**Molecular detection of periodontopathic bacteria in synovial fluid**

**Language:** English

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**Introduction**
Rheumatoid diseases and periodontitis share similar features of inflammatory disorders. It is supposed that bacteria, including periodontopathogens could influence the progression of rheumatic diseases.

**Objectives**
In order to evaluate this possible association we established a specific and sensitive method based on molecular techniques to detect 5 major periodontopathogens, Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola, Tannerella forsythensis in synovial fluid.

**Material and Methods**
**Molecularbiological assessment of bacteria in synovial fluid**

1ml fresh synovial fluid was diluted in 1 x phosphat buffered saline. The cells were precipitated by centrifugation for 10min, 5000xg. For every sample 100 cells of E.coli strain XL2B were co-prepared in the same tube as a positive control for DNA isolation. Preparation of bacterial DNA was carried out using the blood extraction kit (QuiaGen) according to manufacturer's instruction for gram negative bacteria.

Cell pellets were vortexed vigorously and incubated in lysis buffer ATL containing proteinase K at 56°C overnight. After addition of one volume buffer AL the samples were incubated at 70°C for 10min. 200 µl of ethanol was added to the samples, vortexed and the samples were applied to a QIAamp Spin Column were the DNA is bound. After two washing steps (buffer AW1 and AW2) the DNA bound to the column is dried by centrifugation. The DNA was eluted twice with 50µl buffer AE supplied within in the kit. Long-term storage of DNA is possible at -20°C.

**Positive control for DNA isolation**

For each patient sample a positive control PCR was performed for detection of the E.coli strain XL2B.
PCR:

10pmol upper primer: 5’ ttg att aca agg atg acg acg 3’
10pmol lower primer: 5’ cgc gca ctc cag gcc ttt g 3’
0.2mM dNTP
1.5mM MgCl2
0.5U Taq-Polymerase (Invitrogen, Karlsruhe, Germany)
5mM Betain

Cycling:

5min 95°C
30sec 95°C/
45sec 57°C / 35 cycles
60sec 72°C /
5min 72°C

Specific PCR for 5 periodontopathogens

Specific PCR for each periodontopathogen was carried out using primers specific for 16S rRNA genes:

**Actinobacillus actinomycetemcomitans**
upper: 5’ aaa ccc atc tct gag ttc ttc 3’
lower: 5’ atg cca act tga cgt taa at 3’

**Porphyromonas gingivalis**
upper: 5’ agg cag ctt gcc ata ctg cg 3’
lower: 5’ act gtt agc aac tac cga tgt 3’

**Prevotella intermedia**
upper: 5’ caa aga ttc atc gga 3’
lower: 5’ gcc ggt cct tat tgc aag 3’

**Treponema denticola**
upper: 5’ taa tac cga atg tca ttc aca t 3’
lower: 5’ tca aag aag cat tcc cat ttc ttc tta 3’

**Tannerella forsythensis**
upper: 5’ gcg tat gta acc tgc ccg ca 3’
lower: 5’ tgc ttc agt gtc agt tat acc t 3’

**PCR-sample**

<table>
<thead>
<tr>
<th>Amplification profile</th>
<th>Annealing temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>10pmol upper and lower primer</td>
<td>5min 95°</td>
</tr>
<tr>
<td>PCR buffer supplied with the enzyme</td>
<td>30sec 92°C</td>
</tr>
<tr>
<td>0.2mM dNTP</td>
<td>45sec x°C / 35 cycles</td>
</tr>
<tr>
<td>1.5mM MgCl2</td>
<td>60sec 72°C</td>
</tr>
<tr>
<td>0.5U Taq-Polymerase (Invitrogen, Karlsruhe, Germany)</td>
<td>5min 72°C</td>
</tr>
<tr>
<td>1% formamide</td>
<td>hold 4°C</td>
</tr>
</tbody>
</table>

