesses (median 1.6; 25th–75th percentile, 0.16–6.52). Similar results were obtained with endodontic bacteria (odds ratio, 7.71; 95% confidence interval, 1.28–46.4;  $P=0.017$ ). After adjustment with age and sex as confounders, both associations remained significant ( $P=0.003$  and  $P=0.008$ , respectively, logistic regression). There was also a link between

periodontal bacteria and periapical abscess (odds ratio, 7.00; 95% confidence interval, 1.14–43.0;  $P=0.046$ , Fisher exact test), but this did not remain significant after adjustment ( $P=0.115$ , logistic regression). No other associations between other bacteria in thrombus samples and dental findings were found.

## Discussion

Our results showed that oral bacterial DNA could be detected in coronary thrombus aspirates of MI. Bacterial DNA typical for endodontic infection, mainly oral viridans streptococci, was measured in 78.2% of thrombus aspirates, and periodontal pathogens were measured in 34.7%. A recent study by Ohki et al<sup>18</sup> reported similar percentages of periodontal bacteria found in the thrombus aspirates of MI patients.

The major bacteria found here in thrombus aspirates belonged to the viridans oral streptococci group. Viridans streptococcus is a pseudotaxonomic non-Linnean term for a group of human commensals most commonly found in the oral cavity. Traditionally, 6 groups have been classified as viridans streptococci: the *Str mitis* group, *Str sanguinis* group,

**Table 3. Association of Dental Findings With Oral Bacteria Found in Patients With ST-Segment–Elevation Myocardial Infarction Undergoing Primary Percutaneous Coronary Intervention**

	Presence of Dental Finding	n	Endodontic Bacteria,*		<i>Streptococcus</i> sp. Mainly <i>Str mitis</i> Group,† n (%)			Periodontal Bacteria,‡			
			n (%)	OR (95% CI)	P	OR (95% CI)	P	n (%)	OR (95% CI)	P	
All§		30	19 (63.3)			17 (56.7)			9 (30.0)		
Fillings	Yes	26	17 (65.4)	1.89 (0.23–15.74)	0.611	15 (57.7)	1.36 (0.17–11.23)	0.773	8 (30.8)	1.60 (0.14–18.0)	1.000
	No	4	2 (50.0)			2 (50.0)			1 (25.0)		
Root canal treatments	Yes	20	12 (60)	0.64 (0.13–3.25)	0.589	11 (55)	0.82 (0.17–3.81)	1.000	6 (30)	1.27 (0.24–6.82)	1.000
	No	10	7 (70)			6 (60)			3 (30)		
Furcation lesions	Yes	19	13 (68.4)	1.81 (0.39–8.35)	0.696	12 (63.2)	2.06 (0.46–9.30)	0.454	8 (42.1)	7.27 (0.77–68.9)	0.100
	No	11	6 (54.5)			5 (45.5)			1 (9.1)		
Vertical pockets	Yes	15	10 (66.7)	1.33 (0.30–5.92)	1.000	10 (66.7)	2.29 (0.52–10.01)	0.462	5 (33.3)	1.56 (0.31–7.82)	1.000
	No	15	9 (60.0)			7 (46.7)			4 (26.7)		
Periapical abscess	Yes	14	12 (85.7)	7.71 (1.28–46.4)	0.026	12 (85.7)	13.2 (2.11–82.5)	0.004	7 (50)	7.00 (1.14–43.0)	0.046
	No	16	7 (43.8)			5 (31.3)			2 (12.5)		
Caries lesions	Yes	13	9 (69.2)	1.58 (0.34–7.22)	0.708	8 (61.5)	1.42 (0.33–6.17)	0.721	4 (30.8)	1.0 (0.2–5.0)	1.000
	No	17	10 (58.8)			9 (52.9)			5 (29.4)		
Root canal treatment with periapical abscess	Yes	10	8 (80)	3.27 (0.55–19.5)	0.246	8 (80)	4.89 (0.82–29.1)	0.119	5 (50)	3.25 (0.61–17.3)	0.204
	No	20	11 (55)			9 (45)			4 (20)		
Residual roots	Yes	3	2 (66.7)	1.18 (0.09–14.69)	1.000	2 (66.7)	1.60 (0.13–19.84)	1.000	1 (33.3)	2.13 (0.12–38.48)	1.000
	No	27	17 (63.0)			15 (55.6)			8 (29.6)		
Pericoronary lesions	Yes	2	2 (100)	1×10 <sup>9</sup> (0.00)	0.520	2 (100)	1×10 <sup>9</sup> (0.00)	0.492	0 (0)	0.000 (0.000)	1.000
	No	28	17 (60.7)			15 (53.6)			9 (32.1)		

CI indicates confidence interval; and OR, odds ratio. *P* values were calculated with the Fisher exact test.

