Isolation of *Staphylococcus aureus* from Raw Fish in Relation to Culture Methods

Etsuko SAITO¹, Nanako YOSHIDA², Junichi KAWANO³*, Akira SHIMIZU² and Shizunobu IGIMI³

¹Department of Bioresource and Agrobiosciences, Graduate School of Science and Technology and ²Department of Microbiology and Immunology, Faculty of Agriculture, Kobe University, 1–1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657–8501 and ³Division of Biomedical Food Research, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan

(Received 10 March 2010/Accepted 28 September 2010/Published online in J-STAGE 12 October 2010)

**ABSTRACT.** Five hundred and fifty fish samples from various stages in the course of distribution in Hyogo Prefecture (209 retailed in super markets, 173 obtained from fishery cooperatives at a harbor, 91 caught by trawling and 77 caught by rod fishing) were examined for contamination with *Staphylococcus aureus* (*S. aureus*). *S. aureus* was detected in 41 (19.6%) of the retail fish samples and 46 (26.6%) of the samples from the fishery cooperatives. No *S. aureus* was isolated from the live fish (91 trawled and 77 fished by rod). With regard to the retail fish, the contamination rate of processed fish (26.0%) was significantly higher than that of unprocessed fish (14.2%). For 88 samples, the efficacy of the selective medium was compared using Baird-Parker agar and mannitol salt agar supplemented with egg yolk (MSEY agar) by the direct plate and enrichment culture methods. Using the direct culture method, the *S. aureus* positive rate with the Baird-Parker agar (30.7%) was significantly higher (P<0.01) than that with the MSEY agar (6.8%). The enrichment culture method remarkably raised the *S. aureus* detection rate. Seventy-eight (85.7%) of 91 isolates belonged to the human ecovar. Sixty-two (68.1%) of the 91 isolates had some enterotoxin genes, including 44 (48.4%) with the *sea* gene. These data showed that the fish were contaminated with *S. aureus* after landing and that Baird-Parker agar had an advantage in detecting *S. aureus* with a direct plate culture.

**KEY WORDS:** biotype, distribution, enterotoxin, raw fish, *Staphylococcus aureus.*

---

Staphylococcal food poisoning is caused by staphylococcal enterotoxins (SEs), which are mostly produced by *S. aureus*, and is an important intradietetic intoxication in the world. The number of staphylococcal food poisoning incidences in Japan has decreased in accordance with improvement of equipment for food production, distribution systems and consciousness regarding food sanitation. Fifty to 100 cases of *S. aureus* contamination of retail fish [30, 33, 38] and no reports on live fish. It is necessary to investigate the route of *S. aureus* contamination to prevent staphylococcal food poisoning due to consumption of fish.

In Japan, there is no standard operating procedure for detecting *S. aureus* from foods, and the procedure varies depending on the inspection agency. According to the food sanitation inspection guidelines [39], most agencies have adopted the direct culture method using mannitol salt agar supplemented with 3% egg yolk emulsion (MSEY agar). In contrast, the enriched culture method using Baird-Parker agar is widely used in other countries and is recommended by the International Organization for Standardization (ISO) in Switzerland [13] and Bacteriological Analytical Manual (BAM) [2] of the Food and Drug Administration (FDA) in the United States. In the modern era of expanding globalization of food distribution, the method of detecting *S. aureus* in Japan needs to conform to international standards.

In this report, we examined *S. aureus* contamination of fish at various stages of commercial distribution in Hyogo Prefecture, including fish retailed in supermarkets, fish obtained from fishery cooperatives at a fishing harbor, i.e., wholesale fish markets, and fish caught by trawling and rod fishing, and the characteristics of *S. aureus* isolates by phenotypic and genotypic tests. In addition, we evaluated the efficacy of MSEY and Baird-Parker agar in detecting *S. aureus* with fish samples.

