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Application of pulsed-field gel electrophoresis to the epidemiological characterization of *Staphylococcus intermedius* implicated in a food-related outbreak

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SUMMARY

An outbreak of food intoxication involving over 265 cases in western United States occurred in October 1991. *Staphylococcus intermedius* was implicated as the aetiologic agent. Representative outbreak isolates (five clinical and ten from foods) produced type A enterotoxin. DNA fragments generated by four restriction endonucleases and analysed by pulsed-field gel electrophoresis (PFGE) provided definitive evidence that all isolates from nine different counties in California and Nevada were derived from a single strain. The PFGE pattern of these outbreak isolates was distinct from those of a heterogeneous collection of seven *S. intermedius* strains of veterinary origin and five unrelated *S. aureus* laboratory strains. The data show a significant PFGE pattern heterogeneity not only among members of different *Staphylococcus* species but also within members of the same species and even the same enterotoxin type. The results indicate that PFGE is a valuable strain-specific discriminator for the epidemiological characterization of *S. intermedius*. To our knowledge, this represents the first documented foodborne outbreak caused by *S. intermedius*. These findings suggest that the presence of *S. intermedius* and other species such as *S. hyicus* in food should be reason for concern.

INTRODUCTION

Staphylococcal food poisoning, a commonly reported foodborne illness, is caused by the ingestion of preformed toxin produced in foods. With exceptions [1] the enterotoxin production is associated with coagulase-positive rather than coagulase-negative *Staphylococcus* species. Among the coagulase-positive species (*S. aureus*, *S. intermedius*, *S. lyticus*, *S. delphi*) *S. aureus* has been almost exclusively implicated in human foodborne illness.

S. intermedius is now recognized as a common component of veterinary flora and as a veterinary pathogen [2, 3]. Although the ecology and epidemiology of the organism are not clearly defined, it is rarely isolated from humans [4] except from infections resulting from animal bites [5]. It is, however, occasionally found in raw and processed foods [6]. This species was previously classified as *S. aureus* biotypes

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E and F, but it can be readily differentiated from *S. aureus* on the basis of certain biochemical reactions and cell-wall components [7–9]. The enterotoxigenicity of *S. intermedius* was previously established [5, 10, 11] but its association with food poisoning outbreaks has not been reported.

A staphylococcal food poisoning outbreak in southwestern United States in October 1991 involved more than 265 cases in nine counties and incriminated 'butter-blend spread' products [12]. Because of the widespread occurrence of staphylococci in the environment and the frequent encounter of more than one strain or species in a food, the specific strain or type which occurred in the food must be identified in order to determine the aetiology of the illness and trace the source of infection. Among the techniques used to differentiate strains (phage-typing, plasmid composition, antibiotic susceptibility patterns and biochemical reactions) chromosomal restriction fragment length polymorphism has proved useful in other bacterial systems as an excellent strain-specific discriminatory tool.

Evidence is presented here to establish the aetiological agent of this outbreak as *S. intermedius* and to document the usefulness of DNA fingerprinting, using PFGE, in molecular epidemiological studies of staphylococcal foodborne illnesses.

MATERIALS AND METHODS

Bacterial strains

The *Staphylococcus* strains used, their sources, and the enterotoxins they produced are listed in Table 1.

Species identification

Staphylococci were isolated from the incriminated butter-blend products at the Los Angeles District laboratory of the Food and Drug Administration, and from foods and clinical specimens obtained from patients by the Nevada State Health Department. Although a few coagulase-negative staphylococci (e.g., *S. hominis*) were isolated from the incriminated foods, most isolates were coagulase-positive and tentatively identified as *S. intermedius* by the Gram-positive Identification (GPI) Test Kit (bioMérieux Vitek, Inc., Hazelwood, MO). The GPI identification was confirmed by testing the isolates for mannitol fermentation (aerobically and anaerobically) and acetylmethyl carbinol production. The species identity of the clinical isolates from Nevada was also confirmed by examining their cellular fatty acid profiles.

Reagents and instrumentation

Restriction endonucleases were purchased from GIBCO BRL (Gaithersburg, MD); lambda concatemer size standards were purchased from New England Biolabs (Beverly, MA). Proteinase K, lysostaphin and all other chemicals and supplies were obtained from Sigma Chemical Co. (St Louis, MO). The TECRA™ Staphylococcal Enterotoxin Visual Immunoassay kit (International Bioproducts, Redmond, WA) was used to serotype and quantitate enterotoxins. ELISA absorbance was measured on a TECAN microplate reader (Hillsborough, NC). Pulsed-field gel electrophoresis (PFGE) was conducted by using the BioRad CHEF DR-II (BioRad Laboratories, Richmond, CA).

