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Isolation and characterization of *Streptococcus mitis* from blood of child with osteomyelitis

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Objectives. Osteomyelitis is an inflammatory process accompanied by bone destruction that is caused by bacterial infection, with most child cases showing a haematogenous origin and metaphysis of the long bones. The aim of the present study was to characterize streptococcal strains isolated from the blood of a child diagnosed with osteomyelitis in a long bone and investigate the biological properties related to virulence of strains associated with osteomyelitis.

Methods. Blood isolate species were determined based on the 16S rRNA sequence. Next, the blood isolates were analysed for phagocytosis susceptibility by polymorphonuclear leukocytes, platelet aggregation, inhibitory effects on osteoblastic cells, and their properties of adhesion with cells, and compared to the reference strain *Streptococcus mitis* ATCC49456.

Results. The blood isolates were found to be a single clone (named SA1101), which was determined to be *S. mitis*. The phagocytosis susceptibility of SA1101 was significantly lower than that of ATCC49456, while its platelet aggregation rate was higher. Furthermore, SA1101 showed an inhibitory effect toward the growth of osteoblastic cells and had greater properties of adhesion to those cells as compared to ATCC49456.

Conclusions. These results suggest that *S. mitis* SA1101 is a possible etiological agent and caused osteomyelitis in the present case.

Introduction

Osteomyelitis is an inflammatory process accompanied by bone destruction that is caused by bacterial infection and has been classified into three categories based on pathogenesis; secondary to a contiguous focus of infection, secondary to vascular insufficiency, and haematogenous origin[1,2]. Most cases of osteomyelitis that occur in children have a haematogenous origin and affect metaphysis of the long bones, such as the femur, tibia, and humerus, with *Staphylococcus aureus* generally considered the most common bacterial pathogen related to the disease[3]. In addition, group A β-haemolytic *Streptococcus* and *Streptococcus pneumoniae* are also considered to be major pathogens for osteomyelitis[1,4]. On the other hand, oral streptococci are rarely reported as possible pathogens[5].

Oral streptococci are able to invade the bloodstream and cause several kinds of systemic diseases, among which subacute infective endocarditis associated with dental procedures is well known to be related to the mitis group of species[6]. The mitis group is composed of *Streptococcus sanguinis*, *Streptococcus parasanguinis*, *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus pneumoniae*, and the 16S rRNA sequences of *S. mitis*, *S. oralis* and *S. pneumoniae* exhibit a greater than 99% homology with each other[7]. Of those, *S. mitis* is known to be a pioneering oral microorganism in infants, and a significant number of clones of this species inhabit the mucosa of the oral cavity and pharynx[8]. Although the bacterium is regarded as a leading causative agent of infective endocarditis[9], only a limited number of reports have

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shown its association with other types of disease[10]. *S. mitis* is generally considered to be the most common oral streptococcal species causing bacteremia in children and has been associated with serious complications[8].

In the present study, we analysed an *S. mitis* strain isolated from blood and dental plaque specimens obtained from a 7-year-old girl diagnosed with osteomyelitis in a long bone. The origin of the pathogenic bacteria and properties related to the pathogenicity of osteomyelitis were considered.

Materials and methods

Subject

A 7-year-old girl came to the Children's Hospital, University of Helsinki, because of pain in both ankles, which had lasted for several weeks and was stronger in the right, causing a right-side limp. Suspicion of osteomyelitis in the right tarsus arose on the basis of clinical symptoms that consisted of pain, tenderness on palpation, and redness and swelling, as well as changes found by magnetic resonance imaging, and results of laboratory tests (CRP elevated to 30). A blood sample was obtained and inoculated into both aerobic SN and anaerobic FA BacT/Alert bottles (bioMérieux, Durham, NC, USA), then incubated at 37°C in a BacT/Alert 240 blood culture analyzer (bioMérieux) with automatic monitoring. Samples in bottles that showed positive were inoculated onto chocolate agar and incubated at 35°C in air with 5% CO_2, as well as onto fastidious anaerobe agar (FAA) and incubated at 37°C under anaerobic conditions. All bacterial colonies had a uniform morphology. Bacterial masses were collected from the plates and stored at −70°C until use. Biopsy specimens were taken from the tibia and fibula, and then incubated on chocolate agar, blood agar containing oxolinic acid and colistin, and FAA, according to an established methodology[11]. Streptococci belonging to the *Streptococcus viridans* group were isolated from a blood sample (later designated as SA1101), whereas the tibia and fibula specimens were negative. The diagnosis of osteomyelitis was confirmed and treatment with clindamycin was started. The symptoms quickly subsided and the patient was discharged after receiving 4 days of i.v. clindamycin. After an additional 8 weeks of oral clindamycin (40 mg/kg/day), the patient was asymptomatic and the results of laboratory tests were within normal limits.

