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Molecular subtyping of *Staphylococcus aureus* from an outbreak associated with a food handler

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SUMMARY

On 6 May 2000, a staphylococcal food poisoning outbreak occurred at a high school, affecting 10 of the 356 students who attended the breakfast. Twenty-seven *Staphylococcus aureus* isolates, producing enterotoxin A (SEA), SEB-, or non-SEA-E, were recovered from 7 patients, 2 food handlers and left-overs. To investigate the outbreak, we genotyped the isolates by using pulsed-field gel electrophoresis (PFGE) and three PCR-based techniques: inter-IS256 PCR typing, protein A gene (*spa*) typing, and coagulase gene restriction profile (CRP) analysis. Our results show that PFGE was the most discriminatory technique, whereas the three PCR-based techniques were insufficient in the discriminatory power to distinguish the *S. aureus* isolates from the outbreak. Based on the enterotoxin-producing types and the results of genotyping, three distinct types of strains (A1111, B2221 and N3221) were designated. Both the A1111 and B2221 strains were found in the specimens from the patients and a hand lesion of a food handler, suggesting that the source of contamination for the outbreak was most likely originated from a food handler.

INTRODUCTION

Food poisoning caused by *Staphylococcus aureus* is characterized by nausea, vomiting, abdominal pain and diarrhoea, with a short incubation period of 30 min to 8 h after ingesting the contaminated food [1]. Enterotoxins produced by this bacterium are believed to be wholly responsible for the food poisoning symptoms [2]. Staphylococcal food poisoning outbreaks are ranked highly among food-borne disease outbreaks in many countries [3, 4]. In Taiwan, *S. aureus* has contributed to 30% of the food-borne disease outbreaks from 1986 to 1995 [5]. In recent years, this organism is still one of the major causes of food-borne disease outbreaks even though the incidence rate has decreased [6].

Analysing *S. aureus* isolates from a food-borne disease outbreak by highly discriminatory typing techniques provides useful clonality information for identifying the aetiological agent, tracing the source of contamination and leading to prevention and control measures. Ideal typing method with rapidity, ease of use, low cost, reproducibility and high discriminatory power is being pursued by microbiologists for routinely typing *S. aureus* isolates from outbreaks. To date, numerous genotyping techniques have been developed for *S. aureus* [7–13]. Among these techniques, PFGE has been demonstrated to have the advantages in discriminatory power and reproducibility, but its major drawbacks are labour-intensive and time-consuming [14]. Several PCR-based techniques, such as inter-IS256 PCR typing [7], protein A gene (*spa*) typing [8], and coagulase gene restriction profile (CRP) analysis [9], are rapid and easy to perform, but their usefulness in distinguishing *S. aureus* isolates from food-borne outbreaks still needs to be evaluated.

On 6 May 2000, a food-borne disease outbreak

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occurred at a high school in Taichung County, Taiwan. Ten of the 356 students who attended breakfast developed symptoms of vomiting, abdominal pain and diarrhoea 2–3 h later. The suspected food was salad bread baked by a local bakery. Twenty-seven *S. aureus* isolates which produced enterotoxin A (SEA), SEB or non-SEA-E were recovered from the stool or vomit of seven patients, nose and wound swabs from two food handlers, and the left-overs. To investigate the outbreak, we characterized the *S. aureus* isolates by PFGE, inter-IS256 PCR typing, *spa* typing and CRP analysis. Our results show that PFGE is the most discriminatory method for molecular epidemiologic study of the outbreak, and the source of contamination was most likely a food handler.

MATERIALS AND METHODS

Bacterial isolation

Taichung County Health authorities collected 6 stool swabs and 4 vomit specimens from the 10 patients, and 4 nasal swabs and 1 wound swab from 4 food handlers. The specimens were sent to the Laboratory of the Third Branch Office, Centre for Disease Control, Taiwan, for bacterial pathogen examination. The left-overs were sent to the Food and Drug National Laboratories for microbial testing. For isolation of *S. aureus*, the specimens were cultured on Baird–Parker agar plates (Merck Taiwan Ltd., Taichung City, Taiwan) and incubated at 35 °C for 24 h. Two colonies from each plate were chosen and subcultured on nutrient agar plates (Eiken Chemical Co., Tokyo, Japan). Bacterial isolates were tested using staphylase agglutination testing kits (Oxoid Unipath, Hants, U.K.). Positive results were identified as *S. aureus*. A total of 24 *S. aureus* isolates were recovered from 7 patients and 2 food handlers. The Food and Drug National Laboratories provided three *S. aureus* isolates recovered from the left-overs. The sources of the 27 isolates are shown in Table 1. The bacterial isolates were stored in 15% glycerol and kept at −70 °C until use.

