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Characterization of Staphylococcus Species Isolated From Raw Milk with Special Reference to the Enterotoxigenic Coagulase-Positive Types

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CHARACTERIZATION OF *STAPHYLOCOCCUS* SPECIES ISOLATED FROM RAW MILK, WITH SPECIAL REFERENCE TO THE ENTEROTOXIGENIC COAGULASE-POSITIVE TYPES

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Numerous reports from many parts of the world have described the occurrence of outbreaks of staphylococcus food poisoning. Some studies have incriminated raw milk as a reservoir of the staphylococcus organisms as the vehicle of transmission. Recently Cheddar cheese has been implicated as the contaminating agent in food poisoning outbreaks traced to dairy products.

The frequency of outbreaks of staphylococcus food poisoning has stimulated interest in the characterization of members of the staphylococcus genus. The strains particularly important are those capable of producing an enterotoxin.

In order to determine the strains of staphylococcus organisms isolated from raw milk which are a possible causative agent of food poisoning outbreaks, various physiological tests were systematically conducted. The physiological characteristics studied were coagulase production, gelatin hydrolysis, mannitol fermentation, pigmentation, hemolysis production, antibiotic sensitivity and bacteriophage typing. These tests are of particular interest in evaluating the organisms isolated from raw milk.

REVIEW OF LITERATURE

The staphylococci are members of a large group of micrococci, many species of which are saprophytes and are similar morphologically.

The organism that is primarily responsible for staphylococcal food poisoning is the enterotoxigenic Staphylococcus aureus. The classification as found in Bergey's Manual (2) is as follows:

Order IV: Eubacteriales Buchanan 1917
Family : Micrococceaceae
Genus II: Staphylococcus
Species : aureus

The Staphylococcus species liquify gelatin and ferment carbohydrates, particularly mannitol, glucose, lactose, and sucrose with the production of lactic acid but no gas. These organisms do not form indole, but nitrates are reduced to nitrites and milk is acidified and sometimes coagulated. Strains of the Staphylococcus species develop a golden-yellow water-insoluble pigment while others are non-pigmented. Staphylococcus species are Gram positive spheres in pairs or irregular clusters. They are aerobic and facultatively anaerobic. The cells are non-sporeforming and usually are non-motile. Dack (8) indicated in his study that Staphylococcus species are among the more heat resistant of the non-sporeforming organisms. The thermal death point of the staphylococci is approximately 60°C for 30 to 60 minutes.

The nature of food poisonings may be chemical, parasitic or of bacterial origin. The bacterial type of food poisoning signifies

illness caused by the ingestion of some particle of food, containing sufficient numbers of organisms to produce a toxin.

Tanner and Tanner (28) indicated that food poisoning caused by Staphylococcus species is probably the most common of all food poisonings. The discovery of this organism as a cause of food poisoning explained many outbreaks of gastroenteritis. Diagnosis of these outbreaks was difficult because other agents were formerly sought. Staphylococcus organisms were found but were ignored because the search was being made for members of the Salmonella genus.

Dack (8) pointed out that Staphylococcus species are common microorganisms and are so prevalent that foods can not be protected against them. They are present in the throat, on the skin and in the air, thus having ready access to food. Staphylococcus organisms may grow rapidly in some foods and produce an enterotoxin when suitable temperatures are provided. Such foods as milk, cheese and cream are sometimes involved. Dolman (11) indicated that the term "enterotoxin" was adopted because the toxic substance produced seemed to exert its most conspicuous effects upon the gastrointestinal canal, or enteron, of man.

The number of Staphylococcus species that are capable of producing enterotoxigenic coagulase has been studied by Elek (13). He stated that there was no knowledge of the exact number of staphylococcus strains required in a food product to cause food poisoning. On the other hand, Frazier (20) found that the enterotoxin was produced only

after growth of Staphylococcus species to several million per gram. Dack (8) supported these observations and showed that various kinds of foods involved in staphylococcal food poisoning usually contained hundreds of millions of organisms per gram. Dewberry (9), and Jordon and Burrows (25), pointed out that there is now general agreement that food poisoning is caused by pathogenic coagulase-positive, hemolytic, pigmented Staphylococcus species. Enterotoxigenic strains are generally accepted as indicative of their pathogenicity. The results of experimental research by Evans and Niven (16) and Evans et al. (15) supported the conclusions that "all enterotoxigenic strains produce coagulase, but not all coagulase-positive strains produce enterotoxin."

