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Enterotoxin Production and DNA Fingerprinting of *Staphylococcus aureus* Isolated from Diverse Samples by Pulsed-Field Gel Electrophoresis

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Staphylococcus aureus is an important animal and human pathogen implicated in a variety of disease including food-poisoning caused by staphyloccal enterotoxins (SEs). In order to investigate the difference in genomic types and to monitor the transmission of *S. aureus* isolates, a total of 25 *S. aureus* isolates from different sources were determined for their genotypic characteristics by pulsed-field gel electrophoresis (PFGE) in addition to their ability to enterotoxin production and antibiotic resistance patterns in this study. All the isolates were susceptible to amikacin, and the resistance pattern to ampicillin and penicillin were most common among 14 different patterns. Eleven of 24 isolates produced one of three SEs, SEA, SEC or SED. Sixteen representative PFGE patterns were obtained by *SmaI* restriction fragments of *S. aureus* isolates. Analysis of dendrogram based on PFGE band patterns suggested that food-poisoning outbreaks be caused by the diverse sources of food, of which their raw materials were infected with *S. aureus*. Also, it could be concluded that PFGE was a powerful tool for epidemiological tracing of infection source for food-initiated outbreaks.

Key Words: *Staphylococcus aureus*, Enterotoxins, PFGE

INTRODUCTION

Staphylococcus aureus is a gram-positive pathogen responsible for a wide range of human and animal disease, including septicemia, endocarditis and pneumonia, and wound, bone and joint infections. The ability of this bacterium to successfully persist within hosts is largely due to the presence of virulence factors that promote adhesion, acquisition of nutrients and evasion of host immunologic responses (Monday and Bochach, 1999). Among these is the pyrogenic exotoxin (PT) family, which is comprised of several biologically related proteins expressed by *S. aureus*. Staphylococcal enterotoxins (SEs), unlike the other members of the PT family, have the unique ability to induce staphylococcal food poisoning, a common type of gastroenteritidis. The SEs have been divided into nine major antigenic types (SEA to SEE, SEG to SEJ) on the base of their antigenicities (Kotb, 1995; Munson et al., 1998). Recently, methicillin-resistant *S. aureus* (MRSA) has become a widely recognized nosocomial cause of morbidity and mortality throughout the world (Deplano et al., 2000).

Several investigators have described DNA-based techniques for typing *S. aureus* stains, including PCR using Tn*916* target gene (Cuny and Witte, 1996), 16S-23S rDNA gene spacer (Gurtler and Barrie, 1995) and ribotyping (Richardson et al., 1994). Also, there was a growing consensus that restriction fragment length polymorphism (RFLP) using pulsed-field gel electrophoresis (PFGE) was the method of choice in the clinical laboratory for typing *S. aureus* (Bannerman et al., 1995). The objective of this study was to determine the genotypic characterization of *S. aureus* isolates from the diverse sources including mastitic milk from cow, food-poisoning cases, abscesses lesions of pig and chicken by PFGE.

MATERIALS AND METHODS

1. Bacterial strains

A total of 25 *S. aureus* isolates were tested in this study (Table 1). Nine isolates were from the mastitic milk sam-

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Table 1. Characteristics of 25 *S. aureus* isolates in this study

Isolate	Origin	Source	Enterotoxin	PFGE type
SA01	Mastitic milk	Cow farms		5
SA02				5
SA03				11
SA04				11
SA05			D	10
SA06				2
SA07				9
SA08				9
SA09				9
SA10	Abscess lesion	Swine farms	A	12
SA11			A	12
SA12			A	12
SA13			A	12
SA14	Chicken	Slaughterhouses		16
SA15	Pork		D	4
SA16			A	8
SA17			D	3
SA18			D	8
SA19	Food	Food-poisoning		14
SA20			A	12
SA21			AC	7
SA22				6
SA23				1
SA24				13
Reference	ATCC 25923	Clinical		15

ples from 9 different farms. Six isolates were from food-poisoning cases. Also, nine isolates were from abscess lesions of diseased pig from swine farms or from those of chicken and pork from slaughterhouses. *S. aureus* ATCC 25923 was included as reference strain. All the isolates were collected between 2002 and 2003 and identified by means of API ID 32 Staph (bioMerieux, France).