A negative control containing water instead of DNA was performed for each periodontopathogen. Positive controls consisting of varying concentrations of bacterial DNA were carried out for verification of method specific detection limit. DNA for positive controls was kindly supplied by Prof. JK Kook, Chosun University, Korea. The sensitivity of the amplification was analysed by means of dilution series ranging from 10.000, 1.000, 100 and 10 copies/reaction. As little as 10 copies per amplification were detectable for all five bacteria.
**Evaluation of periodontopathic bacteria in subgingival pockets**

**DNA-isolation**

Paper points for collection of subgingival samples were used to bind periodontopathogens of the deepest pockets of each quadrant. Preparation of bacterial DNA was carried out using the QIAamp DNA Mini Kit (Quiagen). The paper points were incubated with 180 µl ATL-buffer and 20 µl proteinase K and incubated at 70°C for 10 min. 200 µl buffer Al was added and the mixture was incubated at 96°C for 5 min. The mixture (without paper points) was applied to a QIAamp Spin Column and washed twice with buffer AW1 and AW2. The DNA was solved in 400 µl AE-buffer and stored at -20°C.

**Multiplex-PCR**

For specific amplification of Actinobacillus actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Tannerella forsythensis (Tf), and Treponema denticola (Td) the micro-Ident® test of HAIN Diagnostik based on alkaline phosphatase mediated staining reaction was used. 

Mastermix provided in the micro-Ident® test (containing buffer, biotynilated primer, DNA for positive control), 2U Taq-polymerase (Eppendorf), and 5 µl of isolated bacterial DNA were mixed. PCR was performed (5 min 95°C; 10 cycles: 30 sec 95°C, 2 min 58°C; 20 cycles: 25 sec 95°C, 40 sec 53°C, 40 sec 70°C; 8 min 70°C). 

The quality of PCR product was checked by agarosegel electrophoresis.

**Bacteria specific hybridization**

20 µl of the PCR product were mixed with 20 µl of the denaturation solution in the well of the tray and incubated at room temperature for 5 min. 1 ml prewarmed (45°C) hybridization buffer was added to the sample. A strip (hybridized with DNA sequences of each bacteria as well as a positive control) was placed in the well of the tray. The tray was incubated at 45°C for 30 min in a shaking water bath.

After complete aspiration of hybridization buffer 1 ml of stringent wash solution was added and incubated at 45°C for 15 min. The strip was washed once with 1 ml rinse solution for 1 min and 1 ml of conjugate solution was added (room temperature for 30 min).

After washing twice with 2 ml rinse solution and once with 1 ml distilled water 1 ml of substrate solution was added. The substrate incubation time varied between 3 and 20 min and the occurrence of bacteria was evaluated visually by means of colored bands. Two positive controls for amplification reaction and for conjugate were included in the test.

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### Results

**Detection of periodontopathogens in synovial fluid**

Lane 100bp marker (Invitrogen, Karlsruhe, Germany)

Lane 1:

Lane Dilutions of bacteria specific standard DNA

Lane 2-5:
Lane 2: 10,000 copies
Lane 3: 1,000 copies
Lane 4: 100 copies
Lane 5: 10 copies
Lane 6-15: Bacteria specific PCR using DNA isolated from synovial fluid of all patients suffering from rheumatoid arthritis or juvenile arthritis

Lane 6: patient 1, JIA, female, 14 years
Lane 7: patient 2, JIA, female, 14 years
Lane 8: patient 3, JIA, male, 16 years, 1. sample
Lane 9: patient 3, JIA, male, 16 years, 2. sample
Lane 10: patient 5, JIA, female, 15 years
Lane 11: patient 6, JIA, female, 17 years
Lane 12: patient 7, RA, female, 33 years
Lane 13: patient 8, RA, male, 53 years
Lane 14: patient 9, JIA, female, 9 years
Lane 15: patient 10, RA, male, 67 years
Lane 16: negative control containing water

