Detection of Genotype associated with Disease Activity and Development of Probe

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국문요약

질환활성과 관련된 유전자형 검색 및 탐색자 개발

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질환활성과 관련된 세균의 분포 및 유전자형을 탐색하고자 구강농양 및 골수염의 급성감염 환자와 진료실 및 실험실의 정상인을 대상으로 시료를 채취하여 포도상구균을 분리 및 동정을 시행하고, 특성을 규명하였으며, plasmid 및 염색체유전자를 분리하여 제한효소로 처리후 전기영동을 실시하고 분리된 plasmid로 탐색자를 제작하여 dot blot을 시행하였다.

대부분의 급성환자에서 분리된 포도상구균은 *S. lugdunensis*와 *S. aureus*이었으나, 진료실 및 실험실에서는 coagulase 음성 staphylococci가 분리되었다. 급성환자에서 분리된 포도상구균은 ampicillin과 penicillin에 내성을 보였다. 분리된 *S. lugdunensis*균주중 네 균주는 δ형의 용혈소를 생산하였다. Plasmid를 분리한 결과 *S. lugdunensis*균주중 세 균주는 약 6.5 kilobases이었으나 *S. aureus*는 약 4.3 kilobases 정도 크기의 band를 보였다. *S. lugdunensis*에서 분리된 plasmid로 제작한 탐색자로 dot blot를 시행한 결과 치과 영역에서 분리한 plasmid를 갖는 균주는 양성반응을 보였다. 염색체유전자의 유전자형을 분석한 결과 δ형의 용혈소를 생산한 네 균주의 *S. lugdunensis*는 유사한 유전자형을 보였다. 이러한 연구결과 질환의 진행에 *S. lugdunensis*가 중요한 역할을 하는 것으로 생각되고, 치과영역에 존재하는 plasmid는 공통적인 유전자 서열을 갖는 것으로 추정된다.

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INTRODUCTION

Oral infections are induced by pathogenic microorganisms[1-4]. The staphylococci are gram-positive spherical cells, usually arranged in grapelike irregular clusters. *Staphylococcus aureus*(*S. aureus*) is coagulase-positive, is major pathogen for human and produces a number of cytolytic toxins including four hemolysins (α, β, γ, δ). These hemolysins lyse red blood cells[5].

Staphylococcus lugdunensis (*S. lugdunensis*) was identified as a pathogenic species of coagulase negative staphylococcus and an occasional but not rare cause of severe infections, such as infective endocarditis after dental extraction, bacteremia, osteomyelitis, peritonitis and soft tissue infections[6-9].

Dental manipulations are a major cause of the bacteremias that lead to infection of the valvular tissues. In patients with congenital heart disease and artificial heart valve, antibiotic prophylactic measures should be made. But the appearance of resistant strains may decrease the efficacy of treatment[2,4].

Our previous study showed the presense of *S. aureus* in dental clinic[10]. But there was no report about the *S. lugdunensis* isolation in dentistry. The detection and monitoring of such bacteria may be necessary for both research and clinical diagnostic purposes and might be of a great help to make a therapeutic decision or to predict active sites.

There were several trials to detect pathogenic microorganisms by immunofluorescence assay or DNA hybridization method[11-14].

DNA probe technology seems to be both a sensitive and specific assay and may alleviate the concern for transport of fastidious microorganisms. The purpose of this study is to isolate and to characterize the staphylococci associated with disease activity.

MATERIALS AND METHODS

Isolation. Staphylococci were sampled from the patients with oral abscess and osteomyelitis and healthy person with a cotton applicator wet in normal saline, and inoculated to blood agar plate directly. After overnight incubation at 37℃ with 10% CO_2, staphylococci was isolated in blood agar plate (Korea Media, Seoul, Korea), identified with Gram stain, hemolysis pattern, catalase and coagulase, and commercial biochemical test(Baxter, West Sacramento, USA).

Hemolytic activity on blood agar plate. Bacteria were grown on trypticase soy(TS) agar plates supplemented with 5% sheep blood (Green cross Co., Seoul. Korea) and were incubated for 24 hour at 37℃ under aerobic conditions. δ-hemolysin forms a narrow zone of complete hemolysis with blurred edges on sheep blood agar plate and complete clearing of the partial zone of δ-hemolysis formed by a *S. aureus* strain.

