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δ-Hemolysin Like Gene of *Staphylococcus lugdunensis* in Acute Oral Infection Have Partial Homology with δ-Hemolysin Gene of *Staphylococcus aureus*

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To investigate the distribution and hemolytic activity of staphylococci in acute oral infection, staphylococci were isolated from the patients with acute oral infection and healthy persons, hemolytic activity was measured on sheep blood agar plates, and DNA-DNA hybridization was preformed with δ-hemolysin gene probe of S. aureus *under low stringent condition or high stringent condition. The isolation ratio of* S. lugdunensis *in patients was higher than that of healthy persons. Four strains of* S. lugdunensis *had δ-like hemolytic activity, but two strains did not. In dot blot analysis,* S. lugdunensis *was hybridized with δ-hemolysin probe of* S. aureus *under low stringent condition, but weakly hybridized with δ-hemolysin probe under high stringent condition. These results suggest that* S. lugdunensis *is an important pathogen in acute oral infection and δ-hemolysin gene of* S. lugdunensis *have partial homology with δ-hemolysin gene of* S. aureus.

Key words: *staphylococci, hemolysin, gene, infection,* S. aureus.

Introduction

The genus staphylococcus is currently composed of nonmotile, catalase-positive, facultatively anaerobic, Gram-positive cocci. In the last decade, the number of validly published species in the genus staphylococcus has increased from a few to 24 (Freney *et al.*, 1988). *Staphylococcus aureus* (*S. aureus*) is known that a major pathogen for various human infection including dental field. It produces a wide range of extracellular and cell bound proteins which are potentially important as virulence factors. Those are α-,β-,γ-,and δ-hemolysins, coagulase, leucocidin, enterotoxins, and protein A (Bramley *et al.*, 1989).

Staphylococcus lugdunensis(*S. lugdunensis*) is a newly identified pathogenic species of staphylococci and an occasional but not rare cause of severe infections, such as infective endocarditis after dental extraction, bacteremia, osteomyelitis, peritonitis and soft tissue infections (Lee *et al.*, 1987; Barker *et al.*, 1991; Vandenesch *et al.*, 1991; Etienne *et al.*, 1989; Ludlam and Phillips, 1989). Vandenesch *et al.* (1993) reported that *S. lugdunensis* produces a hemolysin with phenotypic properties similar to *S. aureus* δ-hemolysins and *S. lugdunensis* δ-like hemolysin gene shares homology with δ-hemolysin gene of *S. aureus*. δ-Hemolysin is a potential virulence factor having a detergent-like activity on various cell membranes.

Our previous study showed the presence of *S. aureus* in dental clinic(Kim *et al.*, 1993). More recently, we found that *S. lugdunensis* is related to acute oral infection(Kim *et al.*, 1994). But their roles in pathogenesis of oral infection remain uncertain. To examine the roles of staphylococci in acute oral infection, staphylococci were isolated from the patients with acute oral infection and healthy persons, hemolytic activity was measured on sheep blood agar plates, and DNA-DNA hybridization was preformed with δ-hemolysin gene probe of *S. aureus* under low stringent condition or high stringent condition(Vandenesch *et al.*, 1991).

Material and Methods

Isolation

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Table 1. DNA probes synthesized in hybridization experiments

Type	δ-hemolysin
Gene	*hld*
Species	*Staphylococcus aureus*
Sequence	5'-TATCGACACAGTGAA-3'
Size	15-mer oligonucleotide
Reference	Vandenesh *et al.* (1991)

Table 2. Proportion of staphylococci in patients with acute infection and healthy persons

Species	Patients	Healthy persons
S. aureus	3/10 (30%)	8/22 (36%)
S. lugdunensis	6/10 (60%)	0/22 (0%)
S. cohnii	1/10 (10%)	0/22 (0%)
CNS (others)	0/10 (0%)	14/22 (64%)

CNS: Coagulase Negative Staphylococcus

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 were isolated in blood agar plate (Korea Media, Seoul, Korea), identified with Gram stain, hemolytic pattern, catalase and coagulase, and commercial biochemical test (Baxter, West Sacramento, USA).