**MATERIALS AND METHODS**

**Samples:** From July 2005 to September 2007, a total of 550 samples from various species of fish were examined,
including 209 samples (51 prawns, 40 squids, 30 horse mackerels, 28 sauries, 24 sardines, 19 mackerels, 6 yellowtails, 4 sea breams, 2 Spanish mackerels, 1 tuna, 1 salmon, 1 trevally, 1 sea bass and 1 oyster) obtained from 40 retail shops, 173 samples (17 horse mackerels, 16 gobies, 16 sea breams, 13 sand borers, 9 soles, 9 globefishes, 9 lizardfishes, 7 squids, 6 lefteye flounders, 5 white croakers, 5 black rockfishes, 4 octopuses, 4 hairtails, 4 congers, 4 shrimps, 3 herrings, 3 black scrapers, 2 righteye flounders, 2 rays, 2 barracudas, 2 threadtail flatfishes, 2 stone fishes, 2 bartic flatfishes, 2 halfbeaks, 2 blend banded soles, 2 sea basses, 2 surfperches, 2 rivulatuses, 2 conger pikes, 2 gurnards, 1 greenling, 1 angler, 1 frogfish, 1 sardine, 1 rockfish, 1 bonito, 1 crab, 1 largescale blackfish, 1 mackerel, 1 shark, 1 sea cucumber, 1 wart perch, and 1 striped mullet) from 3 fish markets, 91 samples (9 horse mackerels, 8 shrimps, 7 congers, 7 soles, 6 octopuses, 5 gobies, 5 wart perches, 5 squids, 3 sea breams, 3 sharks, 3 white croakers, 3 rays, 3 sand borers, 2 gurnards, 2 globe fishes, 2 squillas, 2 lefteye flounders, 2 sardines, 2 goat fishes, 2 barracudas, 2 conger pikes, 2 bartic flatheads, 1 hairtail, 1 Japanese ice fish, 1 black crocker, 1 stone fish, 1 largescale fish, and 1 angler) caught by trawl fishing and 77 samples (44 horse mackerel, 14 mackerels, 6 sea breams, 5 sardines, 3 pearl-spot charises, 2 filefishes, 1 rock fishes, 1 wrasse and 1 largescale blackfish) caught by rod fishing. Samples were purchased at retail shops in Hyogo Prefecture, obtained at fish markets in Hyogo Prefecture and caught by trawling or rod fishing in Hyogo Prefecture. Samples purchased at the retail shops included 96 processed fish, such as fillets and bony parts, and 113 unprocessed fish.

**Isolation of S. aureus:** Samples were taken by swabbing the surfaces of fish with culture swabs (EZ II, Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.), which are paired sterile cotton swabs. All samples were subjected to the enrichment culture method. Namely, the swabs were inoculated in trypticease soy broth (TSB, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1% sodium pyruvate and 0.5% dipotassium phosphate and 6.5% NaCl and incubated at 30°C for 24 hr. A drop of these broth cultures was plated onto mannitol salt agar (Nissui) supplemented with 3.0% egg-yolk emulsion (MSEY agar), smeared with a spreader and incubated at 30°C for 48 hr.

For 88 samples obtained from fish markets from January to March in 2006, the efficacy of the selective media was compared with Baird-Parker agar (Oxoid Ltd., Cambridge, England) supplemented with 1.5% egg-yolk emulsion and 0.01% potassium tellurite as directed by the manufacturer and MSEY agar by the direct plate culture and enrichment culture methods. The samples were collected with EZ II culture swabs; one swab was streaked on MSEY and Baird-Parker agar. Another swab was inoculated onto the modified TSB mentioned above. A drop of these broth cultures was plated onto MSEY and Baird-Parker agar and incubated at 30°C for 48 hr. Typical colonies were black or gray in color surrounded by a clear zone, whereas they were yellow and egg-yolk positive in MSEY agar.

Suspect colonies, which revealed acidification of mannitol and positive results for the egg-yolk reaction, were subjected to identification procedures. Colonies with coagulase-positive, Gram-positive cocci and that were catalase-positive were selected as possibly being S. aureus and identified by PCR for the Sa442 gene specific for S. aureus using the primer set SAU-1 and SAU-2 [11]. The following PCR program was used: denaturing at 94°C for 45 sec, annealing at 61°C for 1 min and extension at 72°C for 2 min for 30 cycles to completion; the final cycle consisted of extension at 72°C for 5 min.

**Biotyping:** S. aureus isolates were subjected to biotyping by the method of Devries [7] using the following 4 characteristics: production of staphylokinase, production of β-hemolysin, coagulation of bovine plasma and the type of growth on crystal violet agar.