Antibiotic susceptibility test. The antibiotic susceptibility was determined according to the size of inhibition-zone following the National Committee of Clinical Laboratory Standards (NCCLS).

Cadmium-resistance tesing. The resistance to cadmium acetate(Junsei Chemical Co., Tokyo, Japan) were determined by broth using Muller-Hinton medium.

Isolation and detection of plasmid. The staphylococci were examined for their plasmid content by modified boiling method of Dunkle and Sippel[15]. The prepared plasmid was used in the following analysis. About 5 μl of each sample digested with *Hin*d III was electrophoresed to detect the presence of plasmid DNA in 0.7% agarose gel. After electrophoresis, the gel was stained with ethidium bromide, des-

tained with water and visualized on the UV transilluminator.

Restriction endonuclease analsysis. Total genomic DNA was isolated bacterial genomic DNA and digested with *Hin*d III[16]. Each bacterial strain was grown to the mid-logarithmic growth phase in 5 ml of Luria-Bertani medium (LB). 1.5 ml of the culture was spinned in a microcentrifuge for 2 min and the supernatant was discarded. The cell pellets were washed with 1 ml of TE(50 mM Tis, 2 mM EDTA, pH 8.0). To extract the DNA, we resuspended pellet with 500 μl TE buffer by repeated pipetting, and added 200 μl of lysostaphin(0.1 mg/ml in 10 mM Tris-Cl, pH 8.0). After 1 hour of incubation at 37℃, we added 5 μl of proteinase K(10 mg/ml in D.W. : Sigma) and 30 μl of 10 % sodium dodesyl sulfate. After 1 hour of incubation at 37℃, we added 700 μl of phenol-chloroform-isoamyl alcohol(25 : 24 : 1, V/V/V), mixed thoroughly, and spinned 4 to 5 min in a microfuge. The aqueous, viscous supernatant was collected into a fresh microcentrifuge tube, mixed with an equal volume of chloroform-isoamyl alcohol (24 : 1, V/V), extracted thoroughly, and spinned in a microfuge for 5 min. The supernatant was transferred to a fresh tube with 0.6 volume isopropanol to precipitate the nucleic acids, shaked the tube back and forth until a stringy white DNA precipitate becomes clearly visible, and the pellet was transferred to a fresh tube with 70 % ethanol by hooking it onto the end of a micropipet that has been heat-sealed and bent in a Bunsen flame. After washing the DNA with 70 % ethanol, we redissolved the pellet in 100 μl TE buffer.

Digestion. We used 10 units of restriction endonuclease *Hin*d III (KOSCO Biotech, Sungnam city, Kyungki-do, Korea) to digest 1 μg of DNA in the appropriate buffer, as recommended by the manufacturer. We incubated the mixture at 37℃ in a water bath for one hour. Finally, we added loading solution to the mixture.

Agarose gel electrophoresis. Electrophoresis was carried out in a custom-made horizontal gel apparatus(16.0×16.0×0.5 cm), with use of 0.7 % agarose(Sigma) in TAE buffer. The sample was electrophoresed for 30min with 0.5 μg//ml ethidium bromide and destained it in distilled water for 2 hours.

Dot blots. The DNA was blotted onto a positively charged nylon membrane(Boehringer mannheim, West Germany). Blotted DNA was denatured for 15−30 minutes at 120℃. Nylon membrane was prehybridized in box at least 20 ml hybridization solution(5×SSC, 1 % blocking reagent, 0.1% n-lauroylsarcosine, 0.02 % SDS) per 100 ㎠ membrane of hybridization solution containing 5 μl of freshly denatured labelled DNA per ml. The membranes were incubated for at lesat 6 hours at 68℃, redistributed the solution occasionally.

Probe preparation. The plasmid DNA of *S. lugdunensis* was digested with *Pst*I and electrophoresed. Electrophoresed plasmid DNA was eluted with Gene-Clean kit(Beringer Mannheim, West Germany) and denatured by heating in a boiling for 10 minutes and chilled quickly on ice. The denatured DNA was labelled with random primed DNA labelling with nonradioactive digoxigenin-dUTP(DIG DNA labelling and detection kit, Beringer Mannheim, West Germany). 1 μl of freshly denatured DNA, 2 μl of hexanucleotide mixture and 2 μl of dNTP labelling mixture was added to a Eppendorf tube on ice. This tube was spinned and incubated for at least 60 minutes at 37℃.

