Enterotoxigenicity of \textit{Staphylococcus aureus} Cultures Isolated from Acute Cases of Bovine Mastitis

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To determine whether staphylococci causing bovine mastitis are potential causes of human intoxications, 142 cultures identified as etiological agents of acute cases and 18 cultures causing chronic cases of staphylococcal mastitis were obtained from investigators in the United States and Canada, examined microscopically, and tested for carbohydrate utilization, terminal pH, catalase, coagulase, egg yolk hydrolysis, gelatin hydrolysis, cytochrome oxidase, urease production, nitrate reduction, micrococal nuclease, phage type, and enterotoxin production. Three cultures were not confirmed as \textit{Staphylococcus aureus}. Of the 157 \textit{S. aureus} cultures, 23 produced staphylococcal enterotoxins. Although a direct relationship between staphylococcal mastitis and outbreaks of staphylococcal food poisoning was not proved, results indicated that staphylococcal infections of the bovine mammary gland represent a significant reservoir of enterotoxigenic strains of \textit{S. aureus}.

\textit{Staphylococcus aureus} is frequently present in milk and manufactured dairy products. Such products have been involved in outbreaks of staphylococcal food poisoning (1, 14, 15, 20). Since it has been established that \textit{S. aureus} is one of the principal etiological agents of bovine mastitis (8), it might be assumed that some relationship exists between staphylococcal mastitis and food poisoning outbreaks attributed to manufactured dairy products. However, a direct relationship between these two conditions has not been established.

In an attempt to explore the relationship between staphylococci causing bovine mastitis and the potential for human intoxications, \textit{S. aureus} cultures from validated cases of acute staphylococcal mastitis were solicited from investigators in the United States and Canada. One hundred and forty-two cultures identified as the etiological agents of acute cases and 18 cultures causing chronic cases of staphylococcal mastitis were received.

\textbf{MATERIALS AND METHODS}

Cultures were subjected to the following morphological, cultural, and serological examinations to verify their classification and to determine their enterotoxigenicity and phage type.

\textbf{Microscopic examination.} A simple stain with 1\% aqueous solution of crystal violet was used.

\textbf{Carbohydrate utilization.} Utilization of glucose and mannitol was tested by the method of Mossel (16), except the tests were terminated after incubation for 5 days at 35 C.

\textbf{Terminal pH.} The test was performed in 2\% glucose broth (4); pH was determined electrometrically after incubation for 7 days at 35 C.

\textbf{Catalase.} Growth from Trypticase soy agar slant was emulsified in 30\% H\textsubscript{2}O\textsubscript{2}.

\textbf{Coagulase.} Brain Heart Infusion cultures (18- to 24-hr) were tested with rabbit plasma and rabbit plasma containing 0.1\% ethylenediaminetetraacetic acid by the method of Baer (3).

\textbf{Egg yolk hydrolysis.} Plates of tellurite polymyxin egg yolk agar (12) were examined after 48 hr at 35 C for zones of clearing or precipitation surrounding points of inoculum.

\textbf{Gelatin hydrolysis.} Gelatin hydrolysis was tested by plate method (17) after 48 hr at 35 C.

\textbf{Cytochrome oxidase.} The Steel modification (18) of Kovac's method was used to test for cytochrome oxidase.

\textbf{Urease production.} Slants of Christensen's agar (11) were incubated as long as 7 days at 35 C.

\textbf{Nitrate reduction.} Daily tests from indole-nitrite medium (Difco, prepared according to instructions of manufacturer), incubated for as long as 7 days at 35 C, were made with reagents specified in Manual of Microbiological Methods (17).

\textbf{Micrococal nuclease.} Cell-free extracts of boiled, Brain Heart Infusion cultures were assayed by the method of Chesbro and Auburn (10), modified by changing the CaCl\textsubscript{2} concentration to 10 mM and adjusting the reaction mixture to pH 9.5 (14). Cul-
cultures were grown on a rotary shaker 18 to 24 hr at 37 C, and each extract was assayed in triplicate.

**Staphylococcus typing.** Cultures were typed with a set of 22 basic phages recommended by the Subcommittee on Phage Typing of Staphylococcus, Nomenclature Committee, International Association of Microbiological Societies (7), by the method of Blair and Carr (6).

**Enterotoxin testing.** Culture filtrates were tested for identifiable S. aureus enterotoxins by the microslide gel diffusion method described by Casman and Bennett (9).

**RESULTS AND DISCUSSION**

Eighteen cultures from chronic mastitis infections and 142 cultures from acute infections were received and tested. Of the 160 cultures, 3 could not be confirmed as S. aureus. Two were identified as Sarcina sp. and one as S. epidermidis.

The 157 S. aureus cultures were subdivided into eight types on the basis of their biochemical features. All cultures were catalase-positive, cytochrome oxidase-negative, coagulase-positive, utilized glucose anaerobically, reduced K2TeO4, and produced heat-stable nuclease. In addition, most cultures liquefied gelatin, utilized mannitol anaerobically, hydrolyzed egg yolk, produced urease, and reduced nitrate. All cultures attained a terminal pH in 2% glucose broth in the range pH 4.0 to 4.6; for most, the pH was 4.5. The features in which variants differed from typical S. aureus (Bergey's Manual, 7th ed.) and the frequency of strain occurrence in acute and chronic infections are shown in Table 1.

