Detection of *Mycoplasma salivarium* and *Mycoplasma fermentans* in synovial fluids of temporomandibular joints of patients with disorders in the joints

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Abstract

Thirty-six synovial fluid samples of temporomandibular joints were obtained from 33 patients with pain and anterior disk displacement (closed lock) in the joints. DNAs were prepared from the samples and amplified by a PCR-based assay specific for *Mycoplasma salivarium* or *Mycoplasma fermentans*. Of the 36 samples, five (14%), three (8%), and 19 (53%) were positive for *M. salivarium*, *M. fermentans* and both, respectively.

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1. Introduction

Most patients with disorders in the temporomandibular joint (TMJ) are annoyed by long-standing pain and dysfunction in the TMJ. The signs and symptoms in many of these cases are considered to be due to osteoarthritis (OA). OA is characterized by progressive degradation of articular cartilage. It is postulated that this process is caused by loss of a balance between proteases/cytokines and their inhibitors, which plays an important role in normal cartilage homeostasis [1,2]. We are interested in knowing whether or not exogenous stimuli, especially, oral microorganisms, participate in the pathogenesis of OA.

*Mycoplasma salivarium* is a member of the human normal microbial flora, and preferentially resides in dental plaques and gingival sulci [3,4]. Based on the incidence and viable counts of the organism in the normal and pathological human oral cavity or gingival crevice [3–5], antibody response to the organism [4,5], and its biological and immunological activities [5–8], the organism is considered to play an etiological role in some cases of oral infections, including periodontal diseases [3–5,7].

In a previous study [5], it was shown that the incidence of *M. salivarium* in the oral cavity (12/12 (100%) vs. 12/20 (60%); \(P < 0.01\), analyzed by chi-square test) and metabolism-inhibitory (MI) anti-
body titers to the organism (median: 106 vs. <16; P<0.05, analyzed by Wilcoxon’s rank sum test) were significantly higher in subjects with arthrosis temporomandibularis (AT) than controls.

Although the number of subjects was small, we speculated that *M. salivarium* might participate in the inflammatory process of disorders in the TMJ. To further confirm this conclusion, in the present study attempts were made to detect *M. salivarium* DNA from synovial fluid (SF) of patients with TMJ disorders.

2. Materials and methods

2.1. Subjects and SF samples

Subjects were 33 patients (30 females and three males, ranging in age from 15 to 59 years) afflicted with pain and anterior disk displacement (closed lock) in the TMJ, who visited the Department of Oral Surgery II at the Dental Hospital of Hokkaido University. The preauricular region was shaved, if necessary, and the area was surgically disinfected. By puncturing the skin with a disposable 23-gauge needle, physiological saline solution (2 ml) was injected into the superior joint space, and then the fluid (SF sample) was aspirated. SF samples were obtained from the left or right TMJ in 30 subjects, and both TMJs in three subjects.

2.2. DNA preparation from SF samples and mycoplasma cells

SF samples (200 μl) were mixed with 800 μl of HMW buffer (10 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA, 150 mM NaCl, 0.1% (w/v) SDS and 100 μg ml⁻¹ proteinase K) and incubated at 50°C for 2 h. The DNA was extracted twice with an equal volume of phenol saturated with TE buffer (10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA), once with phenol-chloroform-isoamyl alcohol (24:1, v/v). The aqueous phase was made to 0.3 M sodium acetate, and the DNA was precipitated with two volumes of absolute ethanol. The precipitate was suspended in TE buffer. The amount of DNA was determined spectrophotometrically by taking an optical density of 1 at 260 nm as 50 μg ml⁻¹ [9].

*M. salivarium* ATCC 23064 and *Mycoplasma fermentans* ATCC 19989 were propagated in the liquid medium of Hayflick [10] supplemented with 1% (w/v) L-arginine-HCl and D-glucose, respectively. When there was a rise (*M. salivarium*) or fall (*M. fermentans*) in pH of 1 unit, cells were harvested by centrifugation of the cultures at 15000×g for 15 min, washed three times, and suspended in phosphate-buffered saline (pH 7.2). The cells (10 mg of protein) of *M. salivarium* or *M. fermentans* were suspended in 1.0 ml of HMW buffer and were treated as described above. The DNA was suspended in 50 μl of TE buffer and was treated with DNase-free RNase A (50 μg ml⁻¹). After another extraction with phenol-chloroform-isoamyl alcohol and ethanolic precipitation, the DNA was suspended in TE buffer.

2.3. Amplification

PCR was performed with thermostable ExTaq DNA polymerase (Takara Biomedicals, Otsu, Japan) in an automated DNA thermal cycler (PC-700; ASTEC, Fukuoka, Japan). To prevent contamination, a strict spatial separation of the different technical steps involved in PCR was maintained, and the recommendations of Kwok and Higuchi [11] were followed.

The Takara Mycoplasma Detection Set (Takara), which was developed to detect mycoplasma infection in cell cultures on the basis of the DNA sequence of the 16S-23S spacer region in the rRNA operon [12], was used to screen for mycoplasmas in SF samples. The manufacturer’s instructions recommend amplifying mycoplasmal DNA by two rounds of PCR, the first round with primers F1 and R1 and the second round with primers F2 and R1. However, in this study, amplification was performed only by the first round of PCR. In compensation, the amplification cycle was increased from 30 to 45 cycles, the same cycle as that in the PCR-based assay for *M. fermentans* [13].