2. Antibiotic susceptibility test

Sensitivity to antibiotics was determined by the standardized agar diffusion test (Bauer et al., 1966) on Muller Hinton agar (Merck, Germany) using following antibiotic Sensi-Disc (BBL, USA): amikacin (30 μg), amoxicillin (20 μg), ampicillin (10 μg), cephalothin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), oxacillin (1 μg), penicillin (10 U), sulfamethoxazole-trimethoprim (23.75/1.25 μg) and tetracycline (30 μg).

3. Detection of enterotoxigenicity

The types of enterotoxins (SEA-SED) produced by *S. aureus* isolates were determined by the use of SET-RPLA kit (Denka Seiken, Japan). For enterotoxin detection, after incubation of the isolates in Trypticase soy broth (MERCK, Germany) for 18~20 h, the culture was centrifuged and the supernatant fraction was used for assay.

4. PFGE

PFGE was done by a procedure described by Suh and Song (2005) and Jorgensen et al. (1996) with some modifications. Colonies grown on Tryptic soy agar (MERCK, Germany) were suspended in cell suspension buffer with cell density adjusted to a range of 12 %T using colorimeter (bioMerieux, USA). The agarose plugs incubated in tubes containing lysozyme (5 mg/ml) and lysostaphin (50 μg/ml) for overnight at 37℃ were transferred to proteinase K (5 mg/ml) for another overnight at 50℃. The plugs digested with 50 U of *SmaI* (Bioneer, Korea) for overnight at 26℃ were run on a 1% agaroge gel (Takara, Japan) in 0.5X TBE buffer with CHEF-mapper system (Bio-Rad, USA) under the following conditions: temperature 13℃; initial switch time, 0.47 s; final switch time, 1 m; run time, 24 h. A dendrogram was constructed with Analysis software (Biometra, Germany). The patterns were compared by means of the Dice coefficient of band-based similarity by unweighted pair group method using averages (UPGMA).

RESULTS

Except for an isolate, SA24, which was an only isolate susceptible to all antibiotics tested, all isolates were resistant to ampicillin, 91.7% (22/24) to penicillin and 41.7% (10/24) to kanamycin (Table 2). Also, all isolates expressed susceptibility to amikacin. A total of 24 *S. aureus* isolates were represented by 14 resistance patterns. AmPe and AmPeKaGm resistance patterns were the most common among them. Other 12 resistance patterns included one isolate each. None of the isolates was resistant to oxacillin, which was used for the test of resistance to methicillin.

Using the SET-RPLA kit, 44% of *S. aureus* isolates showed the ability to produce the enterotoxins. Only one isolate was positive to SEA and SEC. All eight isolates from the source of pigs were positive to SEA or SED.

Table 2. Antibiotic resistance patterns of 24 *S. aureus* isolates

Drug resistance no.	Resistance patterns [a]	No. (%) of isolates	
8	Am Pe Gm Ce Te Ac Ne Ka	1	4.2
7	Am Pe Gm Cp Te Sx Ka	1	8.3
	Am Pe Gm Cp Ac Sx Ka	1	
6	Am Pe Gm Cp Ac Ka	1	12.5
	Am Pe Gm Cp Sx Ka	1	
	Am Pe Gm Te Ne Ka	1	
5	Am Pe Te Ne Ka	1	4.2
4	Am Pe Ka Gm	2	16.6
	Am Pe Ka Te	1	
	Am Pe Ac Ne	1	
3	Am Pe Ac	1	8.3
	Am Pe Te	1	
2	Am Pe	9	37.5
1	Am	1	4.2
0	Sensitive to all	1	4.2
Total		24	100

[a] Am, ampicillin; Pe, penicillin; Gm, gentamicin; Ce, cephalothin, Te, tetracycline; Ac, amoxicillin; Ne, neomycin; Ka, kanamycin; Cp, ciprofloxacin; Sx, sulfamethoxazole-trimethoprim. *S. aureus* ATCC 25923 was not included in this test