Immunological detection. After blocking, binding of antibody conjugated to hybridized DIG-labelled DNA occurred in the first step of the detection reaction. The color reaction

was initiated at alkaline pH by the addition of colorless X-phosphate and NBT. A blue precipitate started to form in a few minutes and continued for up to 1 day.

RESULTS

S. aureus, S. lugdunensis and *Staphylococcus cohnii*(*S. cohnii*) were isolated from the patients with acute infection but the isolation ratio of *S. lugdunensis* in the patients with abscess was higher than that of osteomyelitis(Table 1).

Most of staphylococci in patients with infection were *S. lugdunensis* and *S. aureus* but 34 % of staphylococci in healthy person in dental clinic and laboratory was *S. aureus.*

All staphylococci showed the positive reaction to Voges-Proskauer, PNP-β-D-Galactopyranoside, micrococcus screen, optochin, trehalose fermentation, arginine and mannose fermentation and showed the negative reaction to PNP-β-D-glucuronide and raffinose fermentation(Table 2).

Four strains of *S. lugdunensis* showed the susceptibility to novobiocin but two strains did not(Table 2).

All strains of *S. lugdunensis* showed the positive reaction to ornithine decarboxylase and lactose fermentation and showed the negative reaction to 40 % bile esculin and urease(Table 2). All staphylococci showed the resistance to ampicillin and penicillin, *S. lugdunensis* was also resistant to cephalothin, oxacillin and clindamycin, and some strains produced the δ-like hemolysin(Table 3). 67 % of the clinical isolates of *S. lugdunensis* gave a distinct, clear zone of synergistic, complete hemolysis when tested against the β-hemolysin of *S. aureus* (Fig. 1). In the analysis of plasmid, there was a clear bnad about 6.5 kilobases in three strains of *S. lugdunensis* isolated from patients with infection, and a clear band around 4.3 kilobases in four strains of *S. aureus* in dentistry, and two bands were observed in three strains of *S. aureus* in healthy person.

There were positive reactions among staphylococci with plasmid in dentistry(Fig. 2). Four strains of staphylococci with plasmid isolated from the patients with infection showed the resistance to oxacillin, three strains of staphylococci with plasmid isolated from the patients with infection showed the resistance to tetracycline, and all strains of staphylococci with plasmid showed cadmium-resistance(Table 3).

In the dot blots assay, the plasmid DNA probe of *S. lugdunensis* was able to hybridize with strain isolated from the patients with acute infection(Fig. 3). The genomic pattern of *S. lugdunensis* with δ-like hemolysin showed similar genomic pattern by restriction endonuclease analysis with *Hin*d III(Fig 4 and 5).

Table 1. Proportion of staphylococci in patients with acute infection

	Patients		Healthy Person	
	abscess	osteomyelitis	Dental	Clinic Laboratory
S. aureus	2/6	1/4	2/10	6/12
S. lugdunensis	4/6	2/4	0	0
S. cohnii	0	1/4	0	0
CNS	0	0	8/10	6/12

CNS : Coagulase negative staphylococci

Table 2. Characterization of staphylococci isolated from patients with acute oral infection.

Bac.	PYR	OC	NOV	CV	VP	BE	PGT	LAC	MS	PGR	OPT	URE	TRE	NIT	IDX	PHO	ARG	RAF	MNS
1. *Sl* (Abscess)	+	+	−	+	+	−	+	+	+	−	+	−	+	−	+	−	+	−	+
2. *Sa* (Osteomyelitis)	−	−	−	−	+	−	+	+	+	−	+	+	+	+	+	+	+	−	+
3. *Sc* (Osteomyelitis)	+	+	+	+	+	+	+	−	+	−	+	−	+	−	−	−	+	−	+
4. *Sl* (Osteomyelitis)	+	+	+	+	+	−	+	+	+	−	+	−	+	−	−	−	+	−	+
5. *Sa* (Abscess)	+	−	−	+	+	−	+	+	+	−	+	−	+	−	+	−	+	−	+
6. *S1* (Osteomyelitis)	−	+	−	−	+	−	+	+	+	−	+	−	+	+	+	+	+	−	+
7. *Sl* (Abscess	+	+	+	+	+	−	+	+	+	−	+	−	−	−	+	−	+	−	+
8. *Sl* (Abscess)	+	+	−	+	+	−	+	+	+	−	+	−	+	−	+	−	+	−	+
9. *Sl* (Abscess)	+	+	−	+	+	−	+	+	+	−	+	−	+	−	+	−	+	−	+
10. *Sa* (Abscess)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
11. *Sa* (Healthy person in Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
12. *Sa* (Healthy person in Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
13. *Sa* (Healthy person in Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
14. *Sa* (Healthy person in Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
15. *Sa* (Healthy person in Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
16. *Sa* (Healthy person in Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
17. *Sa* (Healthy person in Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+
18. *Sa* (Dental Clinic)	−	−	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	−	+

