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Prevalence and Characterization of *Staphylococcus aureus* and Enterotoxigenic *Staphylococcus aureus* in Retail Raw Chicken Meat Throughout Japan

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ABSTRACT. A total of 444 samples of raw chicken meat (thighs, breasts, wings, livers, gizzards, hearts and ovaries) that retailed at 145 different supermarkets in 47 prefectures in Japan were examined for contamination with *Staphylococcus aureus* in association with its enterotoxigenicity. *S. aureus* was isolated from 292 (65.8%) of the samples, and from 131 of the 145 supermarkets. There was no significant difference in the detection rate of *S. aureus* according to the type of meat examined. About 80% of 714 isolates belonged to the poultry (57.1%) and human biotypes (22.1%). Seventy-eight (21.7%) of 360 isolates were enterotoxigenic and isolated from 78 samples in 53 supermarkets in 31 prefectures. Staphylococcal enterotoxins (SEs) produced were SEB (50 isolates), SEA (14), SEC (8), SED (2), SEA+SEB (2), and SEA+SEC (2). Most of the enterotoxigenic isolates belonged to the human and poultry biotypes, coagulase type VII, VIII or IV, and were lysed by phages of group III. Identical SE types, biotypes, coagulase types and pulsed-field gel electrophoresis (PFGE) patterns were shown in isolates from different types of meat at the same supermarket and from samples taken from different supermarkets in the same prefectures or in isolates from samples obtained from several different prefectures. Among the 50 SEB-producing isolates, 27 yielded three similar PFGE patterns that differed by only a few fragments, suggesting that they were closely related genetically. The three patterns were found in isolates of samples that retailed at 17 supermarkets in 11 prefectures, indicating that they may be disseminated among raw chicken meat in Japan.

KEY WORDS: biotype, coagulase type, pulsed-field gel electrophoresis, *Staphylococcus aureus*, staphylococcal enterotoxin.

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Staphylococcal food poisoning caused by enterotoxin-producing *Staphylococcus aureus* is an important food-borne disease throughout the world. In several countries, the foods that most frequently cause this type of food poisoning are red meat, poultry, and their products [7, 28]. In Japan, the most common type of foods causing this poisoning are typical Japanese-style processed foods composed mainly of rice, i.e., rice balls (nigirimeshi), fried bean curd stuffed with vinegared rice (inarizushi), and a luncheon dish composed of various foods accompanied with rice (bento) [8, 16, 25]. Raw meat and meat products have infrequently been involved in staphylococcal food poisoning.

It has been reported in various countries that most raw fresh and frozen poultry, both of chicken and turkey, are frequently contaminated with *S. aureus* [27]. Consumption of raw chicken meat and chicken products has been on the increase in Japan. To prevent food poisoning, it is important to determine how much actual contamination with enterotoxigenic *S. aureus* in retail raw chicken meat occurs. In Japan [21, 24] and China [11], several studies have been published on the incidence and characteristics of enterotoxigenic *S. aureus* in retail raw chicken meat. However, the strains studied in these reports were isolated from samples obtained from a limited number of districts. In addition, information regarding the detailed characteristics of the isolates was insufficient.

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The present survey was conducted to isolate *S. aureus* from raw chicken meat collected from all 47 prefectures of Japan in order to ascertain the recent status of the contamination of commercial raw chicken meat. In addition, the present study aimed to characterize enterotoxigenic *S. aureus* isolates by phenotypic and genotypic methods.

MATERIALS AND METHODS

Samples: Four hundred and forty-four raw chicken meat samples (tray packaged or not) used in this study were purchased from 145 different supermarkets in 47 prefectures throughout Japan during the period from May 2002 through August 2003. The numbers of samples of each meat type were as follows: thighs (n=114), breasts (n=51), wings (n=148), livers (n=94), gizzards (n=31), hearts (n=3), and ovaries (n=3).

Isolation of S. aureus: The samples were taken by vigorous swabbing of the surface of the meat with sterile cotton swabs. The swabs were streaked directly on mannitol salt agar (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 3% fresh egg yolk emulsion, which was incubated at 37°C for 48 hr (direct plate culture method). One to five typical colonies (mannitol-positive and egg yolk factor-positive) on an agar plate were picked. The same swabs were transfered also to 5-m*l* heart infusion broth (Nissui) containing 7% NaCl and it was incubated for 24 hr at 37°C. Subsequently, 10 μl of the broth cultures were plated onto

the above-mentioned mannitol salt agar plate, which was incubated at 37°C for 48 hr (enrichment culture method). One colony only on a plate agar was picked. Gram-positive cocci and catalase-positive isolates were subjected to biochemical tests. *S. aureus* was identified by the coagulase test and other biochemical tests described by Devriese and Hájek [5].