Three months prior to the osteomyelitis episode, the patient had visited a dentist, at which time multiple carious lesions and a fistula derived from severe dental caries in the mandibular left second primary molar were identified. Due to management problems, the dental treatment was postponed until 2 months after treatment for osteomyelitis, then dental plaque specimens were collected, from which 100 strains were isolated on Mitis-salivarius (MS) agar (Difco Laboratories, Detroit, MI, USA) plates (two of which were later designated as SA1201 and SA1202). In addition, dental plaque specimens were collected again at a recall examination 1 year later, from which 100 strains were isolated using the same method (one of which was later designated as SA1301). The study protocol was approved by the Uusimaa Ethics Committee for Gynaecology and Obstetrics, Pediatrics and Psychiatry, and informed consent was obtained from the patient and her parents for this study.

Bacterial strains

In addition to the bacterial strains isolated from the blood (SA1101) and dental plaque (SA1201, SA1202, and SA1301) specimens of the present patient, *S. mitis* ATCC49456 and *S. pneumoniae* R6, reference strains for each species, were also used. All strains were grown in Brain Heart Infusion (BHI) broth (Difco) and on Mitis-Salivarius (MS) agar (Difco).

Determination of species isolated from specimens

To determine the species in the obtained specimens, we first extracted genomic DNA using a conventional method. Briefly, bacterial cells were collected in a micro-centrifuge tube and incubated by *N*-acetylmuramidase SG (Seikagaku Corp., Tokyo, Japan) and lysozyme (Wako Pure Chemical Industries, Osaka

Japan). Then, genomic DNA was extracted using a Gentra Puregene Yeast/Bact. Kit B (QIAGEN, Tokyo, Japan), according to the manufacturer's instructions. For the blood isolates, approximately 1.5 kbp of a 16S rRNA gene fragment, which encompasses the conserved region of the 16S rRNA gene, was amplified by PCR with Ampli*Taq* Gold polymerase (Applied Biosystems, Foster City, CA, USA) using the primers 8UA (5′-AGA GTT TGAT CCT GGC TCA G-3′) and 1540R (5′-AAG GAG GTG ATC CAG CC-3′)[12]. The PCR products were separated by electrophoresis on a 0.7% agarose gel and amplified DNA fragments were extracted from the gel, using a QIAEX gel extraction kit (Qiagen, Düsseldorf, Germany). The DNA was then directly cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA), after which the nucleotide sequence was determined using a dye-terminator reaction with a DNA sequencing system (373-18 DNA sequencer; Applied Biosystems) and an ABI PRISM kit. Data analyses were performed using Gene Works software (IntelliGenetics, Mountain View, CA, USA). The 16S rRNA gene sequences obtained were compared with those available in the GenBank/EMBL/DDBJ databases using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/). As for the dental plaque specimens, all isolated strains were screened using broad-range PCR by targeting the shorter part of the 16S rRNA gene fragment (approximately 500 bp) amplified by PCR with Ampli*Taq* Gold polymerase using the primers PA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and PD (5′-GTA TTA CCG CGG CTG CTG-3′)[13]. After selecting the strains by fingerprinting pattern analysis described later, approximately 1.5 kbp of the 16S rRNA gene fragments of the selected strains were amplified and the sequences were determined, as described above.