Distinct numbers of between 8 and 12 bands (>49 kb) and 16 representative patterns were obtained by PFGE of *SmaI* restriction fragments of *S. aureus* isolates (Fig. 1). Twenty-five isolates were grouped into 3 main clusters (Fig. 2). Cluster I contained 6 isolates, which included 3 from samples of mastitic milk (PFGE type 2 and 5), 2 from the pork of slaughterhouses (PFGE type 3 and 4) and 1 from food-poisoning case (PFGE type 1). Those two isolates from the mastitic milk showed an identical pattern each other (PFGE type 5). Fifteen of 25 isolates (60%) were included in Cluster II. Four isolates from the abscess legions of pigs from different farms had an identical band pattern with an isolate SA20 (PFGE type 12), which was isolated from food-poisoning case. Also, isolates from milk samples produce very similar PFGE patterns each other (PFGE type 9, 10 and 11). Four isolates were represented by 4 PFGE types (type 13 to 16) in cluster III. A reference strain, *S. aureus* ATCC 25923 (PFGE type 15) was included in this cluster, which showed similar band patterns with those from food-poisoning cases. An isolate, SA14, originated from chicken was most remotely related from all other isolates in this study.

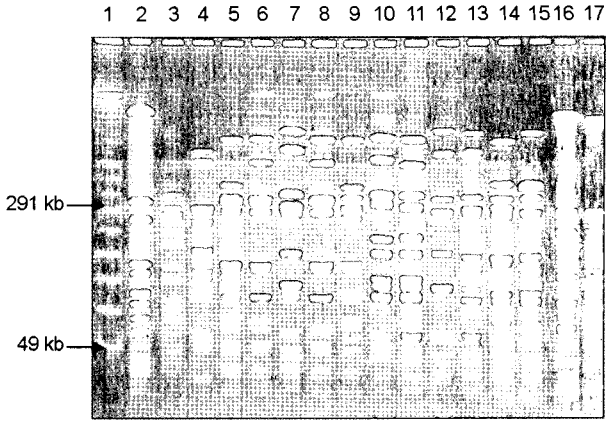

Fig. 1. PFGE patterns of *SmaI* digests of total DNA from representative *S. aureus* Isolates. Lane 1, lambda ladder marker; Lane 2, ATCC 25923; Lane 3, SA14; Lane 4, SA02; Lane 5, SA04; Lane 6, SA05; Lane 7, SA06; Lane 8, SA07; Lane 9, SA10; Lane 10, SA15; Lane 11, SA17; Lane 12, SA18; Lane 13, SA23; Lane 14, SA21; Lane 15, SA22; Lane 16, SA19; Lane 17, SA24.

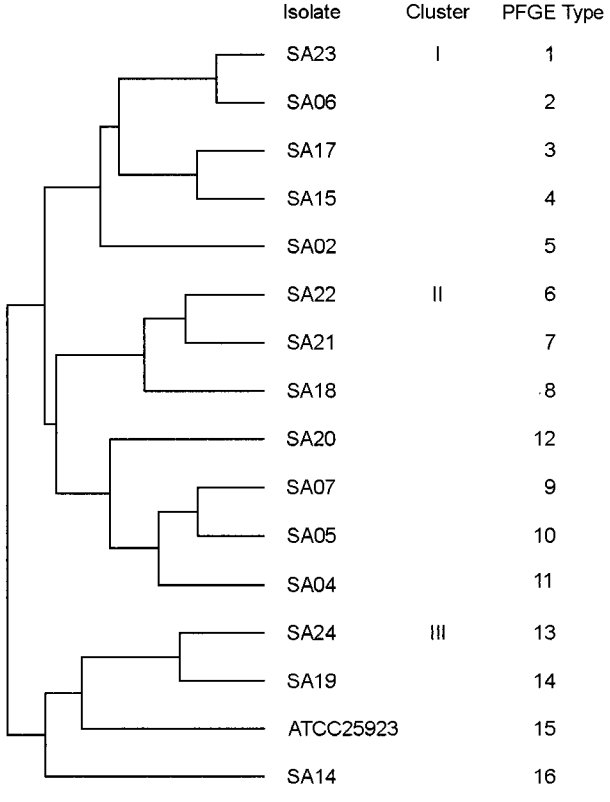

Fig. 2. Dendrogram of 16 representative PFGE types for *S. aureus* isolates.