Hemolytic activity on blood agar plates

Bacteria were grown on trypticase soy (TS) agar plates supplemented with 5% sheep erythrocytes (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 erythrocyte agar plates and complete clearing of the partial zone of δ-hemolysis formed by a *S. aureus* strain (Hébert and Hancock, 1985).

Preparation of total genomic DNA

Total genomic DNA was isolated bacterial genomic DNA. 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 Tris, 2 mM EDTA, pH 8.0). To extract the DNA, the pellets were resuspended with 500 μl TE buffer by repeated pipetting, and 200 μl of lysostaphin (0.1 mg/ml in 10 M Tris-Cl, pH 8.0) was added. After 1 hour of incubation at 37℃, 5 μl of proteinase K (10 mg/ml in D.W: Sigma) and 30 μl of 10 % sodium dodesyl sulfate were added. After 1 hour of incubation at 37℃, 700 μl of phenol-chloroform-isoamyl alcohol (25:24:1, V/V/V) was added, 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, the tube was shaked 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 frame. After washing the DNA with 70% ethanol, the pellet was redissolved in 100 μl TE buffer.

Probe preparation

The 15-mer δ-hemolysin probe of *S. aureus* was synthesized and labeled with nonradioactive digoxigenin-dUTP (DIG DNA tailing and detection kit, Beringer Mannheim, West Germany) (Table 1). 100 pmol of oligonucleotide, 2 μl of hexanucleotide mixture and 2 μl of dNTP labeling mixture was added to a Eppendorf tube on ice. This tube was spinned and incubated for 15 minutes at 37℃.

Dot blot analysis

Freshly denatured genomic DNA was blotted onto a positively charged nylon membrane (Boehringer mannheim, West Germany). Nylon membrane was prehybridized in a box at least 20 μl hybridization solution (5X SSC, 1% blocking reagent, 0.1% n-lauroylsarcosine, 0.02% SDS) and hy-

Table 3. Major hemolytic pattern of staphylococci isolated from the patients with infection and healthy person.

Strain no.	Species	Source	Major hemolytic pattern
1	*Staphylococcus lugdunensis*	Abscess	δ
4	*Staphylococcus lugdunensis*	Osteomyelitis	δ
6	*Staphylococcus lugdunensis*	Osteomyelitis	δ
7	*Staphylococcus lugdunensis*	Abscess	α
8	*Staphylococcus lugdunensis*	Abscess	α
9	*Staphylococcus lugdunensis*	Abscess	δ
10	*Staphylococcus aureus*	Abscess	β
12	*Staphylococcus aureus*	Healthy person	β
13	*Staphylococcus aureus*	Healthy person	β
15	*Staphylococcus aureus*	Healthy person	β

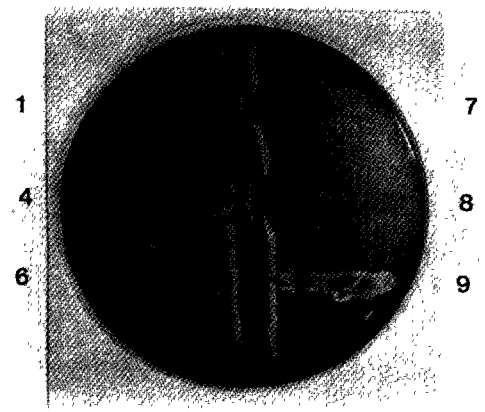

Fig. 1. Synergistic hemolysis between a strain of *S. aureus* (vertical streak of growth) and the strains of *S. lugdunensis* (horizontal streak of growth). Four strains (1, 4, 6, and 9) of the *S. lugdunensis* produced a clear zone of synergistic, complete hemolysis within the zone of incomplete hemolysis produced by the δ-hemolysin activity from the *S. aureus*. But two strains (7 and 8) did not.

bridized in hybridization solution containing 5μl of labeled DNA per ml. The membranes were incubated for at least 6 hours under low stringent condition or high stringent condition.