Attempts have been made by many investigators to solve the problem of distinguishing enterotoxigenic Staphylococcus species from those that are not enterotoxigenic. Hussemann and Tanner (24) accepted the views put forward by many investigators that the physiological tests were not sufficient to separate food poisoning staphylococcus strains from others. Coagulase production and hemolytic properties were considered important.

Evans (14) found that the best indicator of pathogenic potentiality has been the ability to coagulate blood plasma. Other characteristics of some importance were mannitol fermentation, orange pigmentation, and the ability to grow in the presence of 7.5% sodium chloride. According to Pinner and Voldrich (26), pigment formation and its importance in determining the pathogenicity of Staphylococcus

species is apparently an unsound criterion. They were able to show that non-pathogenic S. albus, S. citreus, and S. roseus split off from highly pathogenic S. aureus. These changes in pigmentation occurred often and were a transient matter.

Stritar and Jordon (27) stated that it was difficult for an investigator to be certain of the previous history of a culture. A culture from incriminated food might not be the one which formed the enterotoxin. As a conclusion, pertaining to the physiological tests, Chapman et al. (5) considered the following points as important:

1. A knowledge of the hemolytic activity of staphylococci is insufficient as a simple serologic test of pathogenicity of species.

2. The coagulase activity is an important adjunct to the hemolysis test. Coagulase strains are usually pathogenic regardless of their hemolytic activity.

3. Coagulase-positive strains are usually pathogenic, regardless of the color produced on solid media.

4. On the other hand, the importance of the hemolysis test depends on an accurate determination of the color produced by the strain. Hemolytic non-coagulating albus strains are probably non-pathogenic but hemolytic aureus strains are usually pathogenic, regardless of their coagulase.

5. For the serologic recognition of toxic types of staphylococci, it is imperative to use at least the hemolytic, and coagulase activity tests and a careful determination of the color. With a combination of these three reactions it is possible to estimate the toxicity of a strain with a high degree of precision.

Continued studies of the staphylococcus strain characteristics and isolation methods have led to the development of selective plating

media for the detection of staphylococci. Of special concern are those organisms suspected of being pathogenic or possibly involved in cases of food poisoning.

Chapman (3), (4) has proposed several selective media for Staphylococcus species that have some value in differentiating between coagulase-positive and coagulase-negative strains. The selective action of these media is based on a high sodium chloride content and the use of mannitol as an energy source. Zebovitz et al. (30) pointed out that Tellurite-glycine Agar had been developed and employed for the quantitative detection of coagulase-positive staphylococci. The medium is more selective than any medium that had been previously developed and proposed. The selectivity of this medium is based upon the use of potassium tellurite, glycine and lithium chloride as selective agents, and a final pH of 7.2. The major defect of Tellurite-glycine Agar is the partial inhibition of some coagulase-positive strains, and the failure to inhibit all coagulase-negative strains. The pathogenic coagulase-positive cocci on Tellurite-glycine Agar usually form jet-black colonies within 24 to 36 hours. This medium inhibits the growth of most strains of coagulase-negative cocci and colonies which do develop are generally gray in color. Species of Proteus rarely grow on Tellurite-glycine Agar, but when they do a brown colony is produced. Other organisms as other cocci, coliforms and members of the genera Bacillus and Pseudomonas are inhibited.

Bacteriophages for strains of S. aureus were studied by investigators for the purpose of grouping or classifying the strains.

Fisk (17) (18) developed methods for isolating and cultivating bacteriophages from S. aureus.

Dubos (12) pointed out that

> Phage typing is of especial value in the study of sets of cultures that have been isolated from related sources. Among other applications, the method has been employed to trace the source of staphylococci incriminated in food poisoning.

Phage typing is applicable to the study of coagulase-positive staphylococci, while coagulase-negative strains are not susceptible to the typing phages. A considerable degree of specificity exists among the three groups of phages. As an example, cultures that are susceptible to lysis by group II are not susceptible to lysis by groups I and III.

Foltz et al. (19) in some early studies on bacteriophage typing indicated that a great predominance of the Staphylococcus species isolated from milk, particularly those involving bovine mastitis, were lysed by bacteriophage pattern 42D. Bacteriophage typing has been a useful method to detect and trace the source of food poisoning in dairy and food products.

Staphylococcus species are to be given great consideration when found in milk. Clark et al. (7) found Staphylococcus species to be common in milk and milk products. Heinemann (21) reported that pasteurized skim milk or condensed skim milk have been known to serve as excellent media. Enterotoxigenic strains multiplied rapidly at 95°F and were found to grow over the range of 80°F to 115°F.