Bac, Bacteria : *Sl, Staphylococcus lugdunensis* : *Sa, Staphylococcus aureus* : *Sc, Staphylococcus cohnii* : PYR, L-Pyrrolidonyl-β-Naphthylamide : OC, ornithine decarboxylase : NOV, novobiocin : CV, crystal violet : VP, Voges-Proskauer : BE, 40% bile esculin : PGT, PNP-β-D-Galactopyranoside : LAG, lactose fermentation : MS, micrococcus screen : PGR, PNP-β-D-

glucuronide : OPT, Optochin : URE, Urea : TRE, Trehalose fermentation : NIT, Nitrate reduction : IDX, Indoxyl phosphatase : PHO, Phosphatase : ARG, Arginine : RAF, Raffinose fermentation : MNS, Mannose fermentation

Table 3. Antibiotic resistance, plasmid, cadmium resistanse, hemolysis, of staphylococci isolated from patients with infection and healthy person in dental clinic and laboratory.

Bac. Anti. Resistance	Plasmid Cd. DNA(kb)	Resistance (125vM)	Hemolysin pattern
1. *Sl* OX Am P CF CC (Abscess)	6.5	R	δ
2. *Sa* AM P (Osteomyelitis)	0	R	β
3. *Sc* Ox Am P CF CC (Osteomyelitis)	0	S	α
4. *Sl* OX AM P CF CC (Osteomyelitis)	6.5	R	δ
5. *Sa* AM P (Abscess)	0	S	β
6. *Sl* OX AM P CF CC (Osteomyelitis	6.5	R	δ
7. *Sl* PX AM P CF CC GM (Abscess)	0	R	α
8. *Sl* OX AM P E CF CC GM (Abscess)	0	R	α
9. *Sl* OX AM P CF CC (Abscess)	0	S	δ
10. *Sa* Ox Am P TE (Abscess)	4.3	R	β
11. *Sa* AM P CRO CF TE L C CC CB (Healthy person in Dental Clinic)	0	R	β
12. *Sa* AM P CRO CF TE L FOX CB C CC E VA OX (Healthy person in dental clinic)	4.3 and 1.0	R	β
13. *Sa* AM CB TE (Healthy person in laboratory)	4.3 and 1.0	R	β
14. *Sa* AM CRO (Healthy person in laboratory)	0	R	β
15. *Sa* CRO TE GM L (Healthy person in laboratroy)	4.3 and 1.0	R	β

16. *Sa* AM P CB TE (Healthy person in laboratory)	0	R	β
17. *Sa* AM L (Healthy person in laboratory)	0	R	β
18. *Sa* AM P GM TE AN E (Dental Clinic)	0	R	β

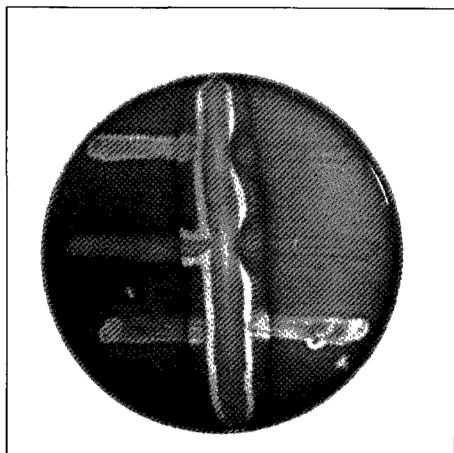

Figure 1. Synergistic hemolysis between a strain of *S. aureus*(vertical streak of growth) and the strains of *S. lugdunensis*(horizontal streak of growth). Four strains of the *S. lugdunensis* produced a clear zone of synergistric, complete hemolysis within the zone of incomplete hemolysis produced by the β-lysin activity from the *S. aureus,* but two strains did not.

