

Characterization of Staphylococci Isolated from Raw Milk

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ABSTRACT

ZEMELMAN, RAÚL (University of Concepcion, Concepcion, Chile), AND LUIS LONGERI. Characterization of staphylococci isolated from raw milk. *Appl. Microbiol.* **13**:167-170. 1965.—To evaluate the pathogenicity of staphylococci from bovine raw milk, the general characteristics of 775 strains isolated from 798 samples of milk were studied. The coagulase test was performed by use of rabbit plasma. Chromogenesis, mannitol fermentation, and gelatin liquefaction were investigated on Chapman's Medium 110, after 48 hr of incubation. Production of β -hemolysin, which has been considered indicative of pathogenic staphylococci of animal origin, was determined by streaking different strains on sheep blood-agar plates in the presence of a strain of Lancefield group B streptococci. Plates were incubated at 37 C for 24 hr, and strong hemolysis was produced in the zone of interaction of β -hemolysin and some substance liberated by streptococcus (CAMP test). Of 404 strains found to be coagulase-positive, 95.8% exhibited a deep-orange pigment, 76.5% produced β -hemolysin, 91.8% fermented mannitol, and 75% liquefied gelatin. Of 371 strains which gave a negative coagulase test, about 16% fermented mannitol and liquefied gelatin; none of these strains produced β -hemolysin. When results are grouped according to pigmentation and coagulase production, β -hemolysin seems to be developed by pathogenic strains of *Staphylococcus aureus* only. If suitability of these tests for investigation of pathogenicity is compared, production of β -hemolysin appears to be the most useful one, since no "false positive" results were found. The use of the CAMP test as a simple and rapid technique to determine production of β -hemolysin by pathogenic strains of animal staphylococci during routine bacteriological work is suggested.

Several papers have been published dealing with the isolation and general characteristics of staphylococci from milk samples. *Staphylococcus aureus* has frequently been found in healthy quarters, but it is most abundant in those affected by some types of mastitis. Moreover, it has been recognized as a common etiological agent of this disease. Kraffenhoft, Adams, and Schipper (1958) reported staphylococci as being the microorganisms most consistently isolated from mastitic and nonmastitic quarters (62 and 76%). Ochi and Katsube (1958) found 42% of the organisms in healthy udders to be staphylococci, and most of these strains were coagulase negative. In mastitic udders, 90% of identified strains were staphylococci. In this instance, approximately half were coagulase-positive.

Some of the most important properties of these microorganisms have been intensively studied to evaluate their potential pathogenicity when they are isolated from bovine milk. Enterotoxigenic strains, especially among coagulase-positive staphylococci, have been recovered

(Evans and Niven, 1950). In addition, routine treatment of streptococcal mastitis with antibiotics may result in the change of the etiological agent, replacing streptococci with strains of staphylococci resistant to these antibacterial drugs.

Some of the characteristics that have been studied in connection with staphylococcal pathogenicity are: coagulase production, mannitol fermentation, gelatin liquefaction, pigmentation, and production of β -hemolysin (Evans, 1948; Clark, Moore, and Nelson, 1961). Data presented by Schalm and Lasmanis (1957) indicate that 90% of coagulase-positive staphylococci isolated from milk can be detected by investigation of β -hemolysin production. Similar results were reported by Loken and Hoyt (1962), who suggested the possibility of using this test to determine potential pathogenicity of staphylococci from milk. The use of washed bovine red blood cells was recommended by Schalm and Woods (1953) to test staphylococci for β -hemolysin production. Clark et al. (1961) employed defibrinated

sheep blood-agar, incubating plates at 37 C for 24 hr, followed by refrigeration for another 24 hr before final examination.

As a result of antibiotic therapy, the frequency of staphylococcal mastitis is increasing in our country. We have considered it of interest to study the more important characteristics of staphylococci isolated from milk.

MATERIALS AND METHODS

A total of 775 strains of staphylococci were isolated from 798 samples of bovine raw milk obtained from individual quarters, including both mastitic and apparently healthy quarters.

To isolate staphylococci, samples of milk were incubated overnight at 37 C and then streaked on Chapman's (1946) Medium 110. After 48 hr of incubation at 37 C, colonies of staphylococci showing different pigmentation were streaked again on this same medium to obtain pure cultures. The strains were submitted to the following tests.