DISCUSSION

Clinical and subclinical staphylococcal mastitis caused by *S. aureus* is of considerable importance worldwide. MRSA is also one of the most important nosocomial pathogens, causing surgical wound infections and abscesses related to injections. Moreover, staphylococcal food-poisoning cases are one of the most common causes of reported food-born diseases (Bunning et al., 1997). So, this study was conducted to monitor the transmission of *S. aureus* isolates from different sources and investigate the difference in their genomic types in addition to phenotypic antibiograms, and to elucidate the most disseminated strains of *S. aureus* using PFGE.

The incidence of MRSA varied considerably from country to country in a pan-European surveillance, ranging from 0.1 to 34.4% (Voss et al., 1994). Jorgensen et al. (1996) reported that all 50 isolates from clinical patient were resistant to penicillin, methicillin and gentamycin, and 82% and 64% of the isolates were resistant to ciprofloxacin and tetracycline, respectively. Kim et al. (2002) reported the high incidence of resistant isolates from mastitic milks to penicillin and ampicillin, ranging from 47.5% to 66.3%. Results of this study showed similar resistance patterns and rates. None of the isolates, however, were resistant to oxacillin, indicating sensitive to methicillin. Also, there was no clear distinction for resistance pattern between isolates from animal and human source. This might be explained by the abusive use of β-lactam antibiotics for therapeutic purpose in animal and human.

The foods that are most often involved in staphylococcal food-poisoning cases differ widely from one country to another. Meat products including especially hams and poultry, milk products including especially cheeses, seafoods and egg products were among the main sources of contamination for those cases (Genigeorgis, 1989; Wieneke et al., 1993). The percent of SE-producing strains also varies considerably from one food to another and from one report to another. Among the *S. aureus* strains isolated from food samples, the percentage of SE strains was estimated to be around 25%. Also, those from cows with mastitis ranged from 0.2% to 43% when SEA to SED were investigated (Rosec et al., 1997; Cardoso et al., 1999), with SEC being predominant among the same source samples (Stephan et al., 2001). Eleven of 23 isolates were enterotoxigenic, with an isolate producing two toxins in this study. It was of interest that all isolates from pig sources were positive to SEA or SED, a similar result of high prevalence of SEA from raw pork by Atanassova et al. (2001). It should be noted that there is, however, a great increase in their estimations when the newly described SEs, such as SEG to SEJ, SEM, and SEM to SEO, are taken into account (Le Loir et al., 2003).

Some authors have shown that in different countries only a few *S. aureus* clones are responsible for most of the cases of bovine mastitis and that these clones have a broad geographic distribution (Fitzgerald et al., 1997; Annemuller et al., 1999). Because six of 9 isolates from mastitic milks of different farms in this study showed an identical or similar pattern and were included in one cluster, it was also suggested that certain common genotype prevail on the farms, a similar result by Lim et al. (2004). On the other hand, 6 isolates from food-poisoning cases showed close genetic relatedness with those from 4 different sources though they were grouped into 3 different clusters. An isolate, SA20, even revealed an identical band pattern with 4 isolates from pigs of different farms. This suggested that food-poisoning outbreaks were caused by the diverse sources of food, of which their raw materials were infected with *S. aureus*. Also, it can be concluded that PFGE is a powerful tool for epidemiological tracing of infection source for food-initiated outbreaks.

REFERENCES

Annemuller C, Lammler C, Zschock M. Genotyping of *Staphylococcus aureus* isolated from bovine mastitis. Vet Microbiol. 1999. 69: 217-224.

Atanassova V, Meindl A, Ring C. Prevalence of *Staphylococcus aureus* and staphylococcal enterotoxins in raw pork and uncooked smoked ham: a comparison of classical culturing detection and RFLP-PCR. Int J Food Microbiol. 2001. 68: 105-113.