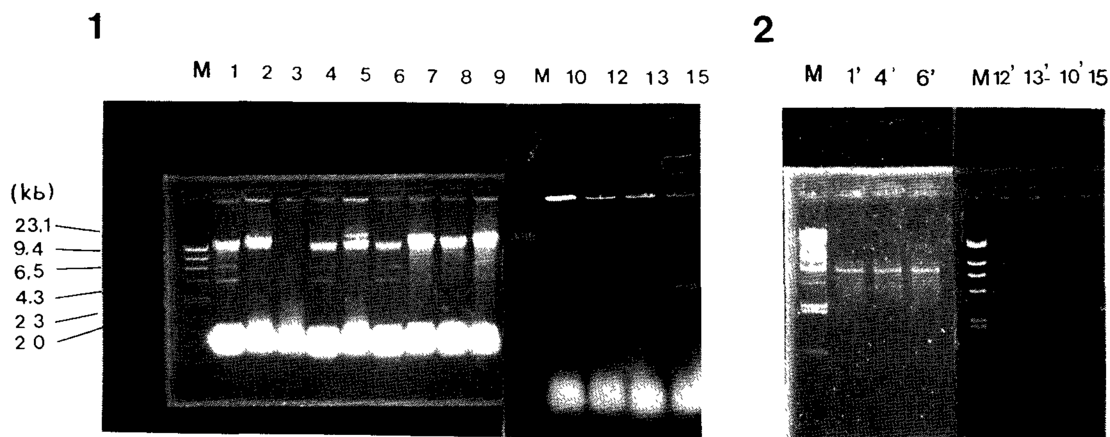

Figure 2. The electrophoresis profile of plasmid DNA. There was a band about 6.5 kb sizes in patient 1, patient 4 and patient 6.
M : Lamda phage DNA size markers in kilobases(kb).
1−10 : Patients with oral abscess and osteomyelitis.
12, 13, 15 : Healthy person in dental clinic and laboratory.
1', 4', 6', 10', 12', 13', 15' : 1, 4, 6, 10, 12, 13 and 15 plasmid DNA digested with *Hin*d III.

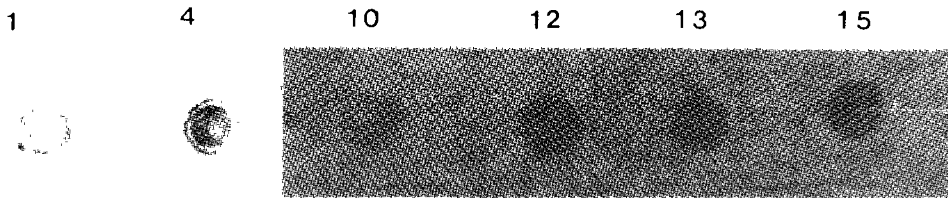

Figure 3. Dot blot hybridization results obtained after hybridization of staphylococci isolated from the patients with infection with its DIG-labelled plasmid DNA probe and detected by colorless X-phosphate and NBT.
1 : *S. lugdunensis* isolated from patient with abscess(strain 1)
4 : *S. lugdunensis* isolated from patient with osteomyelitis(strain 4)
10 : *S. aureus* isolated from patient with abscess(strain 10)
12 : *S. aureus* isolated from patient with healthy person in dental clinic(strain 12)
13 : *S. aureus* isolated from patient with healthy person in laboratory (strain 13)
15 : *S. aureus* isolated from patient with healthy person in laboratory (strain 15)