From information received from those who submitted cultures, it was determined that 86 of the 157 cultures originated from 72 different cows; these cows were distributed among 36 herds. Similar information about the remaining cultures was not provided. Within this group of 86 cultures, multiple cultures were received from 22 of the 36 herds and from 12 of the 72 cows. Phage typing revealed that in 8 of the 22 herds and in 9 of the 12 cows from which multiple cultures were received infection was due to a single phage type; more than one phage type was found among the multiple cultures received from the other 14 herds and 3 cows.

Susceptibility of the 157 S. aureus cultures to phages of the various lytic groups is shown in Table 2. Typable strains were distributed among

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**Table 1. Biochemical types of Staphylococcus aureus causing bovine mastitis, differential features of variants, and the frequency of their occurrence among acute and chronic cases**

| S. aureus type | Features differing from typical S. aureus | No. of cultures causing |
|---------------|------------------------------------------|------------------------|
|               |                                          | Acute infection | Chronic infection |
| Prototype     |                                          | 107            | 14 |
| Variant 1     | Gelatinase —                             | 6              | 0  |
| Variant 2     | Mannitol — b                             | 1              | 0  |
| Variant 3     | Egg yolk —                               | 2              | 3  |
| Variant 4     | Nitrate —                                | 3              | 0  |
| Variant 5     | Mannitol —                               | 16             | 0   |
| Variant 6     | Mannitol 0 b                             | 4              | 0  |
| Variant 7     | Urease —                                 | 0              | 1   |

*As described in Bergey's Manual, 7th ed.

b Mannitol — = not utilized; O = oxidative utilization.

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**Table 2. Staphylococcal aureus cultures and frequency of their occurrence in acute and chronic bovine mastitis**

| Phage lytic group | No. of cultures causing |
|-------------------|------------------------|
|                   | Acute infection | Chronic infection |
| I                 | 29            | 0 |
| III               | 36            | 5 |
| IV                | 21            | 2 |
| I–II              | 1             | 0 |
| I–II–III–M        | 2             | 0 |
| I–III             | 11            | 0 |
| I–III–IV          | 1             | 0 |
| I–III–IV–M        | 7             | 1 |
| III–IV            | 9             | 0 |
| III–M             | 10            | 0 |
| Nontypable        | 7             | 3 |

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**Table 3. Staphylococcus aureus isolated from cases of bovine mastitis**

| Phage group | Phage type | No. of cultures producing enterotoxins | No. of similar types but not enterotoxigenic |
|-------------|------------|----------------------------------------|--------------------------------------------|
|             |            | C | D | C and D |
| I           | 29/52/52A/80 | 4 | 2 | 0 | 21 |
| I           | 52A/80      | 1 | 0 | 0 | 1  |
| I           | 80          | 4 | 0 | 0 | 5  |
| I–III       | 29/42E/77   | 0 | 1 | 0 | 3  |
| I–III       | 52/52A/80/53| 1 | 0 | 0 | 1  |
| III         | 6           | 0 | 4* | 1 | 35 |
| III–M       | 6/7/47/81   | 0 | 3* | 0 | 6  |
| Nontypable  |             | 1 | 1* | 0 | 13 |

*Phages reported in this study were from the Subcommittee on Phage Typing of Staphylococcus, Nomenclature Committee, International Association of Microbiological Societies (7).  

S. aureus cultures were tested for enterotoxigenic potential by the bioassay method described by Casman and Bennett (9). Culture filtrates from 157 S. aureus were tested for enterotoxins. The results are shown in Table 3. The enterotoxins identified were enterotoxin A, B, C1, C2, and C3. For S. aureus produced enterotoxins were identified by the bioassay method described by Casman and Bennett (9).
46 separate phage types. Ten cultures were non-typable at a maximum phage concentration of 1,000 times the routine test dilution, with the set of phages used.

Although the majority of cultures from chronic infections were most susceptible to phages of lytic group III, the sum of features tested suggested that there was no consistent physiological difference between cultures causing acute and those causing chronic infections.

Of the 157 cultures, 23 produced staphylococcal enterotoxins. Eleven produced type C, eleven produced type D, and one produced both types C and D enterotoxins; none produced A or B. Phage types of strains producing enterotoxin are shown in Table 3. Since types of S. aureus in lytic group III are said to be most frequently implicated in food poisoning outbreaks (2, 5, 19), it is interesting to note the high frequency of types in lytic group I among the enterotoxigenic cultures. Enterotoxigenic cultures were contributed by 14 of the 36 identified herds and 22 of the 72 identified cows. It is perhaps noteworthy that in only one case did all cultures of the same phage type from a herd produce enterotoxin.

In three instances, one culture from a herd produced enterotoxin and a similar phage type culture from the same herd did not. In two cases, different phage type cultures from the same herd produced the same type enterotoxin.

Nearly 15% of 157 S. aureus cultures identified as etiological agents of bovine mastitis were shown to produce enterotoxins. The hypothesis of a direct relationship between staphylococcal mastitis and outbreaks of staphylococcal food poisoning remains unproven, but it has been shown that a significant number of S. aureus strains causing bovine mastitis were able to produce enterotoxins. The potential for production of staphylococcal enterotoxins is obvious in those cases in which raw milk containing enterotoxigenic types is poorly held or receives sublethal heat treatment and processing conditions or manner of use of contaminated products allow subsequent growth. The continued high incidence of staphylococcal mastitis and the rather high percentage of enterotoxigenic cultures isolated from validated mastitis cases indicate that greater efforts should be directed toward the prevention of mastitis in the interest of, among other reasons, enhancing the control of foodborne Staphylococcus intoxication. The abnorm-