Fingerprinting pattern analysis

Random amplified polymorphic DNA (RAPD) analysis was performed using Ready-To-Go RAPD analysis beads and primers (GE Healthcare Japan, Tokyo, Japan) by the method described previously[14]. Briefly, PCR was performed using 6 primers (P1, 5′-GGT GCG GGA A-3′; P2, 5′-GTT TCG CTC C-3′; P3, 5′-GTA GAC CCG T-3′; P4, 5′-AAG AGC CCG T-3′; P5, 5′-AAC GCG CAA C-3′; and P6, 5′-CCC GTC AGC A-3′) and comprised 45 cycles of denaturing at 95°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min. Amplicons were separated by electrophoresis on 2% agarose gels to compare the patterns of the bands.

Phagocytosis assays

Phagocytosis susceptibility was analysed using a method described previously[15]. Briefly, the organisms were cultured in brain heart infusion broth (Difco) for 18 h at 37°C. After the bacterial cells were washed, cell concentrations were adjusted with PBS to 1×10^8 CFU/mL. Human peripheral blood (500 μL) was collected from a healthy volunteer and incubated with 500 μL (5×10^7 CFU) of the tested bacteria for 10, 30, 60, 90, 120, and 180 min at 37°C. Interactions between polymorphonuclear leukocytes (PMNs) and the bacteria were observed with a light microscope following Giemsa staining. The rate of phagocytosis is expressed as the mean ratio of phagocytosed PMNs per 100 PMNs, with 500 PMNs examined. The assays were repeated three times and the results are expressed as the mean ± SD.

Platelet aggregation assays

Platelet aggregation assays were performed using whole blood obtained from mice by an impedance method with an aggregometer (Whole-blood aggregometer C540, Baxter Ltd., Tokyo, Japan). The present animal experiments were approved by the institutional animal care and use committee of Osaka University Graduate School of Dentistry. Since the *S. mitis*-derived human platelet aggregation factor was reported to be released into supernatant[16], supernatant samples were extracted from 1×10^9 CFU cells of ATCC49456 and SA1101. Next, the supernatant samples and whole blood obtained from mice (ICR, male, 8 or 9 weeks old, Japan

SLC, Inc., Hamamatsu, Japan) were incubated at 37°C for 5 min, followed by the addition of 4 μg of collagen (type I native collagen fibrils from equine tendons suspended in isotonic glucose solution at pH 2.7; Chrono-log Co., Havertown, PA, USA). The aggregation rate for each strain was calculated using the impedance value obtained with an aggregometer using the following formula. Aggregation rate (%) = [impedance value (Ω) of sample with bacterial cells/impedance value (Ω) of sample without bacterial cells] × 100. The assays were repeated three times and the results are expressed as the mean ± SD.

Effects on osteoblastic cells

MC3T3-E1 osteoblastic cells, a clonal preosteoblastic cell line derived from newborn mouse calvaria (RIKEN, Tsukuba, Japan), were maintained in α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in humidified air with 5% CO_2. MC3T3-E1 cells (1×10^5 cells/well) were seeded on 24-well culture plates, then the medium was replaced with α-MEM without antibiotics. We then quantified bacterial adhesion to MC3T3-E1 cells, as follows. The tested bacterial strains (ATCC49456 and SA1101) were added separately to MC3T3-E1 cells at a multiplicity of infection (MOI) of 100, then incubated for 90 min. External non-adherent bacteria were removed by washing the cells three times with PBS, after which the cells were disrupted by addition of 1000 μL of distilled water and incubated at 37°C for 20 min. Serial dilutions of the disrupted mixture were plated on MS agar plates and incubated for 2 days, then the numbers of adherent and invading organisms were determined. The assays were repeated three times and the results are expressed as the mean ± SD.

Next, the cell proliferation assays were performed for 24 h. In brief, 1×10^5 cells per well were seeded on 24-well culture plates, then the medium was replaced with α-MEM without antibiotics. Next, 1×10^7 CFU of the tested bacterial strains (ATCC49456 and SA1101) were added to the wells at an MOI of 100 and incubated for 3 h, followed by replacement of the α-MEM with antibiotics. The cells were washed with PBS twice at various time points (3, 6, 12, and 24 h) and harvested using 0.25% trypsin-EDTA (Invitrogen). The numbers of cells were counted and their morphology observed. The results of cell growth are expressed as compared to the initial number of cells, which was considered to be 100%. The assays were repeated three times and the results were expressed as the mean ± SD.