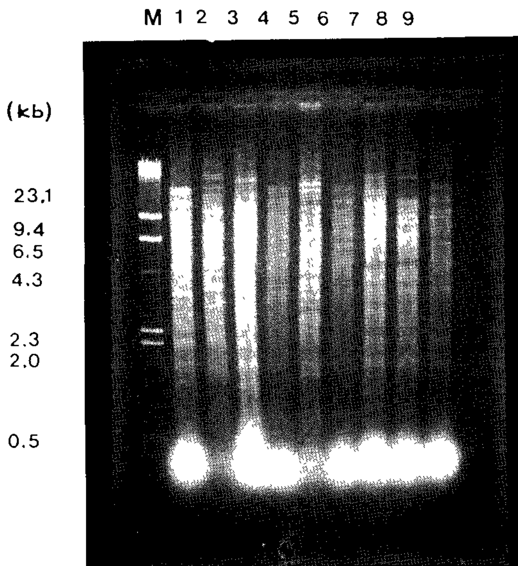

Figure 4. Restriction profile of *Hind* III digests in staphylococci.
There were genetic homologies among 1, 4, 6 and 9.
M : Lamda phage DNA size markers in kilobases(kb).
1−9 : Patients with oral abscess and osteomyelitis.

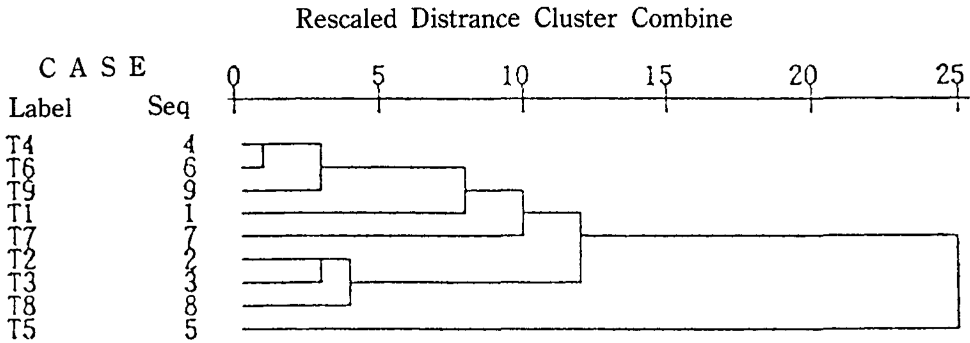

Figure 5. Dendrogram obtained by UPGMA(unweighted pair group method using arithmetric averages) of correlation values of restriction profiles from 9 stains of staphylococci strains.
There were genetic homologies among 1, 4, 6 and 9.
1−9 : Patients with oral abscess and osteomyelitis.

DISCUSSION

S. lugdunensis is gram-positive cocci : occurring singly, in pairs, small clusters, or chains composed of three to five cells. Catalase produced. Coagulase test negative with rabbit plasma. Acid was produced from trehalose and was not produced from raffinose. Esculin test negative[17]. But our results showed the positive reaction to arginine was not consistent with it. These results suggested that *S. lugdunensis* have an diversity. *S. lugdunensis* reported to be a significant opportunistic pathogen in man and common pathogen in clinical infections in a number of countries and to be implicated in native and prosthetic valve endorcarditis, septicemia, brain abscess, and chronic osteoarthritis and infections of soft tissues, bone, peritoneal fluid, and catheters, especially in patients with underlying diseases[7,8,17,18].

In this study, this strain was isolated both abscess and osteomyelitis and *S. aureus* was isolated(Table 1), but *S. aureus* was isloated from dental clinic and the site without infection[10].

S. lugdunensis and *S. cohnii* were only isolated from patients(Tabel 1), and *S. hemolyticus* and *S. epidermidis* were isolated from dental clinic[10]. These results suggested that *S. lugdunensis* and *S. cohnii* might be related to disease activity. *S. aureus* was isolated from the dental clinic in the afternoon and evening, and showed the resistance to oxacillin and vancomycin but most of staphylococci in patients with infection were *S. lugdunensis* and *S. aureus* which showed the resistance to ampicillin and penicillin(Table 3). There results were similar to the resistance to oxacillin in the USA, but showed the contrast to the usual susceptibility to most antistaphylococcal antibiotics including penicillin G[17,18].