**Detection of staphylococcal enterotoxin genes:** The multiplex PCR technique was used to detect enterotoxin genes (sea, seb, sec, see, seg, seh and sei) with Takara Taq (Takara Bio, Inc., Otsu, Shiga, Japan) using primer sets designed by Becker et al. [3] and Omoe et al. [36]. The following PCR program was used in this study: denaturing at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min for 30 cycles to completion; the final cycle consisted of extension at 72°C for 5 min.

As positive controls, the following 5 strains were used: strain 243 (seb), strain FRI-326 (see), strain FRI-361 (sec, seg and sei), strain No.18 (sea) and strain No. 5 (sed and seh). Strains No. 18 and No. 5 were isolated from chicken meat [29].

Production of staphylococcal enterotoxins (SEA, SEB, SEC and SED) was confirmed by the reverse passive latex agglutination (RPLA) method using SET-RPLA (Denka Seiken Co., Ltd., Tokyo, Japan).

**Statistical analysis:** Fisher’s exact test was used to determine the significance of differences between the S. aureus isolation rates in fish species and S. aureus isolation rates with the 2 selective mediums.

**RESULTS**

**Isolation of S. aureus:** S. aureus was detected in a total of 87 (15.8%) of 550 samples examined in this study (Table 1). According to the sample sources, 41 (19.6%) of the 209 retailed fish samples and 46 (26.6%) of the 173 samples obtained in fish markets were positive for S. aureus. None of the 91 trawled or 77 rod-caught fish were positive for S. aureus. Of the 209 retailed fishes, 25 (26.0 %) of the 96 processed fish were positive for S. aureus, while the rate of isolation was significantly lower (P<0.05) for unprocessed fish (14.2%, 16/113). In terms of species, retail prawns were highly contaminated (39.2%, 20/51), followed by yellowtails (33.3%, 2/6) and mackerel (31.6%, 6/19). Of note, samples obtained either by trawling or rod fishing were free of S. aureus contamination.

In the retailed fish, 2 S. aureus colonies with different characteristics were isolated from 4 samples, and a total of
91 isolates were subjected to the following tests.

**Efficacy of the selective medium:** By the direct plate culture isolation method for 88 samples from fish markets, *S. aureus* was detected in 6 (6.8%) and 27 (30.7%) samples using MSEY and Baird-Parker agar, respectively (Table 2), and the difference was significant (P<0.01). By the enrichment culture method, *S. aureus* was detected in 36 (40.9%) samples using MSEY agar and 36 (40.9%) samples using Baird-Parker agar, with no significant difference (P=1.0).

With both MSEY and Baird-Parker agar, the enrichment culture method remarkably increased the detection rate.

**Biotyping:** A total of 91 isolates from 87 *S. aureus*-positive samples were subjected to biotyping. Of these, 78 (85.7%) belonged to the K+β-CV: A biotype (human ecovar), 7 (7.7%) belonged to the K-β-CV: A biotype (bovine ecovar), and 6 (6.6%) belonged to the K-β+C: A biotype (poultry ecovar; Table 3). The human ecovar was the biotype most frequently detected among the isolates from retail fish samples.
fish and those obtained from fish markets.

Detection of staphylococcal enterotoxin genes: Ninety-one isolates were examined for enterotoxigenicity. Of these, 62 isolates (68.1%) had some enterotoxin genes (Table 3). Forty-four isolates (48.4%) harbored the sea gene, followed by seg + sei (5 isolates, 5.5%), seb + seg + sei (4, 4.4%), seb (3, 3.3%), sec (2, 2.2%), sec + seh (2, 2.2%), seh (1, 1.1%) and sea + seg + sei (1, 1.1%) genes. In total, 10 isolates harbored seg + sei with or without other genes. Isolates from the retail fish showed various type of enterotoxin, while most isolates from wholesale fish markets possessed the sea gene. Production of SEA, SEB and SEC from isolates harboring the sea, seb and sec genes, respectively, was confirmed by the RPLA analysis.

DISCUSSION

This paper is the first report on *S. aureus* contamination during commercial distribution of fish. No *S. aureus* was detected in live fish, but contamination in fish from fish markets was evident from the beginning of the distribution process. The contamination rate increased during the course of distribution, as shown by the level of contamination in fish available for retail, particularly when the fish were processed. Given the fact that the human ecovar was the most prevalent biotype, the fish appear to have been contaminated with *S. aureus* from humans engaged in processing operations.