\**Streptococcus* sp. mainly *Str mitis* group, *Str anginosus* group, *Staphylococcus aureus*, *S epidermidis*, *Prevotella intermedia*, and *Parvimonas micra*.

†*Str mitis*, *Str oralis*, *Str gordonii*, *Str sanguinis*, *Str pneumoniae*, *Str salivarius*, *Str thermophilus*, uncultured streptococci, and *Lactobacillus lactis*.

‡*Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Dialister pneumosintes*, and *Treponema denticola*.

§Dental findings are number of cases.

*Str mutans* group, *Str salivarius* group, *Str anginosus* group, and *Str bovis* group.<sup>24</sup> Approximately 98% of oral streptococci belonged to 2 viridans streptococci groups: *Str mitis* and *Str salivarius* groups.<sup>25</sup> Viridans streptococci have traditionally been assumed to be the most important organisms in periapical lesions.<sup>26</sup> Koren et al<sup>11</sup> showed that an abundance of *Veillonella sp.* and *Streptococcus sp.* in the oral cavity was linked to their abundance in carotid atherosclerotic plaques. Oral viridans streptococci have several characteristics that may either induce or maintain the atherosclerotic process. These bacteria are able to attach to different surfaces and generate a biofilm enabling other bacteria to infiltrate the tissue.<sup>27</sup> A recent survey showed that oral viridans group streptococci are capable of invading human aortic endothelial cells and triggering the production of inflammatory cytokines and monocyte chemoattractant proteins.<sup>28</sup> Thrombogenic properties of viridans streptococci have also been investigated. Oral streptococci may initiate or contribute to platelet aggregation in coronaries.<sup>29</sup> A surface protein of *Str gordonii*<sup>30</sup> has been found to bind to the fibrinogen receptor of platelets. These findings suggest that oral viridans streptococci may be more than innocent bystanders with an affinity for inflammatory environments.

To evaluate the pathological significance of our bacteria findings, we also immunohistochemically stained thrombi containing not only platelets but also remnants of ruptured

plaque with monocyte/macrophage receptors CD14 and CD68. CD14 functions as a comolecule for Toll-like receptors that detect conserved microbial patterns and endogenous ligands and play a key role in initiating inflammatory responses.<sup>31</sup> It has been shown that *P gingivalis* and oral streptococci induce proinflammatory cytokine release and accumulation of macrophages through activation of CD14/Toll-like receptor 2 complex.<sup>4,32,33</sup> Moreover, immunostaining of CD68 correlates with the extent of inflammation in atherosclerotic lesion.<sup>34</sup> Thus, the detection of DNA specific to oral pathogens, together with costimulation of CD14 and CD68 in thrombus aspirates, may suggest that these pathogens disseminate into systemic circulation, migrate to coronary plaques, and cause or maintain inflammation. To exclude the possibility of any false staining resulting from endogenous debris or necrotic cells, the chromogenic reporter molecule DAB was used. No staining of DAB was seen.

The oral cavity contains almost 1000 different species, and there is a predominance of streptococci.<sup>27,35</sup> Designing primers and probes for different species of streptococci is challenging because of the high sequence similarities. Moreover, unknown sequences may cause unpredictable cross-reactivity within measurements. Of streptococci, *Str sanguinis* and *Str gordonii*, detected here by measuring streptococcal virulence factors *gtf* P&G, can be found only in the oral cavity.<sup>36</sup>



Thus, all the measurements for *gtf* virulence factor illustrate DNA originating from an oral source. Streptococci are also commonly found in the upper part of the gastrointestinal tract and the respiratory tract.<sup>11</sup> Although the most likely source of bacteria isolated from atherosclerotic samples is the oral cavity, we cannot exclude the possibility of other sources of transient bacteremia such as the gastrointestinal and respiratory tracts.