Statistical analyses

All data are presented as the mean ± SD. Statistical analyses were performed with an unpaired Student's *t*-test using the computational package Prism 4 (GraphPad Software Inc., San Diego, CA, USA).

Results

The blood isolates were found to be a single clone, designated as strain SA1101, which appeared as an α-haemolytic colony on blood agar. Determination of its 16S rRNA sequence (GenBank accession no. AB600875) showed that SA1101 possesses a high homology to both *S. mitis* ATCC49456 (GenBank accession no. DQ303188) (99%) and *S. pneumoniae* R6 (GenBank accession no. AE007317) (99%). On the other hand, microscopic observation of SA1101 did not reveal the appearance of a diplococcus, but rather a long chain, while an optochin test showed that SA1101 was resistant. Thus, we designated strain SA1101 as *S. mitis*.

Determination of the short fragment of the 16S rRNA sequence (approximately 500 bp) in 200 oral isolates obtained from the patient over a 1-year interval demonstrated that 56 strains (21 strains isolated at the time of dental treatment and 35 at the recall examination conducted 1 year later) were likely *S. mitis*, and then confirmed to be *S. mitis* by determination of the entire part of the 16S rRNA sequence as well as analyses of the biological properties. All of the strains could be classified into three patterns by RAPD analyses, with two different patterns identified among strains isolated at the time of dental treatment

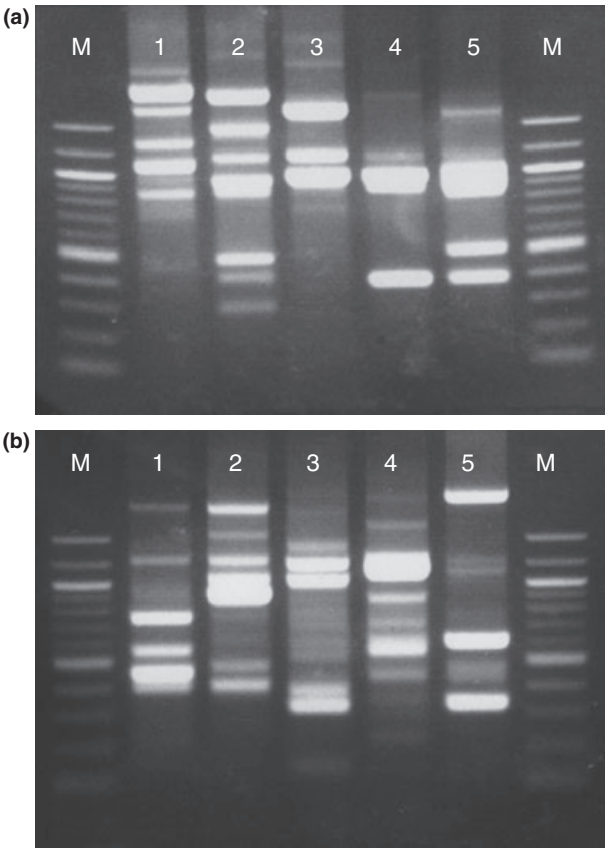

Fig. 1. Comparison of fingerprinting patterns for *Streptococcus mitis* strains analysed using RAPD method. Two representative results obtained using primers 2 (a) and 4 (b) are shown. Lane 1, ATCC49456; lane 2, SA1101; lane 3, SA1201; lane 4, SA1202; lane 5, SA1301. M, molecular size marker (100-bp DNA ladder).

(designated as strains SA1201 and SA1202) and two different patterns for the strains isolated at the time of the recall examination, one of which was consistent with SA1201, while the other was not consistent with any other patterns and designated as SA1301 (Fig. 1). The fingerprinting patterns of SA1201, SA1202, and SA1301 were not consistent with that of SA1101.