The isolation rate of *S. aureus* in the retail fish samples in the present study (19.6%, 41/209) is relatively low compared with previous reports, which had rates ranging from 29.3%–61.7% [30, 33, 38]. Hygienic practices in fish-processing facilities have recently improved, with some facilities adopting measures such as the use of ozone water and ultraviolet sterilizers. The procedures above may have resulted in the lower incidence of *S. aureus* in the present study. The contamination rate of processed fish (26.6%) was significantly higher than that of unprocessed fish (14.3%). In terms of fish species, the highest rate of contamination was 39.2% and was found in prawns (20/51). The prawns examined in this study were all imported and processed using a method similar to that usually performed in Japanese markets. This might be the reason for the high *S. aureus* prevalence in prawns. The rate of isolation in unprocessed Pacific saury (25.0%, 7/28) was significantly higher (P < 0.01) than that in squid (3.1%, 1/32) and horse mackerel (0%, 0/24). In addition to the fact that *S. aureus* was not detected in live fish (0%, 0/168), it appears that *S. aureus* contamination in fish occurs during handling and processing through contact with humans or processing equipment contaminated with *S. aureus*. The differences in the contamination rates between the unprocessed fish may be attributed to sanitary conditions during their transportation.

We obtained fish samples from 3 fish markets, and the *S. aureus*-positive rate differed for each market. This may be because of differences in hygiene control at the individual fish markets. Samples obtained at fish market A in January 2006 were all *S. aureus*-positive. All 39 isolates belonged to the same ecovar (human) and had the same enterotoxin gene (sea), indicating that they were derived from the same contamination source. This suggested the existence of highly contaminated source of *S. aureus* in the market at that time.

The simplified Devriese biotyping system for *S. aureus* has been useful for tracing the origin of this organism in animal food and the food industry [7, 8]. The predominant bio-type determined in this study was the human biotype (85.7%), which characteristically originates from humans. The fact that no live fish were *S. aureus*-positive in this study indicates that the fish were contaminated with *S. aureus* by humans after landing and during the course of distribution.

In the present study, 68.1% of the isolates possessed enterotoxin genes (sea–sei), and sea was the most frequently found gene. Japanese staphylococcal food poisoning is mainly caused by SEA [12, 34]: therefore, it is notable that fish were contaminated with SEA producers.

*S. aureus* possessing seg and sei genes is frequently found in healthy humans and foods [5, 6, 31, 35, 37]. However, there are incidences of food poisoning that might have been caused by *S. aureus* possessing both the seg and sei genes [4, 5, 28, 36]. On the other hand, it has been reported that when SEG and SEI are coexpressed, they interact differently with MHC class II and stimulate completely different subsets of human and mouse T cells, indicating complementary superantigenic activities [9]. Although such a type of *S. aureus* may be derived from healthy humans, its growth and production of SEG and SEI in foods could lead to intoxication. From a public health perspective, it is notable that fish isolates had the seg and sei genes.

In Japan, microorganism tests, including *S. aureus* detection in food, are conducted according to the food sanitation inspection guidelines [39]. These guidelines have been revised several times without sufficient examination of their effects, so they have been reviewed by the exploratory committee of standard methods of microorganism tests of food. The committee indicated that Baird-Parker agar, which is widely used in many countries outside Japan to detect *S. aureus*, and MSEY agar were equally effective in detecting *S. aureus* [40]. Using a direct plate culture method, the *S. aureus*-positive rate with Baird-Parker agar (30.7%) was found to be higher than that with MSEY agar (6.8%), indicating the advantage of Baird-Parker agar when isolating *S. aureus* from fish. This may reflect the efficacy of the sodium pyruvate present in Baird-Parker agar for isolating viable but nonculturable (VBNC) *S. aureus* [13]. It has been suggested that fish are often contaminated with *S. aureus* in this state. Sodium pyruvate has been reported to recover VBNC cells with damaged metabolic pathways [1]. Introducing an enrichment culture method remarkably increased the *S. aureus* detection rate in agar (Table 2), which is consistent with previous reports [32, 33, 38]. In this context, there was no difference in the isolation rate between MSEY (40.9%) and Baird-Parker (40.9%) agar. This suggests that...
the enrichment culture method using enrichment broth containing sodium pyruvate is useful for the detection of *S. aureus* in fish, regardless of which selective media is used.

**REFERENCES**