Hobbs (23) reported that Cheddar cheese played an important role as a contaminating agent in food poisoning outbreaks. Most of the outbreaks have been caused by raw milk or by the handling of the manufactured products. Likewise, Walker et al. (29) pointed out that Colby cheese with its high moisture content, soft body and open texture appeared to be a good environment for the growth of enterotoxigenic Staphylococcus species.

According to Dack (8) the number of reported cases of food poisoning attributed to milk and its products has been low. Hendricks et al. (22) also stated that

> In recent years, with improved supervision in sanitary production and processing of milk and dairy products and increased use of pasteurized milk, the number of reported cases attributed to milk and dairy products has been low.

MATERIALS AND METHODS

In this study the cultures tested were isolated from raw milk. The milk samples were collected from different sources. Fifty-five of the samples were taken from individual cows and thirty-two samples were collected from herds. The samples were taken aseptically, placed in sterile jars and cooled with ice until used.

Enumeration and Isolation

A 0.1 ml portion of milk was taken from the sample and spread over the dried surface of modified Tellurite-glycine Agar (30) in petri plates. After incubation at 37°C for 48 hours, coagulase-positive Staphylococcus species produced smooth, jet-black colonies. Typical colonies were picked from Tellurite-glycine Agar and streaked onto Staphylococcus Medium No. 110 (S-110) in petri plates (10). Pure cultures were transferred into Brain Heart Infusion and incubated at 37°C for 18 hours. These cultures were later used for the different physiological tests.

Physiological Tests

Coagulase Determination

The tube coagulase test was performed, using an 18 hour broth culture of each isolate. A volume of 0.5 ml of the broth culture was added to a serological tube containing 0.5 ml of fresh rabbit plasma, diluted 1:2 with Brain Heart Infusion. As a control, a tube was

inoculated with a known coagulase-positive Staphylococcus species and a tube of uninoculated plasma was included (10). The tubes were incubated at 37°C, and the coagulum was examined after 1, 2, and 3 hours; and re-examined after 18 hours.

Gelatin Hydrolysis

Staphylococcus Medium 110 plates were flooded with a saturated solution of ammonium sulfate. The plates were placed in the 37°C incubator for 15-20 minutes before gelatin hydrolysis was observed. Gelatinolytic colonies showed a clear zone around the colony.

Mannitol Fermentations

Phenol Red Mannitol Salt Agar was prepared from a dehydrated medium containing 7.5% sodium chloride. The plates were streaked across the surface with a loopful of the broth culture. The plates were incubated at 37°C and any yellow zone appearing around the colony within 48 hours was considered positive for acid production.

Pigment Production

The test was determined by observing the colonies on Staphylococcus Medium No. 110 plates. The production of a varying degree of pigmentation, ranging from a deep golden color to those which showed no pigment formation, was recorded.

Hemolysis Production

Sterile Blood Agar Base medium was cooled to 45-50°C and 5% sterile defibrinated rabbit blood was added aseptically. After careful

mixing to avoid the incorporation of air bubbles, the medium was poured into sterile petri plates. The Blood Agar plates were incubated 12 hours at 37°C to insure sterility. A loopful of the broth cultures was streaked across the surface of the plate prepared with the Blood Agar. The plates were incubated at 37°C for 24 hours and refrigerated for another 24 hours before the final examination of the hemolysis activities was recorded. Cultures which showed any hemolysis were considered to be positive.

Antibiotic Sensitivity

The test was performed by using 0.5 ml of a heavy suspension of an 18 hour broth culture poured into a petri dish with 10 ml of Brain Heart Infusion Agar. After the medium solidified, antibiotic sensitivity disks were placed on the surface of the medium. A flamed forceps was used to handle the disks to avoid contamination. The plates were incubated at 37°C for 12 hours or until growth was developed. Zones of inhibition of growth were examined and measured. The antibiotics and the concentrations used were designated by the following letters:

A--Penicillin . . . . . 10 units
B--Chlortetracycline . . . . . 30 μg
C--Oxytetracycline . . . . . 30 μg
D--Bacitracin . . . . . 20 units
E--Chloromycetin . . . . . 30 μg
F--Dihydrostreptomycin . . . . . 100 μg

Bacteriophage Typing

Bacteriophage sensitivity was determined on Brain Heart Infusion Agar inoculated with sufficient bacterial broth culture of staphylococcus isolates to give heavy growth. A very small drop of diluted bacteriophage lysates were placed at designated positions on the surface of the agar plates. After incubation at 30°C for 12 hours, plates were examined for lysis with the aid of a dark background. The 20 typing bacteriophages used in this study were selected for typing staphylococci of human origin.