As for *in vitro* analysis results, the phagocytosis rate of SA1101 was significantly lower than that of ATCC49456 at 10 min after incubation, whereas there were no significant differences between these two strains at time points later than 30 min (Fig. 2). The platelet aggregation rate of SA1101 was 110.2 ± 4.0%, which was significantly greater than that of ATCC49456 (97.2 ± 6.8%) and with no addition of bacteria (100.0 ± 5.8%) ($P <$

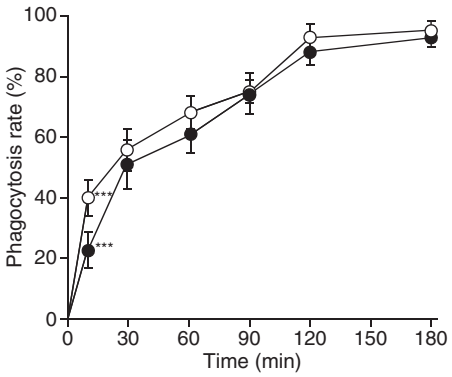

Fig. 2. Transitional changes in phagocytosis rates of SA1101 and ATCC49456. Data are shown as the mean ± SD from five experiments. Shown are changes in the phagocytosis rates of SA1101 (●) and ATCC49456 (○) after 10, 30, 60, 90, 120, and 180 min of incubation. There were statistically significant differences between the strains (***$P < 0.001$).

0.001). In analyses using osteoblastic cells, the number of SA1101 organisms that adhered to the cells was significantly greater than seen with the ATCC49456 organisms (Fig. 3a), while cell growth was prominently inhibited when SA1101 was added to the wells, which was totally different from the results after adding ATCC49456 (Fig. 3b). In addition, the morphology of the osteoblastic cells following SA1101 addition was changed due to growth impairment, which was totally different from that following ATCC49456 addition (Fig. 3c), indicating that SA1101 possesses a greater ability to impair osteoblastic cells as compared to ATCC49456.

Discussion

The first step in the present study was to determine the species of the blood isolate SA1101. Recently, techniques to determine 16S rRNA alignment have been widely applied to specify isolated species. In the present study, the entire 16S rRNA alignment of SA1101 had a high homology with both *S. mitis* and *S. pneumoniae*. It is considered very difficult to discriminate between *S. mitis* and *S. pneumoniae* by comparison of 16S rRNA only, since the sequences of these species are nearly the same[7]. The differing physiological and biochemical properties allowed us to determine that SA1101 was likely *S. mitis* rather than *S. pneumoniae*.

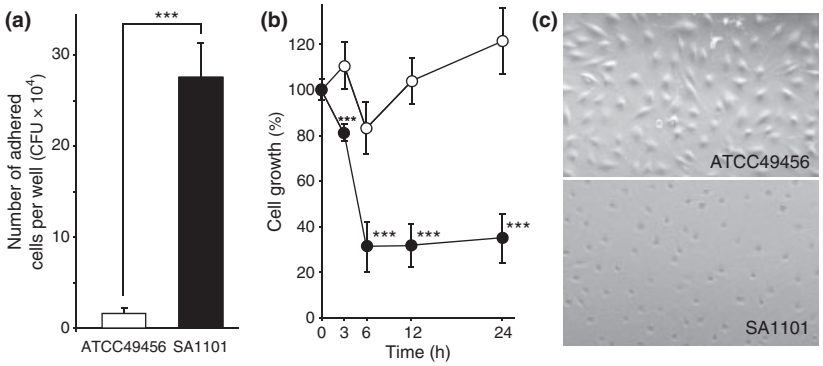

Fig. 3. *In vitro* analyses of SA1101 and ATCC49456 using osteoblastic cells. (a) The number of adhesive cells was counted at 90 min after starting incubation. Data are shown as mean ± SD from three wells. There were statistically significant differences between the strains (***$P < 0.001$). (b) Transitional changes in the relative growth rates of the cells following incubation of SA1101 (●) or ATCC49456 (○) for 3 h. Data are shown as the mean ± SD from three wells (***$P < 0.001$). (c) Microscopic images of cells at 3 h after addition of the strains.