Biotyping: Biotyping was carried out according to the simplified system established by Devriese *et al.* [4, 6, 10], which uses four tests: the production of staphylokinase and *β*-hemolysin, coagulation of bovine plasma within 6 hr, and the type of growth on crystal violet agar.

Detection of SEs: SEs (SEA, SEB, SEC, and SED) were detected by the reversed-passive latex agglutination (RPLA) method with SET-RPLA (Denka Seiken Co., Ltd., Tokyo).

Coagulase typing: Coagulase typing was performed with a coagulase typing kit (Denka Seiken) by neutralizing rabbit antisera specific to the eight coagulase types I to VIII.

Phage typing: Phage typing was performed as described previously [3] by using the 23 phages of the international typing set for human *S. aureus* strains (group I—29, 52, 52A, 79, 80; group II—3A, 3C, 55, 71; group III— 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85; group V—94, 96; miscellaneous—81, 95). Lytic reactions were examined at 100-fold routine test dilution.

PFGE typing: The preparation of chromosomal DNA and cleavage of genomic DNA with *Sma*I (New England BioLabs, Beverly, Mass.) were performed as described previously [23]. PFGE was performed with 1% agarose slab gel (SeaKem GTG, FMC Bioproducts, Rockland, Me.) with a CHEF-DR II system (Bio-Rad Laboratories Inc, Hercules, Calif.) in 0.5 × Tris-borate-EDTA buffer maintained at 14°C. The running parameters used were as follows: initial pulse time, 5 sec; final pulse time, 40 sec; voltage, 6V/cm; and running time, 22 hr. The PFGE patterns were interpreted based on the criteria of Tenover *et al.* [26].

RESULTS

Detection of S. aureus: *S. aureus* was detected in 180 (40.5%) and 292 (65.8%) of the 444 samples by the direct plate culture method and enrichment culture method, respectively (Table 1). Samples positive for *S. aureus* were detected in 47 prefectures and in 131 of 145 different supermarkets investigated.

Biotyping of S. aureus: A total of 714 isolates of *S. aureus* were used for biotyping. Six hundred and two isolates were from 180 positive samples detected by the direct plate culture method. One hundred and twelve isolates were from 112 positive samples detected by the enrichment culture method. Four hundred and eight (57.1%) of the 714 isolates belonged to the poultry, 158 (22.1%) to the human, 64 (9.0%) to K–*β*–CV:C, 45 (6.3%) to K–*β*+CV:A, 38 (5.3%) to K–*β*+CV:C, and 1 (0.1%) to the ovine biotype (Table 2).

Detection of enterotoxigenic S. aureus: Three hundred and sixty isolates from 292 positive samples from 131 dif-

Table 1. Isolation of *S. aureus* from raw chicken meat

Meat type	No. of samples	No. of positive samples	
		Plate culture[a)]	Enrichment culture[a)]
Thigh	114	47 (41.2%)	83 (72.8%)
Breast	51	19 (37.3%)	31 (60.8%)
Wing	148	59 (39.9%)	96 (64.9%)
Liver	94	45 (47.9%)	60 (63.8%)
Gizzard	31	7 (22.6%)	18 (58.1%)
Heart	3	1 (33.3%)	2 (66.7%)
Ovary	3	2 (66.7%)	2 (66.7%)
Total	444	180 (40.5%)	292 (65.8%)

a) Method for isolation is described in the text.

Table 2. Biotypes of *S. aureus* isolates from raw chicken meat

Meat type	No. of isolates	Biotype[a)]					
		Human	Poultry	Ovine	K-*β*+CV:A	K-*β*+CV:C	K-*β*-CV:C
Thigh	191	48	103	1	14	11	14
Breast	65	3	48	0	4	2	8
Wing	241	42	148	0	12	19	20
Liver	171	48	95	0	13	4	11
Gizzard	31	7	14	0	2	2	6
Heart	10	10	0	0	0	0	0
Ovary	5	0	0	0	0	0	5
Total	714	158 (22.1%)	408 (57.1%)	1 (0.1%)	45 (6.3%)	38 (5.3%)	64 (9.0%)

a) Devriese, L.A. *et al.* [4,6,10]. Biotyping pattern (K, staphylokinase; *β*, *β*-hemolysin; BC, bovine plasma coagulation; CV, growth on crystal violet agar type A or C). Human, K+*β*±BC-CV:A/C; Poultry, K-*β*-BC-CV:A; Ovine, K-*β*+BC+CV:C; K-*β*+CV:A, K-*β*+BC-CV:A; K-*β*+CV:C, K-*β*+BC-CV:C; K-*β*-CV:C, K-*β*-BC-CV:C.