It is not currently known how these bacteria end up in atherosclerotic lesions. However, bacteremia originating, for example, from the oral cavity or the gut are more common than has been assumed.<sup>37</sup> Viridans streptococci bacteria are the most common bacteria found in peripheral blood samples after tooth brushing and after dental procedures such as tooth extraction.<sup>38</sup> One of the most common dental operations is root canal treatment. Bacteremia has been observed in 30% to 54% of nonsurgical root canal treatments.<sup>39</sup> Transient bacteremia is usually subclinical but can be demonstrated by blood cultures taken shortly after dental procedures.<sup>40</sup> In the peripheral blood, bacteria are phagocytosed and may subsequently be translocated into the atherosclerotic plaque, or they may end up in the plaque directly through the endothelium or via the vasa vasorum of the coronary artery. Van der Meer et al<sup>41</sup> showed that T cells specific to microbial species can be found in large numbers in peripheral blood and that they are abundant in atherosclerotic plaques. During the development of atherosclerotic lesions, which may take many decades, different microorganisms and different populations of T lymphocytes may contribute to the smoldering inflammatory process that characterizes atherosclerotic tissue. Therefore, repeated transient bacteremias after dental procedures and or other bacterial infections during the lifetime may cause an accumulation of pathogens in atherosclerotic plaques, which may act to boost the inflammatory process and to maintain chronic low-grade inflammation. Rupture of a plaque populated by bacteria with a high affinity for platelets could then lead to thrombus formation and subsequently to an acute coronary event.

Not only bacterial DNA but also whole bacteria cells—even living pathogens—have been detected in atherosclerotic samples.<sup>42–45</sup> In our randomly selected thrombus samples, 3 of 9 cases were found to contain whole (dividing or nondividing) bacteria, whereas various bacteria components and DNA were found in all 9 cases studied. Electron microscopy was used here to evaluate the presence of whole bacteria and bacterial fragments in the samples. Although it is the most commonly used and probably best suited for this purpose, evaluation is based only on morphological characteristics. However, we also performed quantitative PCR in the same samples showing the presence of bacterial DNA. Thus, we can conclude that whole bacterial structures can be detected in thrombus aspirates but that the detection of bacteria in samples by PCR does not provide evidence of whole bacteria but correlates with the presence of bacteria residuals in the area.

The severity of the cardiovascular disease measured as narrowed arteries has been shown to be linked to the severity of ongoing dental infection measured as alveolar bone loss, pathological periodontal pockets, and the number of missing teeth.<sup>17</sup> In addition to infection, the burden has been shown

to be associated with more severe atherosclerosis.<sup>46</sup> However, this has been questioned. As early as in 1999, Thomas et al<sup>47</sup> showed that the distribution of bacterial DNA did not correlate with the severity or extent of disease. In a recent study by Ohki et al,<sup>18</sup> no association was reported between the severity of coronary atherosclerosis and periodontal bacteria. In our study, there was an inverse association between the number of stenotic vessels and dental abscess bacteria, suggesting that bacterial infection may be more important in the vulnerable coronary plaque (Karhunen P et al, unpublished data, 2013). The observed association between the number of stenotic arteries and oral viridans streptococci should be interpreted with caution because the association was a trend and the number of stenosed vessels is only a rough estimate of coronary atherosclerosis.

The limitations of the present study include the fact that not all aspirated thrombi could be studied immunohistochemically because of their small size; in addition, the composition of our specimens was not known. Earlier studies have shown components of atherosclerotic plaque in up to 40% of cases. The thrombus itself is erythrocyte rich (red) in 30% to 40% and contains only platelets (white) in 60% to 65% of cases.<sup>48,49</sup> Panoramic tomographies were not available for all of our patients, limiting the statistical power of the analysis, which is reflected in the wide confidence intervals. The results in regard to the associations should therefore be interpreted with caution. All our patients had acute MI, and no control aspiration samples from healthy coronaries could be obtained. At this point, we cannot estimate whether the bacteria results were from living bacteria inside coronary atheroma or whether they were simply fragments from bacterial DNA engulfed by phagocytic cells from the circulation without any pathological significance.

Our results suggest an association between dental infection and acute coronary events. Because the primary prevention of coronary events is based mainly on lifestyle changes, improvements in dental health and dental care could be a major goal of preventive efforts.

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## Disclosures

None.

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### CLINICAL PERSPECTIVE

Infectious agents, especially bacteria and their components originating from the oral cavity or respiratory tract, have been suggested to contribute to inflammation in the coronary plaque, leading to rupture and the subsequent development of coronary thrombus. This study demonstrated that oral bacterial DNA, especially oral viridans streptococci, can be detected in the coronary thrombus aspirates of patients with ST-segment–elevation myocardial infarction undergoing primary percutaneous coronary intervention (n=101; 76% male; mean age, 63.3 years). Bacterial DNA typical for endodontic infection, mainly oral viridans streptococci, was measured in 78.2% of thrombus aspirates, and periodontal pathogens were measured in 34.7%. Bacteria-like structures and components were detected by transmission electron microscopy and monocyte/macrophage markers for bacteria recognition (CD14) and inflammation (CD68) in thrombus aspirates by immunohistochemistry. In a subgroup of patients with myocardial infarction (n=30) examined with panoramic tomography, a significant association between the presence of periapical abscesses and oral viridans streptococci DNA–positive thrombi was found. Our study confirms earlier studies and suggests that dental infections are implicated in the inflammation and rupture of vulnerable plaques. Repeated transient bacteremia after dental procedures or other bacterial infections during the lifetime may cause entrapment of pathogens in vulnerable atherosclerotic plaques. Improvements in dental health and prophylactic antibiotic treatment before dental procedures may affect the risk of future coronary events.

