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Research Note

Enterotoxin Production by *Staphylococcus aureus* Isolated from Mastitic Cows

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ABSTRACT

Staphylococcus aureus is an important cause of mastitis in cows. The ability of *S. aureus* strains to produce one or more enterotoxins in milk and dairy products is linked to staphylococcal food poisoning. To determine whether staphylococci causing bovine mastitis could cause human foodborne intoxication, the production of staphylococcal enterotoxins A through D (SEA, SEB, SEC, and SED) by 160 *S. aureus* isolates was evaluated with the use of a reverse passive latex agglutination enterotoxin kit. All *S. aureus* strains were isolated over a 9-month period from 2,343 routine submissions of a composite quarter collection of individual mastitic cows at 18 dairy farms in the San Joaquin Valley in California. Prior to enterotoxin detection, isolates were grown by a method that enhances the in vitro synthesis of enterotoxin. Twenty-two of 160 *S. aureus* isolates produced enterotoxin. Seven produced SEC, 12 produced SED, and 3 produced both SEC and SED. None of the isolates produced SEA or SEB.

Staphylococcus aureus is an important bacterial cause of mastitis in cows and is also a cause of foodborne disease in humans. Staphylococcal food poisoning is one of the most economically important foodborne diseases in the United States. It has been estimated that losses in medical expenses and productivity due to *S. aureus* amount to ca. $1,500,000,000 per year *(31)*. The disease is characterized by vomiting and diarrhea within 2 to 6 h after the consumption of contaminated food. The toxic effect of *S. aureus* is related to the action of staphylococcal enterotoxins (SEs) on specific emetic receptors located in the intestinal wall *(19)*. Staphylococcal enterotoxins are thermoresistant, and as little as 94 ng of SEA (in chocolate milk) has been shown to be able to cause illness in susceptible schoolchildren *(9)*.

Outbreaks of *S. aureus* food poisoning have been caused by the consumption of dairy products, including raw milk *(6)*, ultrahigh-temperature (UHT) milk *(10)*, dried skim milk *(8)*, and cheeses *(26)*. Nine major antigenic types of SEs have been characterized: SEA, SEB, SEC (including SEC1, SEC2, and SEC3), SED, SEE, SEG, SEH, SEI, and SEJ *(23, 27)*. The majority of enterotoxigenic isolates have a human origin, and strains producing SEA are implicated in the majority of cases of staphylococcal food poisoning *(18, 33)*. In an attempt to explore the potential of bovine-mastitis-causing staphylococci to cause human intoxication, we tested a collection of *S. aureus* strains isolated from bovine milk for enterotoxin production. *S. aureus* isolates came from routine submissions of a composite quarter collection (a single sample of milk from the four quarters of a cow's udder) of individual mastitic cows at 18 dairy farms in the San Joaquin Valley in California.

MATERIALS AND METHODS

Sampling. Enterotoxin production was determined for 160 isolates of *S. aureus* from the collection of the Milk Quality Laboratory, Veterinary Medicine Teaching and Research Center, University of California, Davis, Calif. The 160 isolates originated from 146 cows that belonged to 18 dairy farms. These isolates were collected over a period of 8 months (April to November 1999) from 2,343 routine submissions of composite quarter collection of individual mastitic cows.

***Staphylococcus* culture and identification.** The culture and identification of the *S. aureus* isolates used in this study was carried out according to the methods described by Bennet *(3)*. Identifications were confirmed by complementary biochemical tests with API 20 STAPH (BioMéhrieux, Marcy-l'Etoile, France), and APILAB Plus software (Version 3.3.3, BioMéhrieux) was used to analyze the results. The first isolation medium was tryptose blood agar containing washed bovine red blood cells (Biological Media Service, School of Veterinary Medicine, University of California, Davis); 1 ml of milk was spread on this medium and incubated at 37°C for 48 h. Creamy grayish white or golden yellow colonies 3 to 5 mm in diameter with distinct zones of hemolysis were

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TABLE 1. *Enterotoxin production by* Staphylococcus aureus *strains isolated at various dairy farms*