Table 3. Detection of enterotoxigenic *S. aureus* isolates from raw chicken meat

Meat type	No. of isolates	No. of enterotoxigenic isolates	Enterotoxin type					
			A	B	C	D	A+B	A+C
Thigh	100	20 (20.0%)	3	12	2		1	2
Breast	36	9 (25.0%)	2	7				
Wing	123	20 (16.3%)	3	15	1		1	
Liver	75	19 (25.3%)	4	13	2			
Gizzard	22	8 (36.4%)	2	2	3	1		
Heart	2	1 (50.0%)		1				
Ovary	2	1 (50.0%)				1		
Total	360	78 (21.7%)	14 (17.9%)	50 (64.1%)	8 (10.3%)	2 (2.6%)	2 (2.6%)	2 (2.6%)

Table 4. Biotypes, coagulase types and phage groups of enterotoxigenic *S. aureus* isolates

		Enterotoxin type						
		A	B	C	D	A+B	A+C	Total
	No. of isolates	14	50	8	2	2	2	78
Biotype	Human	9	24	2		2		37 (47.4%)
	Poultry	5	19	2	2		1	29 (37.2%)
	K-*β*+CV:A		2				1	3 (3.8%)
	K-*β*+CV:C		2	2				4 (5.1%)
	K-*β*-CV:C		3	2				5 (6.4%)
Coagulase type	I							0
	II							0
	III			2				2 (2.9%)
	IV	10				1		11 (15.7%)
	V		2	1				3 (4.3%)
	VI		2					2 (2.9%)
	VII	4	27	2	1	1	2	37 (52.9%)
	VIII		14	1				15 (21.4%)
	Untypable		5	2	1			8 (10.3%)
Phage group	I	1						1 (2.0%)
	II		6					6 (11.8%)
	III	2	18	1	1			22 (43.1%)
	Miscellaneous	1						1 (2.0%)
	Mixed groups[a)]	6	10	2	1	2		21 (41.2%)
	Untypable	4	16	5			2	27 (34.6%)

a) I+II (1 strain), I+III (6), II+III (2), III+V (2), III+Miscellaneous (1), I+II+III (1), I+III+V (3), I+III+Miscellaneous (1), I+III+V+Miscellaneous (2), II+III+V+Miscellaneous (1), I+II+III+V+Miscellaneous (1).

ferent supermarkets in 47 prefectures were examined for enterotoxigenicity. Seventy-eight (21.7%) were enterotoxigenic (Table 3), isolated from 78 samples in 53 supermarkets in 31 prefectures. Fifty (64.1%) isolates were SEB producers, followed by SEA (17.9%), SEC (10.3%), SED (2.6%), SEA+SEB (2.6%), and SEA+SEC (2.6%) producer.

Biotypes, coagulase types and phage groups of enterotoxigenic S. aureus isolates: The biotypes, coagulase types, and phage groups of the 78 enterotoxigenic *S. aureus* isolates examined are summarized in Table 4. Thirty-seven (47.4%) of the 78 isolates belonged to the human biotype, 29 (37.2%) to poultry, five (6.4%) to K–*β*–CV:C, four (5.1%) to K–*β*+CV: C, and three (3.8%) to K–*β*+CV:A.

Thirty-seven (52.9%) isolates belonged to coagulase type VII, 15 (21.4%) to VIII, 11 (15.7%) to IV, three (4.3%) to V, two (2.9%) to III, and two (2.9%) to VI. The other eight (10.3%) were untypable. The combination of SEB and coagulase type VII was most frequently found, as shown in 27 (34.6%) of the 78 isolates. The next most frequent combination was SEB and coagulase type VIII (n=14, 17.9%), followed by SEA and coagulase type IV (n=10, 12.8%).

Fifty-one (65.4%) isolates were phage typable at a 100 × routine test dilution. Forty-three (84.3%) of the 51 typable isolates belonged to group III (n=22) and mixed groups (n=21). Among the mixed groups, all isolates except one were associated with lysis by phages of group III.