## **SUPPLEMENTAL MATERIAL**

Pessi T, Karhunen V, Karjalainen P et al. Bacterial signatures in thrombus aspirates of patients with myocardial infarction

### **Supplemental Methods**

Supplemental Methods 1. Real time quantitative PCR

### **Supplemental Tables**

Supplemental Table 1. Species-specific primers and Taqman probes for real-time qPCR.

### **Supplemental References**



## Supplemental Methods

### Supplemental Methods 1. Real time quantitative PCR

For RT-qPCR bacterial DNA was extracted from the samples using a commercially available QIAamp DNA Mini Kit (Qiagen, California, USA) according to the instructions provided. Table 1 presents the oligonucleotide primers and probes used for real time quantitative PCR (qPCR). The primers and probes were designed and confirmed using BLAST with the National Centre for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov>) and/or Ribosomal Database Project (<http://rdp.cme.msu.edu/probematch/search.jsp>). Presence and amount of human DNA, total bacterial DNA, candidate bacterial DNA for endodontic bacteria (*Streptococcus* sp. mainly *Str. mitis*-group, *Str. mitis*, *Str. oralis*, *Str. sanguinis* & *Str. gordonii*, *Streptococcus anginosus* -group, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Parvimonas micra* and *Prevotella intermedia*) and periodontal bacteria (*Porphyromonas gingivalis*, *Aggregatibacter* (*née* *Actinobacillus*) *actinomycetemcomitans*, *Fusobacterium nucleatum*, *Dialister pneumosintes* and *Treponema denticola*), as well as *Chlamydia pneumoniae* were determined in thrombus aspirates and control arterial blood samples. Specificity and cross reactivity of primers and probes were tested against human DNA, bacteria cultures from clinical samples<sup>1</sup> and reference bacteria from ATCC collection (*Streptococcus mitis* ATCC 49456, *Streptococcus sanguinis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, *Streptococcus anginosus* ATCC 33397, *Prevotella intermedia* ATCC 25611, *Treponema denticola* ATCC 35405, *Dialister pneumosintes* ATCC 33048, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586, *Parvimonas micra* ATCC 33270, *Aggregatibacter actinomycetemcomitans* ATCC 700685, *Porphyromonas gingivalis* ATCC 33277, *Chlamydia* (*née* *Chlamydia*) *pneumoniae* ATCC 53592; LCG Standards AB, Borås, Sweden). The observed concordance varied between 82.2% and 100%. The cut-off values, *i.e.* detection limits were 40 cycles for all candidate bacteria, except for *Streptococcus* sp. mainly *Str. mitis*-group and *F. nucleatum* whose cut off cycle was 37. The cut-off for universal bacterial measurement was 34

cycles. For each real-time PCR, 5  $\mu$ l of a mixture containing 1  $\mu$ l of DNA extract, 1 $\times$  TaqMan Universal Environmental PCR Master Mix (Applied Biosystems, Foster City, Calif., USA), 900 nM (each) sense and antisense primer, and 200 nM TaqMan probe were placed in each well of a 384-well plate. Amplification and detection were performed using the ABI PRISM 7900 sequence detection system (Applied Biosystems) with the following cycle profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 1 min. PCR was performed twice as duplicates of each sample. In uncertain cases analyses were repeated. The critical threshold cycle ( $C_t$ ) is defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial number of template molecules. Relative amounts of these organisms in specimens were calculated by the comparative  $C_t$  method ( $\Delta\Delta C_t$ ,  $\Delta C_t_{\text{sample}} - \Delta C_t_{\text{reference sample}}$ <sup>2</sup>), with a simplification. Briefly, first the differences of  $C_t$  values ( $\Delta C_t$ ) between candidate bacteria and reference gene for each sample were calculated. Secondly, the comparative  $C_t$  ( $\Delta\Delta C_t$ ) for sample (thrombus aspirates) and control samples (blood) was calculated. For the determination of total amounts of bacterial DNA in thrombus, human housekeeping gene, RNaseP (Applied Biosystems) was used as a reference and for amounts of candidate bacterial DNA in thrombus, universal bacteria was used as a reference. The thrombi results were classified into bacteria positive or bacteria negative. Samples were marked positive for candidate bacteria if  $2^{-\Delta\Delta C_t} \geq 2^{3,4}$  or if there was amplified bacterial DNA in the thrombi but not in the control (blood) sample.