Morophological Study

The Gram stain was used and prepared slides were observed using the microscope.

RESULTS AND DISCUSSION

The 87 cultures of Staphylococcus species which were isolated from raw milk were tested for different physiological characteristics. A listing of these characteristics is presented in Table 1.

All isolates were Gram positive cocci occurring singly or in clumps. Slightly higher counts of Staphylococcus species were obtained from Staphylococcus 110 Agar than were obtained when Tellurite-glycine Agar was used. On Staphylococcus 110 medium, the counts ranged from 22 to 30,000 per ml and had a logarithmic average of 1360 per ml. The results obtained on Tellurite-glycine Agar indicated a range of 90 to 21,000 per ml with a logarithmic average of 1150 per ml. This would indicate no real advantage for either medium as a means of enumerating staphylococci.

Zebovitz et al. (30) stated that Tellurite-glycine Agar is superior to other media currently in use for the quantitative detection of coagulase-positive Staphylococcus species. The results of an experiment comparing Tellurite-glycine Agar with Chapman-Stone Agar showed that the Tellurite-glycine Agar partially inhibited some of the coagulase-positive strains, but generally no more than the Chapman-Stone Agar. The majority of the coagulase-negative strains were suppressed by the Tellurite-glycine Agar, but this was not the case when a high salt medium, such as Chapman-Stone Agar, was used.

In this study the staphylococcus counts shown in Table 1 corresponded with the results of Zebovitz et al. (30). The slightly

Table 1. Characteristics of Staphylococcus species isolated from raw milk

Sample no.	Staphylococcus counts		Physiological characteristics				
	Difco S-110	Tellurite-glycine	Coagulase	Gelatin	Mannitol	Pigment	Hemolysis
1C1	2700	2100	-	+	-	White	-
2C1	1200	980	-	-	-	Golden	-
3C1	4800	2920	-	+	+	Golden	+
4C1	1900	980	+	+	+	Golden	+
5C1	1600	1200	-	+	+	Golden	-
6C1	700	500	-	-	+	Golden	-
7C1	16000	9300	-	+	+	Golden	-
8C1	6900	5100	+	+	+	Golden	+
10C1	260	140	-	-	+	Golden	-
11C1	1800	960	-	-	+	Golden	-
12C1	1420	1020	-	-	+	White	-
13C1	28000	17300	-	-	+	Golden	+
14C1	170	950	-	+	+	Golden	+
15C1	110	680	-	-	+	Golden	-
16C1	620	390	-	+	+	White	-
17C1	4510	3980	+	+	+	Golden	+
18C1	2100	1100	-	-	-	Golden	-
19C1	240	1600	-	-	+	White	-
20C1	5200	4700	-	+	+	White	-
21C1	270	190	-	-	+	White	-
24C1	6210	12000	+	+	+	White	+
25C2	540	410	-	+	+	White	-
26C2	120	280	-	-	-	White	-
27C2	980	1200	-	+	+	Golden	-
28C2	270	320	-	-	-	Golden	-
29C2	800	460	-	+	+	Golden	-
30C2	3600	2310	-	-	-	Golden	-
31C2	210	970	-	+	-	Golden	-

Table 1. (continued)

Sample no.	Staphylococcus counts: Difco S-110	Staphylococcus counts: Tellurite-glycine	Physiological characteristics: Coagulase	Gelatin	Mannitol	Pigment	Hemolysis
32C2	7000	1420	-	+	+	White	-
33C2	3000	2420	+	+	+	Golden	+
34C2	2000	1900	-	-	+	Golden	-
35C2	650	1280	-	+	+	Golden	-
36C2	22	150	-	+	+	Golden	-
37C2	22000	16000	+	+	+	Golden	+
38C2	1200	1130	-	+	+	Golden	+
39C2	320	910	+	+	+	Golden	+
40C2	110	280	-	-	+	White	-
41C2	240	210	-	+	-	White	-
42C2	860	1420	-	-	-	Golden	-
43C2	210	280	-	-	+	Golden	-
44C2	3200	1890	-	+	+	Golden	-
45C2	650	450	+	+	+	Golden	+
46C2	710	540	-	+	-	Golden	-
47C2	240	560	-	+	+	Golden	-
48C2	27000	19000	+	+	+	Golden	+
49C2	30000	21000	+	+	+	Golden	+
50C2	1900	2800	+	+	+	Golden	+
51C2	1650	2500	-	-	-	White	-
52C2	420	90	-	-	-	Golden	-
54C2	680	800	+	+	+	Golden	+
55C2	320	250	-	+	-	Golden	-
56C2	25000	13000	-	+	+	Golden	-
57C2	1900	2000	-	+	+	Golden	-
58C2	390	680	-	+	+	Golden	-
59C2	1120	160	-	+	-	White	-
60B	18000	3000	+	+	+	Golden	+