PFGE of enterotoxigenic S. aureus isolates and epidemiology: The 78 enterotoxigenic isolates were further analyzed for their epidemiological relationship by using PFGE. A total of 44 PFGE patterns were identified. SEA-, SEB-,

Table 5. PFGE patterns, biotypes and coagulase types of representative enterotoxigenic *S. aureus* isolates from raw chicken meat

PFGE pattern[a)]	No. of isolates	Enterotoxin type	Biotype	Coagulase type	Prefecture (No. of supermarkets)
1[b)]	2	A	Poultry	IV	Yamanashi (1), Mie (1)
2[b)]	2	A	Human	IV	Fukushima (1), Oita (1)
3[b)]	3	B	Poultry	VIII	Hyogo (2), Hiroshima (1)
4[b)]	12	B	Human	VII	Hokkaido (1), Saitama (1), Hyogo (1), Okayama (3), Kagoshima (2)
	2	B	Human	V	Osaka (1)
	1	B	Human	UT	Okayama (1)
5[b)]	1	B	Human	VII	Kagoshima (1)
	3	B	Poultry	VII	Hiroshima (1), Shimane (2)
	1	B	K–β+CV:C	VII	Aomori (1)
	2	B	K–β–CV:C	VII	Okayama (1), Kagoshima (1)
6[b)]	3	B	Poultry	VIII	Kyoto (1), Hyogo (1)
	1	B	K–β+CV:A	VII	Tochigi (1)
	1	B	K–β+CV:C	VII	Aomori (1)
7[c)]	3	B	Poultry	VIII	Ehime (2)
8[d)]	2	A	Human	IV	Gunma (1)
9[c)]	2	B	Human	VII	Hokkaido (2)

a) PFGE pattern No. corresponds to each of Lane No. in Fig. 1.
b) Isolates of the same PFGE patterns were isolated from samples in different prefectures.
c) Isolates of the same PFGE patterns were isolated from samples in different supermarkets in the same prefectures.
d) Isolates of the same PFGE pattern were isolated from both thigh and liver in the same supermarket.

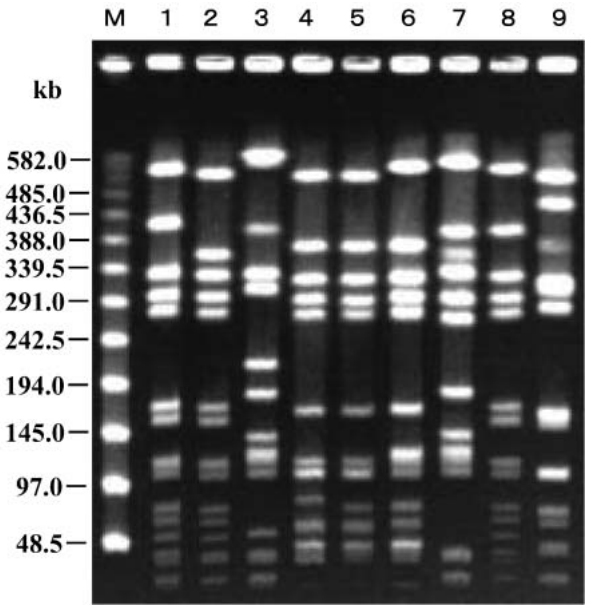

Fig. 1. PFGE of *Sma*I-digested genomic fragments of representative enterotoxigenic *S. aureus* isolates from raw chicken meat. Lane No. at top corresponds to those of PFGE pattern No. in Table 5. M indicates the lambda ladder DNA concatemers used as molecular size markers (kb).

SEC-, SED-, SEA+SEB-, and SEA+SEC-producing isolates yielded 11, 22, 6, 2, 2, and 1 pattern, respectively. As shown in Table 5 and Fig. 1, SEA isolates of PFGE pattern 8, the human biotype, and the coagulase type IV were isolated from both the thigh and liver obtained from the same supermarket in Gunma prefecture. SEB isolates of PFGE pattern 7/poultry biotype/coagulase type VIII, and PFGE pattern 9/human biotype/coagulase type VII, were isolated from samples obtained at different supermarkets in Ehime and Hokkaido, respectively. In addition, identical PFGE patterns (patterns 1 to 6) were found in isolates from several different prefectures (2 to 6), which are located geographically at a great distance from each other. For instance, 15 SEB isolates of PFGE pattern 4/human biotype were isolated from samples obtained from six different prefectures, Hokkaido, Saitama, Osaka, Hyogo, Okayama, and Kagoshima. Twelve of the 15 isolates were coagulase type VII.