Farm no.	No. of samples	No. of isolates	No. of isolates producing enterotoxin					
			SEA	SEB	SEC	SED	SEC + SED	Total
1	531	34	0	0	0	7	1	8
2	373	31	0	0	0	0	0	0
3	256	27	0	0	2	2	0	4
4	231	17	0	0	1	0	0	1
5	428	15	0	0	2	0	1	3
6	108	8	0	0	0	0	0	0
7	69	6	0	0	0	0	0	0
8	63	5	0	0	0	1	0	1
9	51	4	0	0	0	2	0	2
10	54	3	0	0	0	0	0	0
11	24	2	0	0	0	0	0	0
12	35	2	0	0	0	0	0	0
13	14	1	0	0	1	0	0	1
14	33	1	0	0	0	0	1	1
15	14	1	0	0	1	0	0	1
16	16	1	0	0	0	0	0	0
17	29	1	0	0	0	0	0	0
18	14	1	0	0	0	0	0	0
Total	2,343	160	0	0	7	12	3	22

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considered presumptive *S. aureus* colonies. These colonies were selected and tested for cell morphology after Gram staining and for the production of catalase, coagulase, and thermonuclease (TNase). For the determination of coagulase production, lyophilized rabbit plasma with EDTA (BBL Microbiology Systems, Cockeysville, Md.) reconstituted with sterile water was used. Suspected *Staphylococcus* spp. colonies were inoculated into 5-ml test tubes containing 0.5 ml of the reconstituted plasma. Complete clotting after incubation at 37°C within 4 to 24 h was regarded as a positive result. TNase production was determined according to the method described by Ibrahim et al. *(13)*. After 18 h of incubation at 37°C in brain heart infusion (BHI) broth (Difco, BD Diagnostic Systems, Sparks, Md.), cultures were placed into a water bath at 100°C for 15 min to eliminate any nonspecific heat-labile nuclease activity. These cultures were then centrifuged at room temperature for 30 min at ca. 3,000 × *g* before they were tested. One hour before testing, 5-mm wells were prepared on plates containing TNase agar with toluidine blue (Remel, Lenexa, Kans.). Approximately 70 μl of supernatant was then transferred into each well, and the plates were incubated at 37°C for 4 h. The presence of a pink halo surrounding a well was regarded as a positive result.

All gram-positive, catalase-positive, coagulase-positive cocci that produced beta-hemolysis on blood agar and TNase were presumptively identified as *S. aureus* and were then tested with API 20 STAPH for confirmation. Selected colonies were subcultured in BHI broth with 15% glycerol (Difco) for 24 h at 37°C and then frozen at −70°C for storage.

Prior to enterotoxin detection, each of the 160 frozen culture test tubes was thawed at room temperature, and 1 ml of its contents was inoculated into a 10-ml tube of BHI broth and then incubated at 37°C for 24 h. The liquid cultures were subcultured onto tryptose blood agar and incubated at 37°C for 24 h to check for culture purity.

Enterotoxin production. All of the selected isolates were subjected to a procedure that enhances in vitro enterotoxin production *(3)*. Once their purity was confirmed, selected isolates were transferred to slants of BHI agar and incubated at 37°C for 24 h. One loopful of the growth from the slant was transferred to 5-ml tubes of sterile distilled saline solution to obtain a McFarland turbidity value of 1 (ca. 300,000,000 organisms per ml). Four drops of this aqueous culture suspension were spread over the entire surfaces of plates of BHI agar supplemented with phenol red (pH 5.4). The plates were incubated at 37°C for 24 h. When the growth required to induce enterotoxin production had occurred, as indicated by a change in the medium's color from yellow to red-violet (pH 8.2), the plate's content was transferred to 50-ml centrifuge tubes and centrifuged at 32,000 × *g* for 10 min at 5°C to separate the agar and the organisms from the supernatant.

Enterotoxin detection. The supernatant of each of the 160 colonies was tested for SEA, SEB, SEC, and SED with the Staphylococcal Enterotoxin Test Kit (Oxoid, Basingstoke, UK). According to the manufacturers, the sensitivity of this test in the detection of enterotoxins is 0.5 ng/ml. For food extracts, the test's sensitivity can reach 1 ng/g of food matrix. The assay was performed with V-well microtiter plates and with a culture supernatant–to–reagent dilution ratio of 1:2. The plates were stored at room temperature for 24 h to allow latex particles to settle. A diffuse layer at the bottom of the V-well was regarded as a positive result; a tight button of latex particles was regarded as a negative result.