Detection of toxins by RPLA

Staphylococcal isolates were screened for the expression of SEA, SEB, SEC, SED and SEE by reverse passive latex agglutination (RPLA) according to the manufacturer's instructions (Denka Seiken Co., Tokyo, Japan).

Preparation of DNA for genotyping

The *S. aureus* isolates were grown overnight at 37 °C in 5 ml of brain heart infusion broth (Difco Laboratories, Detroit, MI, USA). One millilitre of the overnight bacterial culture was harvested, washed and resuspended in 0·5 ml ET buffer (100 mM EDTA, 10 mM Tris, pH 8·0). The bacterial suspension was then mixed thoroughly with an equal volume of 1·8% low melting agarose (Promega Co., Madison, WI, USA), and then was filled in a 1 ml syringe and allowed to solidify in a refrigerator at 4 °C. The bacterial plugs were then removed from the syringe and incubated in 8 ml ET buffer containing 5 mg/ml lysozyme (Ameresco Co., Solon, OH, USA), 0·1 mg/ml lysostaphin (Sigma Chemical Co., St. Louis, MO, USA) and 0·05% *N*-lauroylsarcosine (Sigma Chemical Co.) with gentle shaking at 37 °C for 18 h. The plugs were then treated with 8 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8·0) containing 0·5 mg/ml proteinase K (Worthington Biochemical Co., Lakewood, NJ, USA) and 1% SDS (Ameresco Co.) with shaking at 50 °C for 18 h. Following the treatment, the plugs were washed with 50 ml TE buffer containing 0·1 mM PMSF (Ameresco Co.) for 1 h at room temperature and twice with 50 ml TE buffer without PMSF. The plugs were then stored in TE buffer at 4 °C until use.

PFGE

The agarose plugs were cut into 1 mm thick slices for PFGE analysis. *Sma*I restriction treatment of the genomic DNA in the slices and separation of the DNA fragments were performed as in previously described procedures [14]. The PFGE pattern was interpreted in accordance with previously published guidelines [15].

Inter-IS256 PCR

A piece of a 3 mm thick slice was cut out of each bacterial agarose plug and melted in 100 μl TE buffer at 65 °C. Five microliters of the DNA solution was used in each PCR reaction with a 20 μl total volume. Amplification of the inter IS256 fragments was performed as described by Deplano and colleagues

Table 1. *The sources, enterotoxin types, genotypes, and strain designations for the 27* S. aureus *isolates*

Isolate code	Source	Enterotoxin type*	PFGE-CRP-*spa*-inter-IS256 PCR type	Strain designation
Sa19159-1	Patient 1, stool	SEA	1-1-1-1	A1111
Sa19159-2	Patient 1, stool	SEA	1-1-1-1	A1111
Sa19160-1	Patient 2, stool	SEA	1-1-1-1	A1111
Sa19160-2	Patient 2, stool	SEA	1-1-1-1	A1111
Sa19161-1	Patient 3, stool	SEA	1-1-1-1	A1111
Sa19161-2	Patient 3, stool	SEA	1-1-1-1	A1111
Sa19163-1	Patient 4, stool	SEA	1-1-1-1	A1111
Sa19163-2	Patient 4, stool	SEA	1-1-1-1	A1111
Sa19164-1	Patient 5, vomit	SEA	1-1-1-1	A1111
Sa19164-2	Patient 5, vomit	SEA	1-1-1-1	A1111
Sa19167-1	Patient 6, stool	SEA	1-1-1-1	A1111
Sa19167-2	Patient 6, stool	SEB	2-2-2-1	B2221
Sa19166-1	Patient 7, vomit	—	3-2-2-1	N3221
Sa19166-2	Patient 7, vomit	—	3-2-2-1	N3221
Sa19162-1	Food handler 1, nose	SEB	2-2-2-1	B2221
Sa19162-2	Food handler 1, nose	SEB	2-2-2-1	B2221
Sa19182-1	Food handler 2, wound	SEA	1-1-1-1	A1111
Sa19182-2	Food handler 2, wound	SEA	1-1-1-1	A1111
Sa19182-3	Food handler 2, wound	SEB	2-2-2-1	B2221
Sa19182-4	Food handler 2, wound	SEB	2-2-2-1	B2221
Sa19182-5	Food handler 2, wound	SEB	2-2-2-1	B2221
Sa19182-6	Food handler 2, wound	SEB	2-2-2-1	B2221
Sa19182-7	Food handler 2, wound	SEB	2-2-2-1	B2221
Sa19182-8	Food handler 2, wound	SEB	2-2-2-1	B2221
FDA-1	Left-over 1	SEA	1-1-1-1	A1111
FDA-2	Left-over 2	SEA	1-1-1-1	A1111
FDA-3	Left-over 3	SEA	1-1-1-1	A1111

* SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; —, no SEA, SEB, SEC, SED, or SEE was detected by the RPLA test.

[7]. The amplified DNA fragments were separated in 2% agarose gels.

spa typing

Preparation of the DNA solution for PCR reaction was performed as in the inter-IS256 PCR description. Amplification of the *spa* gene was performed as described by Frenay and colleagues [8] except that two new primers, primer spa-F 5′-CTCAAGCACCAAA-AGAGGA-3′ and primer spa-R 5′-ATCACCAGGT-TTAACGACATG-3′ were used in the reaction. The PCR reaction consisted of an initial denature step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 35 s 50 °C for 35 s, and 72 °C for 40 s. The PCR products were analysed in 2% agarose gels.

CRP analysis

Preparation of the DNA solution for PCR reaction was performed as in the inter-IS256 PCR description. Amplification of the repeated region of the coagulase gene by PCR was performed as described by Goh and colleagues [9], except that a new forward primer, primer COAG-5 (5′-GGTATTCGTGAATACAAC-GATGGAA-3′), located 40 bp upstream from COAG-2, was used in the reaction with primer COAG-3 (5′-AAAGAAAACCACTCACATCA-3′). Amplification conditions consisted of an initial denature step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 35 s, 55 °C for 35 s, and 72 °C for 2 min. Restriction profiles were determined by digesting the amplified fragment with *Alu*I (Promega Corp.) and separating the DNA fragments in 2·5% agarose gels.

RESULTS

A summary of the sources of isolation, enterotoxin types, genotypes and strain designations is listed in Table 1. Of the 27 isolates tested, 16 produced SEA, 9 produced SEB, and 2 did not produce either SEA to SEE. Three PFGE pulsotypes (Fig. 1a), 2 CRP types