Table 1. *Designations, enterotoxins produced, and sources of* Staphylococcus *strains used in this study*

Strain number	Strain designation	Enterotoxin type	Source
S. intermedius			
1	425–558, 1–2	A	Margarine
2	567–537, 4–2	A	Butter blend
3	567–537, 6–3	A	Butter blend
4	640–679, 9–12	A	Vegetable margarine
5	641–497, 4–1	A	Butter blend
6	641–498, 1–16	A	Butter blend
7	641–499, 1–16	A	Butter blend
8	662–981, 6–1	A	Butter blend
9	662–982, 1–7	A	Butter blend
10	663–846, 3–1	A	Butter blend
11	2085	A	Patient
12	2089	A	Patient
13	2090	A	Patient
14	2091	A	Patient
15	2174	A	Patient
16	87–400	C	Dog bite (human)
17	87–408	–	Dog bite (human)
18	87–335	–	Dog gingiva
19	87–309	C	Dog gingiva
20	88–40	A	Dog wound infection
21	87–312	–	Dog gingiva
22	87–347	–	Dog gingiva
S. aureus			
23	ATCC 13565	A, D	ATCC
24	ATCC 14458	B	ATCC
25	ATCC 19095	B, C, E	ATCC
26	NCTC 10656	A, D	Attrache
27	SEE 326	E	Attrache

Preparation of bacterial plugs

Plugs were prepared by the following modification of the method described by Poddar [13]. Cultures were grown in Luria Bertani broth with agitation at 37 °C until they reached a turbidity of $A_{610} \approx 0{\cdot}6$. From each culture, 5 ml was centrifuged (12000 g for 10 min). The cells were washed once and resuspended in 5·0 ml of ice-cold 10 mM Tris–1·0-M NaCl–50 mM EDTA, pH 8·0. A 1-ml volume of the cell suspension was briefly warmed to 50 °C, mixed with an equal amount of molten 1% PFGE-grade agarose (tempered to 50 °C) and dispensed into moulds. After solidification the plugs were transferred to sterile tubes containing 2·0 ml of lysostaphin solution (50 units/ml in 10 mM Tris HCl–50 mM EDTA, pH 8·0) and incubated at 37 °C for 4 h. The lysostaphin solution was aspirated and 2·0 ml of a Proteinase K solution (2 mg/ml in 0·5 M EDTA–0·1% Sarkosyl, pH 9·0) was added. The plugs were incubated at 55 °C for 8 h. The Proteinase K solution was then replaced with 2·0 ml 10 mM Tris HCl–1 mM EDTA, pH 8·0, containing phenylmethylsulfonyl fluoride (PMSF), freshly added from a 0·1 M stock solution in ethanol, to a final concentration of 1·5 mM. The tubes were agitated gently at room temperature for 1 h; the solution was discarded and the PMSF rinse was

repeated. Finally, the plugs were rinsed with gentle agitation three times (1 h each) with 10 mM Tris HCl–50 mM EDTA, pH 8·0, at room temperature. Plugs were stored at 4 °C in the final wash solution.

Pulsed-field gel electrophoresis

Slices (1–2 mm) from the plugs were aseptically prepared and transferred to 1·5 ml microcentrifuge tubes. The slices were first rinsed with 0·5 ml sterile distilled water for 5 min and then with 1 × digestion buffer (recommended for the enzyme to be used) for 10 min. Finally, the slices were incubated for 12 h in 150 μl of the 1 × buffer containing 20 units of the restriction enzyme. After incubation, the digestion buffer was aspirated and the slices were rinsed 10 min with 0·5 ml 0·5 × Tris HCl-borate-EDTA (pH 8·2) gel running buffer. Slices were transferred to a 1% agarose gel and electrophoresed using the CHEF DR II system. The DNA in the gels was examined after staining with ethidium bromide (1 μg/ml) for 30 min and photographed.