RESULTS

All selected cultures were gram-positive, catalase-positive cocci, produced beta-hemolysis on blood agar, produced heat-stable nuclease, were coagulase positive, and were identified as *S. aureus* by the API 20 STAPH biochemical tests. Twenty-two of 160 isolates (13.8%) produced SEC or SED or both (Table 1). All 22 positive isolates came from different cows. Staphylococcal enterotoxin C was produced by 4.4% (7 of 160) of the isolates, SED was produced by 7.5% (12 of 160), and both SEC and SED were produced by 1.9% (3 of 160). None of the isolates tested produced SEA or SEB. Isolates testing positive for

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SEC came from five farms, isolates testing positive for SED came from four farms, and isolates testing positive for both SEC and SED came from three farms. For 10 of 18 farms (55.5%), at least one sample tested positive for SEC, SED, or both.

DISCUSSION

This study demonstrates that a significant number of *S. aureus* isolates causing bovine mastitis are also able to produce enterotoxins. Our findings are consistent with those of an earlier study *(24)* in which 15% of 157 *S. aureus* isolates from mastitic cows were found to be enterotoxigenic and no isolates produced SEA or SEB. Other investigators have reported variable rates of enterotoxigenicity for mastitic isolates of *S. aureus.* Aarestrup et al. *(1)* detected no enterotoxigenic isolates of *S. aureus* among 160 isolates from bovine mastitis milk. Kenny et al. *(15)* found that 28.6% (72 of 262) of bovine mammary isolates of *S. aureus* produced one or more toxins; one of them produced SEA and seven produced SEB. Adesiyun et al. *(2)* found 53.6% of 134 isolates of *S. aureus* from bovine mastitic milk to be enterotoxigenic, and 23.9% of these isolates produced SEA either alone or in combination with other SEs. Cardoso et al. *(5)* found 42.5% of the isolates they tested to be enterotoxigenic, and Matsunaga et al. *(21)* detected enterotoxin production in 34.5% of 58 *S. aureus* isolates tested. Stephan et al. *(30)* tested 63 isolates obtained from bovine mastitic milk and found that 34 (54%) of these isolates produced enterotoxins. Other earlier studies *(28)* have reported enterotoxigenicity rates ranging from 0 to 56.5% for dairy products and from 16 to 86% for various other raw and cooked foodstuffs.

Different explanations for the different rates of enterotoxigenicity found in different studies have been suggested. These differences may be a reflection of the diversity of isolates and sources of *S. aureus (1, 15).* The source of mastitis isolates (man, animals, pests, insects) may determine a different clinical presentation of mastitis. The combination isolates–clinical presentation (acute, peracute, or chronic) may determine the role of enterotoxigenicity *(21).* The rate of enterotoxigenic *S. aureus* isolates has also been demonstrated to change according to the biovars involved *(7, 14, 20).* The method used *(4, 16)* to detect enterotoxin-producing isolates and differences in ecological reservoirs in various countries *(22)* could have influenced findings regarding enterotoxin production by *S. aureus* of mastitic origin. All enterotoxigenic isolates produced SEC or SED in this study, with the most predominant staphylococcal enterotoxin being SED. Many investigators have reported that *S. aureus* isolates obtained from dairy products of bovine or ovine origin produce high levels of SEC and SED *(7, 15, 17, 25, 32).* However, SEA is the predominant enterotoxin detected in isolates from foods involved in staphylococcal food poisoning *(11, 33).* In this study, none of the enterotoxigenic isolates produced SEA or SEB. It is very unlikely that some of the isolates tested produced undetectable levels of SE, since a sensitive method for the detection of SE detection was used in this study. In fact, the sensitivity of the reverse passive latex agglutination kit has been demonstrated to be equivalent to that of commercially available kits such as the enzyme-linked immunosorbent assay or the polymerase chain reaction technique in the detection of the staphylococcal enterotoxin or its gene in certain foods *(17, 29).*

Although standard milk refrigeration and pasteurization practices are currently widely adopted, the potential for the production of staphylococcal enterotoxin when raw milk containing enterotoxigenic strains is mishandled is obvious, since subsequent heat treatment has no effect on the preformed enterotoxin *(19).* Enterotoxins have in fact been detected in UHT milk *(10)* and in dried skim milk *(8),* and >11,000 people in western Japan were recently poisoned by low-fat milk contaminated by staphylococcal enterotoxin *(12).* Although only SEC and SED were detected in this study, the rather high percentage of *S. aureus* isolates that were enterotoxigenic indicates that veterinary clinicians should make a greater effort to prevent mastitis in the interest of enhancing the control of potential foodborne staphylococcal intoxication.

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