S. mitis is generally known to be a commensal inhabitant of tooth surfaces, as well as the buccal mucous membrane and pharynx[8], thus SA1101 in the present case was speculated to have entered the bloodstream from the oropharynx area. It is also known that severe dental caries can lead to a contiguous focus of infection, allowing pathogenic bacteria to reach the lesion in a haematogenous manner[6]. Therefore, we analysed strains present in dental plaque specimens from the present patient to elucidate the pathway of SA1101 into the bloodstream. The *S. mitis* species is regarded to be comprised of a large variety of clones that are variously distributed among individuals[17]. Based on our results, we speculated that the distribution frequency of *S. mitis* clones that are the same as SA1101 in the oral cavity is extremely low. On the other hand, it is possible that the long-lasting antibiotic treatment administered to our patient successfully eliminated strains that were the same clone as SA1101. It can also be speculated that these strains might have been derived from bacteria inhabiting the pharynx area, while another possibility is that mutational changes might have occurred after invasion of the bloodstream that contributed to disease onset. Additional studies are needed to further investigate these speculations.

Dental caries can lead to destruction of the surface layer of the tooth, resulting in bacterial invasion of the inside space, thus promoting development of a dento-alveolar abscess and infection spreading to other anatomical areas[18]. In the present case, there were no prominent factors such as invasive dental treatment related to invasion of the bloodstream by oral bacteria. It should be noted that possible accumulation of oral bacteria in the abscess that had preceded formation of the fistula formation in the mandibular second primary molar region was likely to be one of the pathways in the present case, as it has been reported that *S. mitis* was frequently found in abscess lesions with a dental origin[19,20]. Since the dentoalveolar infection in the present patient was untreated during the time of active osteomyelitis, it might have been a causal factor. It is unfortunate that we could not analyse oral specimens obtained before dental treatment, which would have provided more information.

Considering the etiology and pathogenesis of osteomyelitis, SA1101 likely possesses several distinctive properties related to virulence, such as resistance to phagocytosis, acceleration of platelet aggregation, adhesion to osteoblastic cells, and inhibition of osteoblast proliferation. Therefore, we performed *in vitro* analyses of SA1101 regarding those properties and compared the results to those of the reference strain ATCC49456. Our phagocytosis assay indicated that the lower susceptibility to phagocytosis of SA1101

might be advantageous for its virulence at a very early stage. Also, the platelet aggregation rate of SA1101 was significantly greater than that of the reference strain. The binding properties to human platelets of *S. mitis* have been reported[21] and appear to be a central process in the pathogenesis of osteomyelitis, thus SA1101 could be a highly virulent strain. In analyses using osteoblastic cells, the number of SA1101 organisms that adhered to the cells was significantly greater and cell growth was prominently inhibited when SA1101 was added to the wells, as compared to the reference strain. In addition, the morphology of the cells following SA1101 addition was changed due to growth impairment, indicating that SA1101 possesses a greater ability to impair osteoblastic cells. Taken together, our results suggest that strain SA1101 was likely responsible for osteomyelitis in the present case, though SA1101 organisms were only isolated from blood specimens and not from samples taken from the osteomyelitis lesions.

In summary, an *S. mitis* strain isolated from the blood of a patient with osteomyelitis may have been the cause of the disease. Few studies have reported *S. mitis* organisms that caused osteomyelitis, however, the present findings indicate their capability to produce osteomyelitis-related lesions. Accumulation of additional reports will help to unveil the etiology of *S. mitis*-related osteomyelitis.

What this paper adds

- Most child cases of osteomyelitis have a haematogenous origin and show metaphysis of the long bones, with *S. mitis* one of the possible pathogens.
- Blood isolates of *S. mitis* obtained in the present case led to our conclusion that it was the pathogen likely responsible for osteomyelitis and it was also speculated that the organisms entered the bloodstream from the oropharynx area.

What this paper is important for paediatric dentists

- Oral streptococci may be pathogens responsible for osteomyelitis. Therefore, prevention of virulent strains entering the bloodstream should be considered by not only providing appropriate oral hygiene instructions, but also performing dental treatments for severe dental caries and periodontal diseases, especially in compromised children.

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