PDR fragments were separated in 1% agarose gel containing 0.5µg/ml ethidium bromide and visualized by UV radiation

a) Actinobacillus actinomycetemcomitans  
b) Porphyromonas gingivalis  
c) Prevotella intermedia  
d) Treponema denticola  
e) Tannerella forsythensis

Detection of subgingival periodontopathogens

<table>
<thead>
<tr>
<th>Patients</th>
<th>A.a. synovial / gingival</th>
<th>P.g. synovial / gingival</th>
<th>P.i. synovial / gingival</th>
<th>T.f. synovial / gingival</th>
<th>T.d. synovial / gingival</th>
</tr>
</thead>
<tbody>
<tr>
<td>patient 1</td>
<td>- /</td>
<td>- /</td>
<td>- /</td>
<td>- /</td>
<td>- /</td>
</tr>
<tr>
<td>JIA, female, 14 years*</td>
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<tr>
<td>patient 2</td>
<td>- / +</td>
<td>+ / +</td>
<td>- / +</td>
<td>- / +</td>
<td>- / +</td>
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<tr>
<td>JIA, female, 14 years</td>
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<tr>
<td>patient 3</td>
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<td>- / -</td>
<td>- / +</td>
<td>- / +</td>
<td>- / +</td>
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<tr>
<td>JIA, male, 16 years, 1. sample</td>
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<tr>
<td>patient 3</td>
<td>- / -</td>
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<td>- / +</td>
<td>- / +</td>
<td>- / +</td>
</tr>
<tr>
<td>JIA, male, 16 years, 2. sample</td>
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<tr>
<td>patient 5</td>
<td>- / -</td>
<td>- / -</td>
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<tr>
<td>JIA, female, 15 years</td>
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<tr>
<td>patient 6</td>
<td>- / -</td>
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</tr>
<tr>
<td>JIA, male, 17 years</td>
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<tr>
<td>patient 7</td>
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<td>- / +</td>
<td>- / +</td>
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<tr>
<td>RA, female, 33 years</td>
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<tr>
<td>patient 8</td>
<td>- / -</td>
<td>- / -</td>
<td>- / -</td>
<td>- / +</td>
<td>- / +</td>
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<tr>
<td>RA, male, 53 years</td>
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<tr>
<td>patient 9</td>
<td>+ / -</td>
<td>- / -</td>
<td>- / -</td>
<td>- / -</td>
<td>- / -</td>
</tr>
<tr>
<td>JIA, female, 9 years</td>
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<tr>
<td>patient 10</td>
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<td>- / -</td>
<td>- / -</td>
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<tr>
<td>RA, male, 67 years**</td>
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</table>
Conclusions

Here we report on the evaluation of a highly sensitive molecular based detection system for the identification of periodontopathogens in synovial fluid. The method was optimized in order to detect up to 10 DNA copies of each periodontopathogen (A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythensis, T. denticola). DNA of A.a. and P.g. was detected in synovial fluid of two different patients suffering from both juvenile idiopathic arthritis and periodontitis. These results represent a first step to investigate a possible connection between the occurrence of oral bacteria in subgingival pockets and their presence in synovial fluid of rheumatic joints. The clinical relevance of the occurrence of periodontopathogens in synovial fluid for the development and/or progression of rheumatic diseases should be investigated in further clinical studies.

This Poster was submitted by Dr. Susanne Schulz.

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Molecular detection of periodontopathogenic bacteria in synovial fluid

Introduction

Rheumatic disorders and periodontitis as inflammatory diseases

Materials and Methods

DNA isolation of periodontopathic bacteria in synovial fluid

Molecular biological assessment of bacteria in synovial fluid

Evaluation of periodontopathic bacteria in subgingival pockets

Results and Discussion

Detection of periodontopathogens in synovial fluid

Detection of subgingival periodontopathogens

Table: Detection of periodontopathogens in synovial fluid

- Detection of subgingival periodontopathogens

- Table: Detection of subgingival periodontopathogens