## Supplemental Tables

Supplement table 1. Species-specific primers and Taqman probes for real-time qPCR.

Primer and probe	Sequence (5'-3')	reference
<i>Streptococcus sp. mainly Str. mitis</i> -group <sup>a)</sup>		1
Forward	CCAGCAGCCGCGGTAATA	
Reverse	CCTGCGCTCGCTTTACG	
Probe	ACGCTCGGGACCTACG	
<i>Str. mitis/Str. oralis</i>		5
Forward	GCCATTGAAGCGGTTACTTTG	
Reverse	CATCCGACATTAACGCAAGTTC	
Probe	ATGATTGAGCGTGGAACGGTGGGT	
<b>Streptococcal virulence factor gftG/gtfP<sup>b)</sup></b>		This study
Forward	TTTACCATGGATGAGCTCAAGCAA	
Reverse	TTGGAGAGCATGAGAGCATG	
Probe	ACGCAGTTCAATATCC	
<i>Streptococcus anginosus</i> –group <sup>c)</sup>		This study
Forward	CCGTAGTGTTTGTGCTAGGTGAAA	
Reverse	CCAGAGACGTAGCTGTTTCGT	
Probe	CCGTAACGATTTCTCG	
<i>Staphylococcus aureus / S. epidermidis</i>		1
Forward	GCGTTTTTCACGTGGAATATC	
Reverse	AATCCAAAACACAAACAAAGACAAGGT	
Probe	ACGTGCCATATTAATTTAC	
<i>Chlamydophila (néé Chlamydia) pneumoniae</i>		6
Forward	ATCCGCTGCTGCAAAC TACT	
Reverse	TGAACCACTCTGCATCGTGTA	
Probe	AGGCCGGGTTAGGTCTATCTACGGCAGT	
<i>Treponema denticola</i>		7
Forward	CCGAATGTGCTCATTACATAAAGGT	
Reverse	GATACCCATCGTTGCCTTGGT	
Probe	ATGGGCCC GCGTCCCATTAGC	
<i>Prevotella intermedia</i>		7
Forward	TCCACCGATGAATCTTTGGTC	
Reverse	ATCCAACCTTCCCTCCACTC	
Probe	CGTCAGATGCCATATGTGGACAACATCG	
<i>Fusobacterium nucleatum</i>		This study
Forward	AGGGTGAACGGCCACAAG	
Reverse	TCTCGGTCCATTGTCCAATATTCC	
Probe	ACACGGCCCTTACTCC	
<i>Aggregatibacter (néé Actinobacillus) actinomycetemcomitans</i>		8
Forward	CAAGTGTGATTAGGTAGTTGGTGG G	
Reverse	CCTTCCTCATCACC GAAAGAA	
Probe	ATCGCTAGCTGGTCTGAGAGGATGGCC	
<i>Dialister pneumosintes</i>		8
Forward	GAGGGGTTTGCGACTGATTA	
Reverse	CCGTCAGAC TTT CGTCCATT	
Probe	CACCAAGCCGACGATCAGTAGCCG	
<i>Porphyromonas gingivalis</i>		8



Forward	TGCAACTTGCCTTACAGAGGG	
Reverse	ACTCGTATCGCCCGTTATTC	
Probe	AGCTGTAAGATAGGCATGCGTCCCATTAGCTA	
<b><i>Parvimonas micra</i></b>		8
Forward	AAACGACGATTAATACCACATGAGAC	
Reverse	ACTGCTGCCTCCCGTAGGA	
Probe	TCAAAGATTTATCGGTGTAAGAAGGGCTCGC	
<b>Universal<sup>d)</sup></b>		8
Forward	TGGAGCATGTGGTTTAATTCGA	
Reverse	TGCGGGACTTAACCCAACA	
Probe	CACGAGCTGACGACA[A/G]CCATGCA	

a) recognition of *Str. mitis*-group (*Str. mitis*, *Str. oralis*, *Str. gordonii*, *Str. sanguinis*, *Str. pneumonia*), *Str. salivarius*, *Str. thermophilus*, uncultured streptococci, *Lactobacillus lactis*

b) recognition of virulence factor for *Str. sanguinis* (gftP) and *Str. gordonii* (gftG)

c) recognition of *Str. anginosus*-group (*Str. anginosus*, *Str. milleri*, *Str. constellatus*, *Str. intermedius*)

d) recognition of total amount of bacterial DNA

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