Relationships Among the Results of Coagulase, Staphylococcal Toxin, and Thermonuclease Tests on Staphylococci from Cow Milk

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Production of staphylococcal alpha- or alpha-beta-toxins correlated well with production of coagulase or thermonuclease (or both) in 203 *Staphylococcus aureus* isolates from milk and should be reliable indicators of *S. aureus* in the absence of *Staphylococcus intermedius*. Failures to produce toxin, tube coagulase, or thermonuclease occurred in only 1 to 2% of *S. aureus*. Evidence of beta- or alpha-beta-toxins was not found among 321 other staphylococci isolated from milk. A few coagulase- or thermonuclease-positive isolates not producing beta- or alpha-beta-toxins were found among the *Staphylococcus hyicus* isolates.

Positive tube coagulase tests have long been accepted as justification for classification of staphylococci of both human and animal origin as *Staphylococcus aureus*. This practice is no longer totally valid in view of recent more definitive methods for classifying staphylococci (11, 20) and the finding of other coagulase-positive species among animal isolates (3, 4, 15, 20, 21). Hajek (6) described a new species, *Staphylococcus intermedius*, which is coagulase positive and produces staphylococcal beta- and delta-toxins (100%) and alpha-toxins (22%). (Staphylococcal hemolysins are herein referred to as alpha, beta, etc., toxins to avoid confusion with the streptococcal hemolysis.)

Little is known of the distribution of *S. intermedius* in milk samples or of its possible association with mastitis. Five isolates were reported among 93 staphylococcal isolates from intramammary infections from dairy cows and 6 from 60 intramammary infections from beef cows (20). In another study, 525 isolates from intramammary infections of beef cows were classified as 141 *S. intermedius*, 151 *Staphylococcus simulans*, 85 *S. aureus*, and small numbers of 10 other staphylococcal species (21). In that study 133 of 241 (55.2%) coagulase-positive isolates were classified as *S. intermedius*, 83 (34.4%) were classified as *S. aureus*, and 15 of 151 (10.0%) were classified as *S. simulans*, a coagulase-negative organism reported to produce staphylococcal beta-toxin (21). Reliance upon coagulase or hemolytic characteristics (or both) to identify *S. aureus* was therefore considered to be unsatisfactory in that study because of the large number of *S. intermedius* isolates. Many of these latter isolates produced a weak maltose reaction, and reclassification as atypical *S. aureus* is being considered (personal communication). Langlois et al. (14) did not find any *S. intermedius* among 581 isolates including 10 staphylococcal species from milk of dairy cows.

Coagulase-positive staphylococci from cow milk usually produce beta-toxin, alone or in combination with alpha-, delta-, or epsilon-toxins, which become evident on properly prepared bovine erythrocyte agar (BEA) plates (2, 3, 5, 7-10, 15-18). Several have emphasized the reliability of beta-toxin production as an indicator of coagulase-positive staphylococci isolated from milk (5, 7-9, 18). Hajek and Marsalek (7) found that 100% of coagulase-positive strains produced beta-toxin, and DeVriese (3) more recently considered the presence of beta-toxin sufficient evidence to

identify *S. aureus*. It has been a common practice in applied bovine mastitis bacteriology to consider the production of beta-toxin as indirect evidence for coagulase production and therefore for identification of an organism as *S. aureus*.

If *S. intermedius* or coagulase-positive *Staphylococcus hyicus* appears with significant frequency in milk samples, or if other species are identified that produce coagulase or beta-toxin, common field laboratory diagnostic practices will need revision. This research was done to investigate the diagnostic significance of staphylococcal hemolytic toxin, tube coagulase, and thermostable nuclease production by staphylococci isolated from milk samples and identified by biochemical means now available (11, 21).

MATERIALS AND METHODS

The staphylococci for this study were obtained from milk samples taken for general surveys (composite samples) or for mastitis diagnosis (quarter samples) from 26 California dairy herds. Milk (0.01 ml) was streaked on BEA plates prepared with washed cow blood cells (2). Colonies of catalase-positive cocci were picked and transferred to a second BEA plate as a test for purity and then transferred to a Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slant and refrigerated at 4°C pending analysis. Revitalization was assured by making transfers on BEA before further testing was done.

Hemolytic toxin activity was determined visually, as recommended previously (2), by an experienced observer after incubation of BEA plates at 37°C for 24 h.

The standard tube coagulase test was done as recommended previously (2) with Difco rabbit plasma and was read at 4 and 24 h.

Thermonuclease tests were performed on both P agar (19) and Mueller-Hinton (M-H) agar plates by an agar overlay modification (1) of the method of Lachica et al. (13).