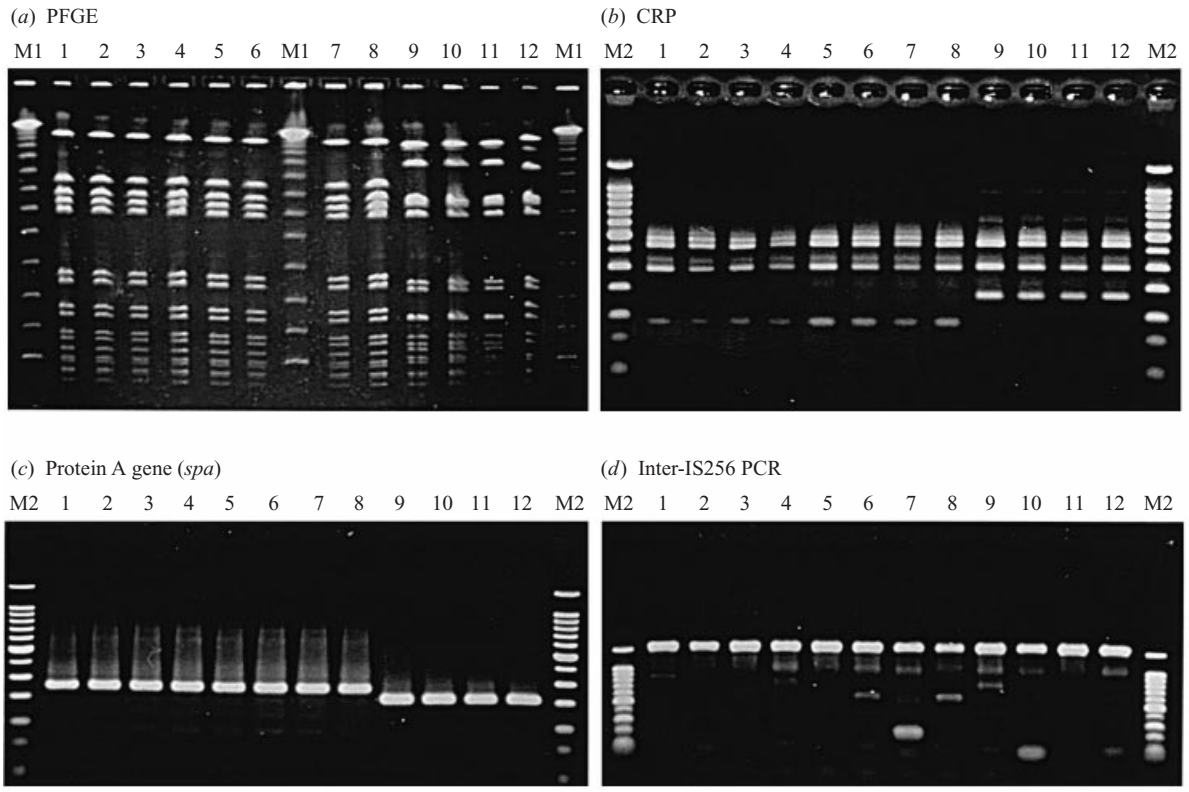

Fig. 1. DNA patterns of PFGE (*a*), CRP (*b*), *spa* (*c*), and inter-IS256 PCR (*d*) for the *S. aureus* isolates. Lane M1, the lambda ladders; lane M2, the 100 bp DNA ladders; lane 1, Sa19159-1 (isolated from patient 1, SEA-producing); lane 2, Sa19160-1 (patient 2, SEA); lane 3, Sa19161-1 (patient 3, SEA); lane 4, Sa19163-1 (patient 4, SEA); lane 5, Sa19164-1 (patient 5, SEA); lane 6, Sa19167-1 (patient 6, SEA); lane 7, Sa19182-1 (food handler 2, SEA); lane 8, FDA-1 (left-over, SEA); lane 9, Sa19167-2 (patient 6, SEB); lane 10, Sa19182-3 (food handler 2, SEB); lane 11, Sa19162-1 (food handler 1, SEB); lane 12, Sa19166-1 (patient 7, non-SEA-E).

(Fig. 1b), 2 *spa* types (Fig. 1c) and 1 inter-IS256 PCR type (Fig. 1d), were identified for the tested isolates. Three distinct types of strains, A1111, B2221 and N3221, were designated on the basis of the enterotoxin types and the results of the genotyping. Strain A1111 type was isolated from stool or vomit of patients 1–6, the hand lesion of food handler 2, and the left-overs. Strain B2221 was recovered from the stool of patient 6, the nose of food handler 1 and the hand lesion of food handler 2. N3221 was isolated from the vomit of patient 7. Of the 8 isolates from the hand lesion of food handler 2, 2 were the strain A1111 and 6 were the B2221.

DISCUSSION

One of the criteria for confirmation of a staphylococcal food poisoning outbreak is isolation of an organism of the same phage type from stool or vomit from two or more ill people [4]. Unfortunately, most laboratories are not equipped to handle phage typing and this technique can be used successfully only if it is performed in an experienced reference laboratory. With the advent of molecular biology, several molecular typing techniques have been recognized as powerful methods in differentiating *S. aureus* strains for the epidemiologic studies of staphylococcal nosocomial infections and food poisoning outbreaks. Among these techniques, PFGE is the most common and has been used as a reference because of its excellent discriminatory power and reproducibility [14, 16–18]. Several PCR-based techniques, such as inter-IS256 PCR typing, *spa* typing, and CRP analysis, have also been developed for rapid typing of *S. aureus* isolates [7–9]. All of these PCR-based techniques offer the advantages of rapidity, low cost and ease of use compared with PFGE. In this study, we used the four above-mentioned methods to analyse the clonality of 27 *S. aureus* isolates from a food poisoning outbreak. Our results show that only PFGE can differentiate the three distinct enterotoxin-producing strains. The three PCR-based techniques showed low discriminatory power for differentiating the isolates and the typing resolution was not even