Among the 50 SEB-producing isolates, 27 (54%) showed three similar PFGE patterns (Fig. 1, patterns 4 to 6) that differed by only a few fragments, suggesting that they were genetically related closely. Interestingly, the three patterns were found in isolates of samples retailed at 17 supermarkets in 11 prefectures. Twenty-two of the 27 isolates were of the human (n=16) and poultry (n=6) biotypes.

DISCUSSION

Detection of *S. aureus* in foods is to some extent dependent on the sampling technique used, as well as the kind of media used for isolation. In the present study, swab samples of the raw chicken meat surface were used to detect *S. aureus*. The organism was cultured with a relatively high frequency from 292 of the 444 samples (65.8%). Although viable counts of *S. aureus* cannot be determined, the swab method is easier and faster to perform. From the standpoint of the risk assessment to humans, however, it will be necessary to investigate the contamination levels (cfu/g) of *S. aureus* in raw chicken meat.

The *S. aureus* contamination rate in retail raw chicken meat was surprisingly high (65.8%) compared with pork

(26.0% and 29.5%) and beef (20% and 25.0%) [11, 24]. The reasons for this difference are not known.

The simplified biotyping system of Devriese [4] for typing *S. aureus* has been useful in tracing the origin of this organism in animal food and in food industry [6, 9, 10, 12, 19]. The raw chicken meat examined in the present study was primarily contaminated by *S. aureus* belonging to the poultry biotype, suggesting that the isolates originate probably from live chickens. In fact, such strains of the poultry biotype had most frequently been found in the nares and skin [20, 22] or intestinal contents (unpublished data) of healthy chickens. The human biotype, which is characteristic of strains of human origin, has frequently been isolated from raw chicken meat, suggesting that the isolates might incidentally be contaminated by workers during the processes handling raw chicken meat. *S. aureus* and enterotoxigenic *S. aureus* strains were frequently detected on the hands of workers in a poultry-processing plant [1].

The frequency of detection of enterotoxigenic *S. aureus* strains in retail raw chicken meat ranges from 11.4 to 31.3%, according to the previous reports [11, 21, 24]. In the present study, 21.7% of the isolates examined were enterotoxigenic. SEB was most frequently found in isolates from raw chicken meat, regardless of the type of meat, and the next in frequency was SEA. A similar tendency was observed in our previous study [21, 24]. With regards to food hygiene, the fact that retail raw chicken meat was frequently contaminated with the two SE types is significant, considering the fact that most staphylococcal food poisoning outbreaks in Japan have been caused by either SEA or SEB [8, 16].

To date, the presence of new types of SEs (SEG through SER) has been reported [2, 17, 18]. The role of these newly described SEs as causative agents of staphylococcal food poisoning still remains undetermined, since no specific assay for their detection is available. In the present study, *S. aureus* isolates were examined for their production of only four classical SEs by SET-RPLA assay. Further work is needed to determine the presence of these new SE genes.

It has been reported that coagulase type VII is the most predominant type responsible for food poisoning in Japan [8, 16, 25]. In the present study, about 53% of the 78 enterotoxigenic isolates were coagulase type VII, followed by types VIII (21.4%) and IV (15.7%), and these three types were most frequently found in raw chicken meat in our previous study [21, 24]. In the past, there have been a few outbreaks of food poisoning caused by combination with coagulase type IV and SEA [14] or coagulase type VIII and SEB [13]. From the standpoint of food hygiene, it is noteworthy that such combinations were frequently found in raw chicken meat in this study.

The majority of isolates from foods implicated in food poisoning incidents are human phage typable, and susceptible to phages of group I or III [15, 28]. Among the enterotoxigenic isolates from raw chicken meat, 82.4% of the phage typable isolates were lysed by the phages of group III of the human typing set.

It is interesting to note that SEB-producing isolates showing identical and similar PFGE patterns (patterns 4 to 6) were found in samples from 11 of 47 prefectures. The reason why these SEB-producing isolates are prevalent in raw chicken meat is unknown. We are now starting a survey of the distribution of SEB of *S. aureus* in humans, poultry, or the environment in poultry slaughterhouses and meat-processing plants.

In conclusion, the present study indicates that retail raw chicken meat in Japan is frequently contaminated with *S. aureus*, and is primarily contaminated by strains belonging to the poultry and human biotypes. Interestingly, the enterotoxigenic *S. aureus* isolates from raw chicken meat frequently produce SEB. To our knowledge, this paper is the first to report on the incidence and characterization of *S. aureus* and enterotoxigenic *S. aureus* in retail raw chicken meat throughout Japan.

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