Final identity of staphylococcal isolates was made biochemically by the methods of Kloos and Schleifer (11) as modified to include species not found in humans (21). Anaerobic mannitol utilization testing was not done. Staphylococcal isolates having at least two of the following characteristics (beta- or alpha-beta-toxin, coagulase positive, thermonuclease positive), and which were maltose positive, were considered to be *S. aureus*. Other staphylococci were examined by the Kloos and Schleifer simplified scheme (11) as modified previously (21).

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RESULTS

By the criteria selected, 203 of the isolates were *S. aureus* (Table 1). The results of the tests for alpha- or alpha-beta-toxin, tube coagulase, and thermonuclease were all positive for 96.6% of the *S. aureus* isolates in this study. The tube coagulase and hemolytic toxin tests were each positive for 99% of the isolates tested. The thermonuclease tests were positive for 98% of the isolates, being identical on M-H and P agar plates.

The results of these tests were also very much as expected for the other staphylococci studied (Table 2). However, four isolates identified as *S. hyicus* had a narrow zone of clear hemolysis, as did one isolate identified as *Staphylococcus sciuri*. Tube coagulase and thermonuclease test results were negative for 242 *S. hyicus* isolates, but varied among 13 isolates that were positive to one or more of these tests. Beta- or alpha-beta-toxin production was not observed among any staphylococci other than *S. aureus*, although one isolate designated *S. simulans* was nuclease positive.

In one instance only, the thermonuclease test was positive on M-H agar and not on P agar.

DISCUSSION

There was a strong association of tube coagulase (99.5%), beta- or alpha-beta-toxin (99%), and thermonuclease (98%) with *S. aureus* isolates from milk. All three tests were positive 97% of the time. Toxin was not observed in only two isolates, and thermonuclease was not observed in only three isolates found to be *S. aureus* (Table 1). All of these isolates producing beta- or alpha-beta-toxin, tube coagulase, or thermonuclease were shown to be *S. aureus*. However (Table 2), some *S. hyicus* isolates are tube coagulase or thermonuclease positive (or both). These organisms do not produce beta- or alpha-beta-toxin and can be differentiated from *S. aureus* on that basis.

Although it seems unlikely that *S. intermedius* will be isolated in large numbers from cow milk, it may be necessary at times to distinguish it from *S. aureus*. At the moment it appears that a test for maltose fermentation would be sufficient for routine differentiation (12, 21).

Since identification of staphylococcal species from cow milk is still not well established on an extensive data base of its own, it is likely that misidentifications do occur. The thermonuclease-positive *S. simulans* isolate in this series may in fact be *S. hyicus*, although it is phosphatase negative, and the four weakly hemolytic *S. hyicus* isolates may in fact be *S. simulans*, although they are phosphatase positive. We did not find coagulase-negative *S. simulans* producing staphylococcal beta-toxin as was previously reported (21).

Observations for production of hemolytic staphylococcal alpha- or alpha-beta-toxin made on good quality BEA plates used for primary inoculation of bovine milk samples provide an easy and reliable presumptive diagnosis for *S. aureus* from milk in the absence of *S. intermedius*. It is important,

TABLE 1. Staphylococcal toxin, tube coagulase, and thermonuclease test results on *S. aureus* isolates from milk

No. of isolates	Alpha- or alpha-beta-toxin	Tube coagulase	Thermonuclease	
			P agar	M-H agar
197	+	+	+	+
2	—	+	+	+
1	+	—	+	+
3	+	+	—	—

TABLE 2. Staphylococcal toxin, tube coagulase, and thermonuclease tests results on 321 other staphylococci isolated from milk

Species	No. of isolates	Hemolytic toxin	Tube coagulase	Thermonuclease	
				P agar	M-H agar
<i>Staphylococcus capitis</i>	1	—	—	—	—
<i>Staphylococcus cohnii</i>	1	—	—	—	—
<i>Staphylococcus epidermidis</i>	7	—	—	—	—
<i>Staphylococcus hyicus</i>	238	—	—	—	—
	4	N ^a	—	—	—
	5	—	+	—	—
	6	—	—	+	+
	1	—	+	+	+
	1	—	—	—	+
<i>Staphylococcus sciuri</i>	4	—	—	—	—
	1	N	—	—	—
<i>Staphylococcus simulans</i>	5	—	—	—	—
	1	—	—	+	+
	6	N	—	—	—
<i>Staphylococcus xylosis</i>	14	—	—	—	—
	3	N	—	—	—
Unknown	17	—	—	—	—
	3	N	—	—	—
<i>Micrococcus</i> spp.	3	—	—	—	—

^a N, Narrow zone of weak hemolysis.

however, that properly prepared plates be used (2), and that good lighting is available for reading by a competent observer.

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