Immunological detection

After blocking, binding of antibody conjugate to hybridized DIG-labeled 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 within a few minutes and continued for up to 1 day.

Fig. 2. Dot blot analysis of DNA from representative isolates of staphylococci. Total DNA was hybridized with DIG-labeled δ-hemolysin probe under low-stringent condition and detected by colorless X-phosphate and NBT. 1, 4 and 6: *S. lugdenensis* isolated from patients with oral osteomyelitis and abscess. 15: *S. aureus* isolated from healthy person.

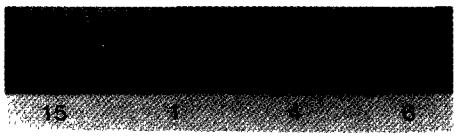

Fig. 3. Dot blot analysis of DNA from four isolates of staphylococci. DNA was hybridized with DIG-labeled δ-hemolysin DNA probe under high-stringent condition and detected by colorless X-phosphate and NBT. 1, 4 and 6: *S. lugdunensis* isolated from patients with oral osteomyelitis and abscess. 15: *S. aureus* isolated from healthy person.

Results

S. aureus, *S. lugdunensis* and *Staphylococcus cohnii* (*S. Cohnii*) were isolated from the patients with acute infection. The isolation ratio of *S. lugdunensis* in the patients with infection was higher than that of healthy persons, but the isolation ratio of *S. aureus* in the patients with infection were similar with healthy person (Table 2).

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*, but 33% of the *S. lugdunensis* were negative in this test (Fig. 1).

In the dot blot analysis, *S. lugdunensis* was able to hybridize with δ-hemolysin probe of *S. aureus* under low stringent condition (Fig 2), but *S. lugdunensis* was weakly hybridized with δ-hemolysin probe under high stringent condition (Fig. 3).

Discussion

S. lugdunensis are Gram-positive cocci; occurring singly, in pairs, small clusters, or chains composed of three to five cells. Catalase produced. Coagulase test negative with human plasma. *S. lugdunensis* reported to be a significant opportunistic pathogen in man and commom pathogen in clinical infections in a number of countries and to be implicated in native and prosthetic valve endocarditis, septicemia, brain abscess, and chronic osteoarthritis and infections of soft tissues, bone, peritoneal fluid, and catheters, especially in patients with underlying diseases (Lee *et al.*, 1987; Etienne *et al.*, 1986; Etienne *et al.*, 1989., Kim *et al.*, 1993).

In this study, the isolation ratio of *S. lugdunensis* in the patients with acute oral infection was higher than that of healthy persons, but the isolation ratio of *S. aureus* in the patients with infection was similar with healthy person (Table 2). These results suggest that *S. lugdunensis* may be one of the important pathogen in acute oral infection (Kim *et al.*, 1994).

Our findings demonstrated that hemolysin similar to δ-hemolysin of *S. aureus* were produced by 67% of *S. lugdunensis*. The δ-Like hemolysin is one of the major toxin of *S. lugdunensis*. Hébert reported that 95% of *S. lugdunensis* produced δ-like hemolysin (Hébert, 1990). δ-like hemolysin may be distinguished from other hemolysins by its heat stability, neutralization by lecithin and serum, and by its pattern of activity towards erythrocytes from various species. δ-Hemolysin can damage a variety of cells by its detergent-like action on cell membranes. Unlike *S. aureus* δ-hemolysin, which displays a wide variety of activity affecting most cell type, the δ-like hemolysin of *S. lugdunensis* was preferentially active against rabbit erythrocytes (Fitton *et al.*, 1980; Vandenesch *et al.*, 1991).

In the dot blot analysis, *S. lugdunensis* DNA was able to hybridize with δ-hemolysin gene probe of *S. aureus* under low stringent condition (Fig 2). These results show that sequences homologous to δ -hemolysin probe of *S. aureus* are present in *S. lugdunensis*. However, a somewhat weaker hybridization signals were observed with the δ-hemolysin probe under high stringent condition (Fig 3). These results suggest that δ-hemolysin gene of *S. lugdunensis* have partial homology with δ-hemolysin gene of *S. aureus*.