Bannerman TL, Hancock GA, Tenover FC, Miller JM. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. J Clin Microbiol. 1995. 33: 551 -555.

Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin

Pathol. 1966. 45: 493-496.

Bunning VK, Lindsay JA, Archer DL. Chronic health effects of microbial foodborne disease. World Health Stat Q. 1997. 50: 51-56.

Cardoso HF, Silva N, Sena MJ, Carmo LS. Production of enterotoxins and toxic shock syndrome toxin by *Staphylococcus aureus* isolated from bovine mastitis in Brazil. Lett Appl Microbiol. 1999. 29: 347-349.

Cuny C, Witte W. Typing of *Staphylococcus aureus* by PCR for DNA sequences flanked by transposon Tn*916* target region and ribosomal binding site. J Clin Microbiol. 1996. 34: 1502 -1505.

Deplano A, Witte W, van Leeuwen WJ, Brun Y, Struelens MJ. Clonal dissemination of epidemic methicillin-resistant *Staphylococcus aureus* in Belgium and neighboring countries. Clin Microbiol Infect. 2000. 6: 239-245.

Fitzgerald JR, Meaney WJ, Hartigan PJ, Smyth CJ, Kapur V. Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. Epidemiol Infect. 1997. 119: 261-269.

Genigeorgis CA. Present state of knowledge on staphylococcal intoxication. Int J Food Microbiol. 1989. 9: 327-360.

Gurtler V, Barrie HD. Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S-23S rDNA spacer regions: characterization of spacer sequences. Microbiology. 1995. 141: 1255-1265.

Jorgensen M, Givney R, Pegler M, Vickery A, Funnell G. Typing multidrug-resistant *Staphylococcus aureus*: conflicting epidemiological data produced by genotypic and phenotypic methods clarified by phylogenetic analysis. J Clin Microbiol. 1996. 34: 398-403.

Kim S, Hong HP, Kim SY, Kwon HI, Lee HM. Molecular typing and detection of enterotoxin by multiplex PCR of *Staphylococcus aureus* isolated from bovine mastitis. Korean J Vet Serv. 2002. 25: 275-283.

Kotb M. Bacterial pyrogenic exotoxins as superantigens. Clin Microbiol Rev. 1995. 8: 411-426.

Le Loir Y, Baron F, Gautier M. *Staphylococcus aureus* and food poisoning. Genet Mol Res. 2003. 2: 63-76.

Lim SK, Joo YS, Moon JS, Lee AR, Nam HM, Wee SH, Koh HB. Molecular typing of enterotoxigenic *Staphylococcus aureus* isolated from bovine mastitis in Korea. J Vet Med Sci. 2004. 66: 581-584.

Monday SR, Bohach GA. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. J Clin Microbiol. 1999. 37: 3411-3414.

Munson SH, Tremaine MT, Betley MJ, Welch RA. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. Infect Immun. 1998. 66: 3337-3348.

Richardson JF, Aparicio P, Marples RR, Cookson BD. Ribotyping of *Staphylococcus aureus*: an assessment using well-defined strains. Epidemiol Infect. 1994. 112: 93-101.

Rosec JP, Guiraud JP, Dalet C, Richard N. Enterotoxin production by staphylococci isolated from foods in France. Int J Food Microbiol. 1997. 35: 213-221.

Stephan R, Annemuller C, Hassan AA, Lammler C. Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. Vet Microbiol. 2001. 78: 373-382.

Suh DK, Song JC. Genotypic characterization of *Salmonella enterica* serotype Enteritidis isolated from food-poisoning cases and chickens by pulsed-field gel electrophoresis. J Exp Biomed Sci. 2005. 11: 9-13.

Voss A, Milatovic D, Wallrauch-Schwarz C, Rosdahl VT, Braveny I. Methicillin-resistant *Staphylococcus aureus* in Europe. Eur J Clin Microbiol Infect Dis. 1994. 13: 50-55.

Wieneke AA, Roberts D, Gilbert RJ. Staphylococcal food poisoning in the United Kingdom, 1969-1990. Epidemiol Infect. 1993. 110: 519-531.