Enterotoxin detection and quantitation

Cultures were grown in 30 ml of brain heart infusion broth (pH 5·5) in 125 ml flasks rotated on a gyrotory shaker for 18–24 h at 35 °C. The cells were removed by centrifugation and culture supernatant fluids were tested for the presence of enterotoxins. Enterotoxins were identified and quantified by the TECRA™ ELISA kit, which consisted of polyvalent (serotype A–E) and monovalent capture antibodies. The presence of preformed heat-denatured toxin in the incriminated food was examined by the urea renaturation method described previously [14].

RESULTS

Toxin studies

All 15 *S. intermedius* isolates selected for further study were enterotoxigenic and produced only staphylococcal enterotoxin A (SEA) (Table 1). The levels of enterotoxin produced by these strains under identical culture conditions were similar, suggesting that a single strain may be involved (data not shown). Of the 7 *S. intermedius* strains of veterinary origin used as controls, 4 were non-enterotoxigenic, 2 produced enterotoxin C, and 1 produced enterotoxin A. All of the five *S. aureus* strains produced one or more enterotoxins (Table 1).

DNA fingerprinting

DNA restriction fragment profiles were analysed to determine the relatedness of the various *S. intermedius* isolates. Four restriction enzymes (Fig. 1. *Sst*II, lanes 1 and 2; *Asc*I, lanes 3 and 4; *Sma*I, lanes 5 and 6; and *Apa*I, lanes 7 and 8) were selected for preliminary analysis of one food isolate (lanes 1, 3, 5, 7) and one clinical isolate (lanes 2, 4, 6, 8) from the outbreak. PFGE was used to resolve the restriction fragments. Although distinctive patterns were obtained with *Asc*I, *Apa*I, *Sma*I, and *Sst*II, the patterns of both isolates were indistinguishable in each case. *Asc*I cleaved the genomic DNA infrequently, with only seven bands visible in the 20–250 kb region (Fig. 1, lanes 3 and 4). *Sst*II, *Sma*I and *Apa*I proved to be better suited for the study of the *S. intermedius* genome, giving about

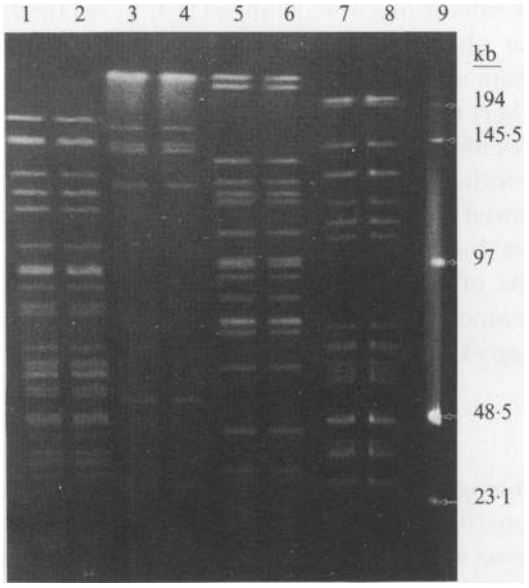

Fig. 1. PFGE profiles of *S. intermedius* food isolate (Table 1, strain 1), lanes 1, 3, 5, and 7, and patient isolate (strain 11), lanes 2, 4, 6, and 8. Lanes 1 and 2 represent *Sst* II digests; lanes 3 and 4, *Asc* I; lanes 5 and 6, *Sma* I; and lanes 7 and 8, *Apa* I digests. Lane 9 is a DNA size marker.