Tube coagulase test. One loopful of culture was emulsified in 0.3 ml of citrated rabbit plasma at a dilution of 1:4 in sterile saline. Tubes were then incubated at 37 C, and final results were observed after 3 hr.

Chromogenesis. As the high sodium chloride content of Chapman's Medium 110 enhances pigmentation of staphylococci, the color of the pigment was observed after 48 hr of incubation on this medium and after exposing the plates to room temperature. Strains were classified in three different groups according to the pigment they produced: those showing golden (ranging from light-cream to deep-orange), white, and yellow-green pigment.

Mannitol fermentation. One drop of bromothymol blue was added to the area from which a colony had been removed for coagulase test, and resulting color was registered. Negative results were indicated by a blue color, and mannitol fermentation was indicated by a change to yellow. Another drop of indicator added to a noninoculated plate was used as control.

Gelatin liquefaction. Stone reaction was conducted on Chapman's Medium 110 after 48 hr of incubation by flooding plates with 5 ml of a saturated solution of ammonium sulfate. After 10 min, clear zones around the colonies were observed in positive cases.

β -Hemolysin production. To determine this hemolysin, the technique described by Christie, Atkins, and Munch-Petersen (1944) was used. It is based on the rapid and strong hemolysis of sheep red blood cells at 37 C in the zone of the plates where β -hemolysin, if produced, encounters some substance liberated by Lancefield group B streptococci, and is known as the CAMP test. This technique has been employed in our country to investigate mastitis-producer streptococci in dairy cattle (Abel, 1953), but it can also be used to determine β -hemolysin if a strain of Lancefield group B streptococcus is employed as the known

factor of the reaction. We have used a serologically identified strain of Lancefield group B streptococcus, which gives a positive CAMP test. This microorganism was streaked on the center of a sheep blood-agar plate in a line dividing the plate in two sections. Staphylococci under test were then streaked at right angles to the streptococcus, but keeping a distance of approximately 1 cm between different staphylococci. Plates were incubated at 37 C, and final results were registered after 24 hr. However, strong hemolysis in the zone of interaction was clearly visible after 6 to 8 hr of incubation. About 12 strains of staphylococci could be tested simultaneously on the same plate, and no refrigeration was required to detect β -hemolysin. In addition, any other hemolysin that might be produced could be easily distinguished from it (Fig. 1).

RESULTS AND DISCUSSION

Table 1 indicates the percentage of strains among coagulase-positive and coagulase-negative staphylococci which showed the following characteristics: golden, white, or yellow-green pigment; production of β -hemolysin; fermentation of mannitol; and liquefaction of gelatin. Staphylococci producing golden pigment represented 95.8% of coagulase-positive strains, 54.2% of the coagulase-negative staphylococci, but pigment production was different in both instances. In fact, pathogenic strains showed a tendency to develop a deep-golden pigment, becoming eventually orange in most instances, whereas non-

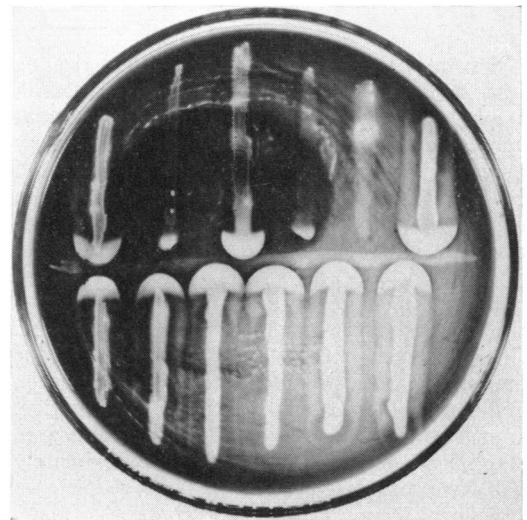


FIG. 1. Sheep blood-agar plate showing detection of β -hemolysin produced by nine strains of staphylococci. Strong hemolysis is observed as semicircular areas in the zone of interaction of β -hemolysin and some substance liberated by streptococcus.

pathogenic strains showed a light-cream pigment. Our results agree with those of Evans (1948), who found that 89% of coagulase-positive staphylococci exhibited an orange pigment. The frequency of isolation of coagulase-positive staphylococcus showing white or yellow-green pigment was very low (2.7 and 1.5%, respectively). These values were 25.1% and 20.8% for coagulase-negative strains.