Conclusions

To investigate the distribution and hemolytic patterns of staphylococci in the patients with acute oral infection such as oral abscess and osteomyelitis, and healthy person, staphylococci was isolated and identified, and hemolytic activity test and dot blot analysis were performed. The isolation ratio of *S. lugdunensis* in the patients was higher than that of healthy persons. 67% of *S. lugdunensis* have δ-like hemolytic activity. In dot blot analysis, *S. lugdunensis* was hybridized with δ-hemolysin probe of *S. aureus* under low stringent condition, but weakly hybridized with δ-hemolysin probe under high stringent condition. These results suggest that *S. lugdunensis* is an important pathogen in acute oral infection and δ-hemolysin gene of *S. lugdunensis* have partial homology with δ-hemolysin gene of *S. aureus*.

References

Barker, K.F., O'Driscoll, J.C. and Bhargava, A.: *Staphylococcus lugdunensis*. *J. Clin. Pathol.* **44:** 873-874, 1991.

Bramley, A.J., Patel, A.H., O'Reilly, M., Foster, R. and Foster, T.J.: Roles of alpha-toxin and beta- toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect. Immun.* **57:** 2489-2494, 1989.

Etienne, J., Fleurette, J., Ninet, J.F., Favet, P. and Gruer, L.D.: Staphylococcal endocarditis after dental infection. *Lancet i* **511,** 1986.

Etienne, J., Pangon, B., Leport, C., Wolf, M., Clair, B., Perronne, C., Brun, Y. and Bure, A.: *Staphylococcus lugdunensis* endocarditis. *Lancet i* **390,** 1989.

Fitton, S.E., Dell, A. and Shaw, W.V.: The amino acid sequence of the delta hemolysin of *Staphylococcus aureus*. *FEBS lett.* **115:** 209-212, 1980.

Freney, J., Brun, Y., Bes, M., Meugnier, H., Grimont, F., Grimont, P.A.D., Nervi, C. and Fleurette, J.: *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schleiferi* sp. nov., two species from human clinical specimens. *Int. Syst. Bacteriol.* **38:** 168-172, 1988.

Hébert, G.A. and Hancock, G.A.: Synergistic hemolysis exhibited by species of staphylococci. *J. Clin. Microbiol.* **22:** 409-415, 1985.

Hébert, G.A.: Hemolysins and other characteristics that help differentiate and biotype *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*. *J. Clin. Microbiol.* **28:** 2425-2431, 1990.

Kim. K.J., Yoo, S.K., Kim, H.J., Kim, S.J., and Um, I. W.: Isolation and characterization of *Micrococcaceae* in dental clinic. *J. Oral Biol.* **17:** 39-43, 1993.

Kim, K.J., Kim, W.S., Kim, J.H., Yoo, S.K., Im, M.K., and Chung, C.P.: Isolation and characterization of staphylococci in acute oral infection. *J. Oral Biol.* **18:** 47-54, 1994.

Lee, D.K., Yim, C.J. and Min, S.K.: Bacteriologic study of oral pyogenic infections. J. Kor. Acad. Oral and Maxillofacial Surg. **13(1):** 285-293, 1987.

Ludlam, H. and Phillips, I.: *Staphylococcus lugdunensis* peritonitis. *Lancet* i:1394, 1989.

Vandenesch, F., Projan, S. J., Kreiswirth, B., Etienne, J. and Novick, R.P.: Agr-related sequences in *Staphylococcus lugdunensis*. *FEMS Microbiol. Letter* **111:** 115-122, 1993.

Vandenesch, F., Storrs, M.J., Poitevin-Later, F., Etienne, J., Courvalin, P. and Fleurette, J.: δ-Like hemolysin produced by *Staphylococcus lugdunensis*. *FEMS Microbiol. Letters* **78:** 65-68, 1991.