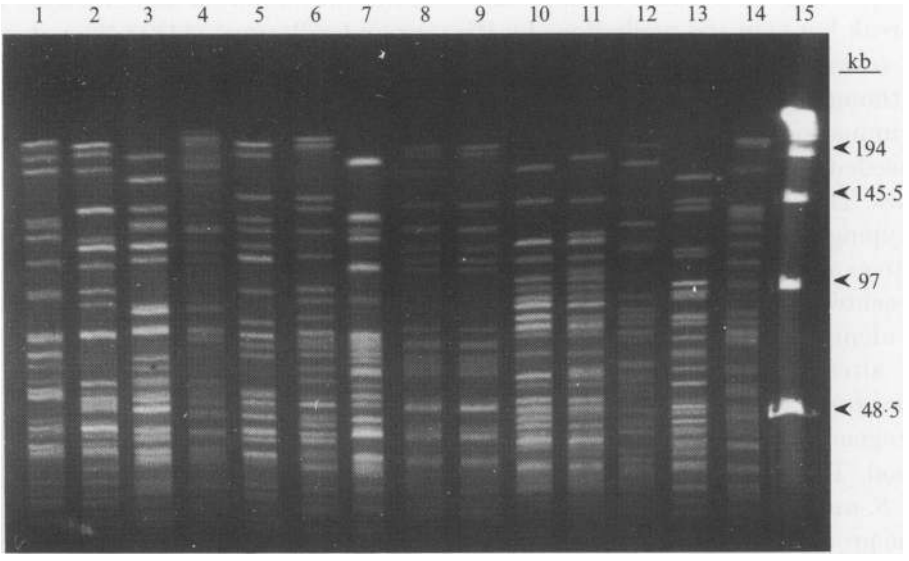

Fig. 2. Comparative *Apa* I PFGE profiles of *S. intermedius* isolates from food, clinical, and veterinary sources, and *S. aureus* strains. Strain numbers correspond to those in Table 1. Lanes 1–7 correspond to strains 16 through 22; lanes 8–9 correspond to strains 1 and 11. Lanes 10–14 correspond to strains 23–27. Lane 15 is a DNA size marker.

26, 22, and 21 bands, respectively, in the 20–250 kb region. *Apa* I was used to compare the genomic arrangement of the 15 representative isolates from the outbreak. No differences were observed in the *Apa* I PFGE patterns of any of the 15 outbreak strains examined using PFGE conditions of 6 V/cm for 18 h, with pulse time ranging from 2 to 7 sec (data not shown). This pattern similarity did

not vary when PFGE conditions were changed to 6 V/cm for 23 h, and pulse range of 2–8 sec (data not shown). Under these two sets of conditions restriction fragments ranging from about 10 to 300 kb were observed to cover essentially the entire genome. Further, the *Apa*I pattern of the outbreak strain (represented by one food and one patient isolate, Fig. 2, lanes 8 and 9) differed substantially from *S. intermedius* of veterinary origin (lanes 1–7) and the *S. aureus* strains (lanes 10–14). The data showed significant genome pattern diversity not only within the genus *Staphylococcus* but also within the species *S. intermedius*. The indistinguishable patterns of the outbreak strains, when taken together with the substantial diversity among the control *S. intermedius* strains, supported the toxin production data, suggesting that a single strain was involved.

DISCUSSION

Enterotoxin-producing staphylococci are significant agents of foodborne intoxication and contribute to large numbers of illnesses every year. Until recently, only *S. aureus* seemed to pose a significant foodborne health hazard; *S. intermedius* was considered more a species of veterinary concern. The enterotoxin production by *S. intermedius* strains [5, 10, 11] and the presence of this organism in raw and processed foods [6] raises the issue about the risks posed by this species. The 1991 outbreak [12] involving butter-blend products resulted in more than 265 cases of illness distributed through nine counties in California and Nevada. This outbreak bore out the predictions by Hirooka and colleagues [11] of the potential of *S. intermedius* to cause foodborne intoxication.

Although there are biochemical and serological methods for species-specific discrimination among the staphylococci, methods for strain specific identification are needed for the accurate tracking of the epidemics and for conducting traceback studies. The molecular typing methods used to characterize staphylococci include ribotyping [15] and PFGE [13, 16]. We used our modification of a PFGE method [13] to examine the genomic relatedness of 15 outbreak-associated isolates, which represented patient and food sources from both states involved. These fingerprints were identical; no differences were seen among them even when the run conditions were altered. The significant differences seen among the fingerprints of *S. intermedius* strains of veterinary origin demonstrate that the species is heterogeneous at the level of genome arrangement and add to the value of the method. The differences seen between strain fingerprints from the outbreak and from *S. aureus* strains reiterate the distinct nature of the *S. intermedius* species.

The production of the same enterotoxin type by all the outbreak strains, and at similar levels, adds to the conclusion that a single strain was involved in the outbreak. These data, along with the PFGE findings, point to a common source of contamination rather than post-process contamination of the food products at points of sale.

Our study underscores the value of the PFGE method for tracking outbreaks by *Staphylococcus* species and confirms the epidemiological findings that this outbreak was indeed caused by an *S. intermedius* strain. Besides *S. aureus*, both *S. intermedius* and *S. hyicus* (also known to produce enterotoxins) [17] should be considered as potential agents of foodborne intoxication.

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