Some SF samples, first screened for mycoplasmas with the Takara Mycoplasma Detection Set, were assayed for DNAs of *M. salivarium* and *M. fermentans* by specific PCR [8,13], and the others were assayed without screening. The 5-μl sample to be analyzed was adjusted to a total volume of 50 μl in 1×ExTaq buffer (Takara) containing final concen-
trations of 200 μM each of dATP, dCTP, dTTP, and dGTP; 20 μM each of primers (sal-1 and sal-2 for *M. salivarium* [8], and RW004 and RW005 for *M. fermentans* [13]); and 0.25 U of ExTaq DNA polymerase. Oligonucleotides synthesized as primers and probes for Southern hybridization (sal-p for *M. salivarium* and RW006 for *M. fermentans*) were purified by HPLC with an Oligo DNA RP4 column.

2.4. Analysis of the amplified products

Amplified samples (7 μl) were analyzed on a 2% agarose gel (Nusieve 3:1; FMC, Rockland, ME, USA) in 0.5×Tris-borate-EDTA buffer containing 5 μg of ethidium bromide per ml. The DNA was visualized by UV fluorescence.

For Southern blotting, the agarose gel was depurinated in 0.25 M HCl, denatured in 0.5 M NaOH-1.5 M NaCl, neutralized by 1 M Tris-HCl (pH 8.0)-2 M NaCl, and transferred to a nylon membrane (Durapore membrane, Nihon Millipore, Yonezawa, Japan) by diffusion blotting in 10×SSC. The membrane was exposed to a UV-cross linker (GS Gene Linker, Bio-Rad Laboratories, Richmond, CA, USA). The membrane was prehybridized in 5×SSC-10×Denhardt’s solution-0.3% SDS-20 mM sodium phosphate buffer (pH 7.2)-500 μg of salmon sperm DNA per ml at 40°C for 3 h. Hybridization was performed for 18 h at 45°C (*M. salivarium*) or at 55°C (*M. fermentans*) in a prehybridization buffer containing 32P-end-labeled oligonucleotide probe. The blot was washed two times (15 min each) at 45°C (*M. salivarium*) or at 55°C (*M. fermentans*) in 1×SSC-1% SDS. The blot was autoradiographed for 4 h on Kodak Royal X-Omat films between intensifying screens at −70°C.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
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<th><em>M. fermentans</em></th>
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3. Results

Sixteen SF samples from 13 female patients were screened for mycoplasmas with the Takara Mycoplasma Detection Set, and 400-bp and 500-bp products were observed in eight of the 16 samples (Fig. 1). Judging from data supplied with the manufacturer’s instructions, the 400-bp and 500-bp products were suspected to be DNAs of *M. salivarium* and *M. fermentans*, respectively. Therefore, the DNAs were amplified by PCR specific for *M. salivarium* or *M. fermentans*, and eight samples were shown to be positive for both *M. salivarium* and *M. fermentans* (Table 1, Figs. 2 and 3).

As expected, *M. salivarium* was detected in TMJs, but detection of *M. fermentans* was absolutely unexpected. Therefore, this result encouraged us to examine 20 more patients.

SF samples from 20 patients were not screened with the Takara Mycoplasma Detection Set, but assayed directly for the DNAs of the two *Mycoplasma* species. Five, three and 11 patients were positive for *M. salivarium*, *M. fermentans* or both *Mycoplasma* species, respectively. Only one patient was negative for both species.

4. Discussion

*M. salivarium* and *M. fermentans* were detected in SF samples from 22 (66%) and 20 (61%) of 33 subjects, respectively. This is the first report that microorganisms were detected in TMJs. We were interested in the coincidence of this finding that *M. salivarium* was present in TMJs of 66% of patients with TMJ disorders and the finding in our previous...
study [5] that nine (64%) of 14 subjects with AT were positive for MI antibodies to the organism. Also, it was surprising that *M. fermentans* was detected in SF samples. This was absolutely unexpected.

*M. salivarium* possesses a variety of biochemical activities considered to facilitate the attack of host tissues and also escape from phagocytosis [5,7,8]. The organism stimulates human gingival fibroblasts and human peripheral blood mononuclear cells to induce interleukin (IL)-6 and IL-8 production [7] and IL-1β and tumor necrosis factor α (TNFα) production [6], respectively.

*M. fermentans* was isolated from 31 (39%) of 79 rheumatoid synovial effusions and three (8%) of 37 effusions obtained from patients without demonstrable rheumatoid arthritis (RA). Immunoglobulin was shown to bind firmly to the organism membrane. Based on these findings, Williams et al. [14] postulated that the organism possibly participated in pathogenesis of RA, since the organism is often present in affected joints in RA and could be the stimulus for production of rheumatoid factors. Thus, considerable attention was paid to the possibility that *M. fermentans* might be a cause of RA. However, this could not be established, because other investigators failed to isolate the organism from rheumatoid synovial effusions by similar methods. Recently, *M. fermentans* was detected in the joints of patients with RA by PCR [15–18], and by both PCR and culture [17].

*M. fermentans* also induces IL-6 and IL-8 production by stimulation of human gingival fibroblasts [7], and IL-1, IL-6 and TNFα production by stimulation of monocytes [19]. Potent natural macrophage stimulators (lipopeptides) were recently separated from *M. fermentans* cells [20].

The presence of these mycoplasmas in TMJs will exert damaging effects on synovium and cartilage by their enzymatic activities and metabolites; will surely affect the balance between cytokines and their inhibitors in TMJs by their cytokine-inducing activities; and could thus be a cause of pathological changes.

Acknowledgments

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References