Production of orange pigment seems to be related to pathogenicity, although the production of white or yellow-green pigments cannot be considered as indicative of nonpathogenic strains. Table 2 shows the percentage of strains positive for the properties studied, among cultures with different pigmentation. Among 104 strains producing white pigment, 11 were coagulase-positive (10.6%), and among 83 strains producing yellow-green pigment, 6 showed this same property (7.2%). Clark et al. (1961) reported that 45% of coagulase-positive strains of staphylococci isolated from milk failed to develop golden pigmentation.

On the basis of these results, it is necessary to emphasize that the possibility of finding coagulase-positive staphylococci which do not produce golden pigment must not be overlooked.

Detection of β -hemolysin is widely used when investigation of staphylococci from animals is required. In the present work, we have tried to assimilate the CAMP test as a simple and rapid technique for its determination. The use of this

method has enabled us to find that 76.5% of strains which are coagulase-positive produce β -hemolysin. Of these, 26 strains were able to produce β -hemolysin only, and the rest showed another type of hemolysin, which was also active on sheep red blood cells at 37 C. However, there was no confusion between these two hemolysins, because β -hemolysin diffused farther from the culture and the strong hemolysis in the zone of interaction was typically candle flame-shaped, with clear-cut margins. The remaining 23.5% of staphylococci were nonhemolytic or produced the other type of hemolysin only.

Elek and Levy (1950) reported that 75% of bovine strains of staphylococci produce β -hemolysin. Clark et al. (1961) found that 96% of bovine strains of staphylococci giving a positive coagulase test result showed this effect. On the other hand, the presence of human staphylococci in cows has been demonstrated (Wallace et al., 1960, 1962) by means of bacteriophage typing. This fact could explain, at least partially, the percentage of staphylococci which are unable to produce β -hemolysin, because human strains rarely show this property. In our work, most of the strains which did not produce β -hemolysin were isolated from a herd periodically submitted to antibiotic therapy. It is possible, therefore, to infer that one selected strain, probably of human origin, had been propagated from cow to cow in this particular herd.

To study the production of β -hemolysin among

TABLE 1. Properties of 775 staphylococcus strains isolated from raw milk as related to coagulase production

Coagulase test	Strains	Per cent positive					
		Pigmentation			β -hemolysin	Mannitol fermentation	Gelatin liquefaction
		Golden	White	Yellow			
Positive	404	95.8	2.7	1.5	76.5	91.8	75
Negative	371	54.2	25.1	20.8	0	16.2	16.7

TABLE 2. Properties of 775 staphylococcus strains isolated from raw milk as related to pigmentation and coagulase production

Pigmentation	Coagulase test	Strain	Per cent positive		
			β -Hemolysin	Mannitol fermentation	Gelatin liquefaction
Golden	Positive	387	79.8	92.8	75.2
	Negative	201	0	17.9	16.4
White	Positive	11	0	72.7	81.8
	Negative	93	0	19.4	26.9
Yellow-green	Positive	6	0	66.7	50.0
	Negative	77	0	7.8	5.2

strains with different pigmentation, we may refer to Table 2, which shows that 79.8% of coagulase-positive staphylococci showing golden pigment exhibited this hemolytic property. It is evident that no positive results were found among coagulase-negative strains, regardless of pigmentation. It appears that production of β -hemolysin characterizes the majority of bovine pathogenic strains of staphylococci showing deep golden pigment.

The CAMP test can be considered a rapid and simple technique which may permit the investigation of β -hemolysin in several strains of staphylococci simultaneously, and it would be of some help in routine investigation of staphylococci from raw milk.

A high percentage of agreement as to a positive test result was found among coagulase-positive strains when mannitol fermentation and gelatin liquefaction tests were conducted (92.8 and 75.2%). However, relatively numerous instances of "false positives" must be taken into account, if these tests are to be used for differentiation between pathogenic and nonpathogenic staphylococci. About 16% of all coagulase-negative strains fermented mannitol and liquefied gelatin, most of them showing light-cream or white pigment. Clark et al. (1961) reported that 84.5% of pathogenic strains fermented mannitol and 88.2% liquefied gelatin.

We may conclude that, of all the characteristics studied in this work, production of β -hemolysin, when compared with the coagulase test, is the property most constantly present in pathogenic strains of animal staphylococci.

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