S. lugdunensis strains are ususually susceptible to most antistaphylococcal antibiotics, including penicillin G, although some stains were reported in the USA to be resistant to oxacillin. But our results showed that all of the staphylococci were resistant to penicillin and ampicillin. *S. lugdunensis* was also resis-

tant to cephalothin, oxacillin and clindamycin, and four strains of *S. lugdunensis* showed the susceptibility to novobiocin but two strains did not(Table 2). These results suggested that the antibioitc resistance to some drugs except penicillin and ampicillin were different among staphylococci. Some strains produced δ-like hemolysin. The difference of antibiotic resistance and hemolytic activity might be a marker of disease activity and resistance to penicillin, ampicillin and cephalothin is very important to the patient with heart defect and artificaial heart because between 50 and 80% of acute infective endocarditis are caused by *S. aureus* and staphylococcal endocarditis was observed after dental extraction and penicillin and ampicillin are the drug of choice for the patient[7,19]. 67 % of the clinical isolates of *S. lugdunensis* gave a distinct, clear zone of synergistic complete hemolysis when tested against the β-hemolysin of *S. aureus*(Fig. 1).

In the analysis of plasmid, there was a clear band about 6.5 kilobases in three strains of *S. lugdunensis* isolated from patients with infection (Fig. 2).

The difference of hemolytic activity might be related to that of genomic pattern(Fig 1 and Table 3). Poitevin-later et al. reported the cadmium-resistance plasmid in *S. lugdunensis*[20] and Etienne et al. reported that small plasmids were found for two thirds of the *S. lugdunensis* strains, the most frequently isolated plasmid had a molecular weight of 3.2 kilobases, and was detected very different origins [21]. But the size of plasmid was different from that of our results(Fig. 2). In our study, three strains of *S. lugdunensis* showed no variations, even for those isolated from patients not exposed to common source and all strains of *S. lugdunensis* showed the resistance to cadmium. These results might be related to racial difference due to food habits[22,23]. Four strains of staphylococci with plasmid isolated from the patients with infection showed the resistance to oxacillin but three strains of staphylococci with plasmid isolated from the patients with infection showed the resistance to tetracycline but the plasmid DNA probe of *S. lugdunensis* was able to hybridize with strain isolated from the patients with acute infection and healthy person in dental clinic and laboratory in dot blot assay. These results suggested that there exist some size differences among plasmid of staphylococci in dentistry including dental clinic[10]. In the analysis of plasmid, there was a clear band about 6.5 kilobases in three strains of *S. lugdunensis* isolated from patients with infection, a clear band around 4.3 kilobases in four strains of *S. aureus* in dentistry, and two bands were observed in three strains of *S. aureus* in healthy person. In dot blot assay, There was positive reaction among staphylococci with plasmid in dentistry. These results showed there were genetic similiarities among staphylococci with plasmid in dentistry. But, there was an additional band in healthy person in dental clinic and laboratory(Fig 2). Further study is needed to clarify the genomic mapping among plasmid DNA. The positive reaction in dot blot assay suggested the transmission of plasmid in dentistry[24].

The genomic pattern of *S. lugdunensis* with δ-like hemolytic activity showed similar genomic pattern(Fig 4 and 5). This result showed the relation between δ-hemolysis and genomic pattern.

These results suggested that specific strain and its genomic pattern might be related to disease activity. Further study is needed to increase the sensitivity of probe based on disease activity.

CONCLUSIONS

To investigate the distribution and genetic pattern of microorganism associated with disease activity, patients with acute infection such as oral abscess and osteomyelitis and healthy person were sampled and staphylococci was isolated, identified and characterized. Plasmid and *Hin*d III-digested bacterial genomic DNA of staphylococci was electrophoresed and dot blot was performed to study the genomic pattern. Most of staphylococci in patients with acute oral infection were *S. lugdunensis* and *S. aureus* and most of staphylococci in healthy person were coagulase negative staphylococci. Most of staphylococci in patients with acute oral infection were showed the resistance to ampicillin and penicillin.

Four strains of *S. lugdunensis* produced δ-like hemolysin.

In the analysis of plasmid, there was a clear band about 6.5 kilobases in three strains of *S. lugdunensis* isolated from patients with infection, a clear band around 4.3 kilobases in four strains of *S. aureus* in dentistry, and two bands were observed in three strains of *S. aureus* in healthy person. There was positive reaction among staphylococci with plasmid in dentistry. In the analysis of genomic pattern, four strains of *S. lugdunensis* with δ-like hemolysin showed the similar genetic pattern with *Hin*d III enzyme digests.

These results showed that *S. lugdunensis* might play a major role in disease activity. There were genetic similliarities among staphylococci with plasmid in dentistry.

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