increased by combining these three PCR-based techniques. CRP analysis has been proved to be insufficient as a sole method for typing of *S. aureus* in a previous study [14]. Inter-IS256 PCR typing, in an inter-laboratory study, has been shown to perform excellently for typing *S. aureus* isolates, comparable to that obtained with PFGE analysis [19]. However, inter-IS256 PCR typing, as well as *spa* typing, did not exhibit effectively discriminatory power for the *S. aureus* isolates typed in this study. Recently, Ribot and colleagues have developed an easy and rapid PFGE protocol for the subtyping of *Campylobacter jejuni* isolates [20]. The whole preparation procedures of the protocol can be done within 5–6 h. Based on the study, it is possible to develop an easy and rapid PFGE protocol to meet the requirement of public health department for routine analysis of *S. aureus* isolates in epidemiologic studies of staphylococcal disease outbreaks.

In this outbreak, all of the patients manifested the symptom of vomiting. Few expressed abdominal pain and diarrhoea in a short incubation period of 2–3 h. In addition, *S. aureus* isolates were recovered from patient specimens, left-overs and two food handlers, indicating that this outbreak could be an incidence of staphylococcal food poisoning. By characterizing the isolates with enterotoxin typing and molecular genotyping, we identified three distinct types of strains, i.e. A1111, B2221 and N3221. Strains A1111 and B2221 types were found in the patient specimens and the hand lesion of a food handler. A1111 was also detected in the left-overs. These data suggest that this was a staphylococcal food poisoning outbreak and the source of contamination was most likely from a food handler who had a wound on his hand when preparing the contaminated food.

According to our experience, multiple *S. aureus* strains were frequently isolated from the faeces of the ill persons from a single suspected staphylococcal food poisoning outbreak. Because the gastrointestinal carriage of *S. aureus* is high in diarrhoeal patients [21], the minority of the isolated strains are usually considered to be background strains and excluded as the aetiological agent for an outbreak. Our results indicate that food can be contaminated by multiple *S. aureus* strains from a food handler and suggest that all *S. aureus* strains isolated from a suspected staphylococcal food poisoning outbreak can be meaningful.

In the beginning, our laboratory identified 11 isolates of the A1111 strain type and 1 isolate of the B2221 strain type from 6 patients and 2 isolates of the B2221 strain type from the nose of food handler 1. These findings did not point out the source of the predominant A1111 strain. In a further investigation, we found that food handler 2, who was not sampled in the first investigation, had a wound on the middle finger of his right hand. Both A1111 and B2221 strains, by a ratio of 1 to 3, were isolated from the wound swab. The food handler had prepared the salad bread. The left-overs from the salad ingredients were detected with *S. aureus* at $1{\cdot}2 \times 10^5$ to $2{\cdot}0 \times 10^6$ c.f.u./g. These findings suggested that food handler 2 was the direct source of the inocula for the food poisoning outbreak. However, the same B2221 strain was also detected in the nose of the food handler 1. The nose is often the source of *S. aureus* [22]. Since the two food handlers worked together, food handler 1 could not be excluded as a source of the B2221 strain.

The ratio of strain A1111 to strain B2221 was 1 to 3 (2 to 6) in the wound specimen from food handler 2, but was 11 to 1 in the specimens from the patients. This change could result from an uneven growth rate in the two strains in the salad because the overwhelming population of A1111 strain found in the patients was in accordance with that found in the food. All of the three tested isolates from the left-overs were the A1111 strain. Many factors such as temperature, water activity, acidity, salt concentration, microbial flora in the foods, etc, can affect the growth of staphylococci [3]. However, the factor that affected the disproportional growth of the two *S. aureus* strains (A1111 and B2221) in this case was unknown.

In conclusion, the results of this study demonstrate that PFGE is a more precise method for molecular typing of *S. aureus* isolates than inter-IS256 PCR typing, *spa* typing and CRP analysis. The three PCR-based techniques are insufficient as a sole subtyping method in distinguishing *S. aureus* isolates. However, PFGE is labour-intensive and time-consuming. An easy and rapid PFGE protocol for *S. aureus* needs to be developed for public health departments undertaking routine subtyping of large numbers of *S. aureus* isolates in epidemiologic investigation of staphylococcal disease outbreaks.

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