Table 1. (continued)

Sample no.	Staphylococcus counts: Difco S-110	Staphylococcus counts: Tellurite-glycine	Physiological characteristics: Coagulase	Gelatin	Mannitol	Pigment	Hemolysis
61B	12000	15000	+	+	+	Golden	+
62B	1400	1010	+	+	+	Golden	+
63B	2010	1270	+	+	+	Golden	+
64B	1750	1400	+	+	+	Golden	+
65B	1140	750	+	+	+	Golden	+
66B	720	1900	-	+	+	White	-
67B	650	1600	+	+	-	White	+
68B	1200	800	+	+	+	Golden	+
69B	380	460	+	+	+	Golden	+
70W	1600	930	+	-	-	Golden	+
71W	1370	860	+	+	+	Golden	+
73W	260	740	-	-	-	White	-
74W	1800	1200	-	-	-	White	-
75W	4340	2100	-	-	-	Golden	-
76W	6000	4500	+	-	-	Golden	+
77W	3040	1800	-	-	-	White	-
79W	3200	4200	+	+	+	White	+
80W	580	210	+	+	+	White	+
81W	960	120	+	+	+	White	+
82W	1210	920	+	-	+	Golden	+
83W	1520	710	-	-	-	White	-
84W	830	450	+	+	-	White	+
85W	2900	3400	+	-	+	White	+
86W	3000	820	+	+	-	White	+
87W	600	110	+	+	+	Golden	+
88W	380	1340	+	+	+	Golden	+
89W	5300	2000	-	-	-	Golden	+

Table 1. (continued)

Sample no.	Staplylococcus counts		Physiological characteristics				
	Difco S-110	Tellurite-glycine	Coagulase	Gelatin	Mannitol	Pigment	Hemolysis
90W	4200	8360	+	+	+	Golden	+
91W	1010	410	+	+	+	White	+
92W	4500	270	+	+	+	White	+
93W	6540	2140	+	+	+	White	+

lower count obtained on Tellurite-glycine Agar was caused by the inhibiting agents which partially inhibited some of the selected coagulase-positive strains and most of the coagulase-negative strains. The selectivity of the Staphylococcus 110 Agar has been found to be satisfactory, however, it allows both coagulase-positive and coagulase-negative staphylococci to grow. For this reason the count of Staphylococcus species on Staphylococcus 110 Agar was slightly higher.

It is apparent from the results in Table 1 that appreciable variability exists among the 87 isolates of Staphylococcus species. This is particularly true with such qualitative tests as coagulase activity, gelatin hydrolysis, mannitol fermentation, pigment formation and blood hemolysis.

The physiological characteristics of the 87 isolates are summarized in Table 2. A large number of isolates varied in one or more of the physiological properties measured. Of the 87 isolates tested

Table 2. Summary of the physiological characteristics of Staphylococcus species isolated from raw milk

	Coagulase		Gelatin		Mannitol		Pigment		Hemolysis	
	+	-	+	-	+	-	Golden	White	+	-
Number of isolates	37	50	58	29	62	25	58	29	42	45
Percent of isolates	43	57	67	33	71	29	67	33	48	52

37 or 43% were coagulase-positive and 50 or 57% were coagulase-negative. Gelatin was liquified by 67%, mannitol fermented by 71% and a golden pigment produced by 67% of the isolates. Regarding hemolysin production, 48% of the isolates showed hemolysis.

The data presented in Table 3 demonstrate that among the 50 coagulase-negative isolates, 48% gave positive results for gelatinolytic action, 60% were positive for mannitol fermentation, 64% produced a golden pigment but only 10% produced hemolysin. These results show a wade variability among the 50 isolates. The hemolysin test was a very weak criterion in this group of coagulase-negative Staphylococcus species. It appears that the classical tests, such as gelatin hydrolysis, mannitol fermentation and pigment formation are of limited value when the hemolytic action is negative.

Table 3. Summary of the physiological characteristics of the coagulase-negative Staphylococcus species isolated

	Gelatin		Mannitol		Pigment		Hemolysis	
	+	-	+	-	Golden	White	+	-
Number of isolates	24	26	30	20	32	18	5	45
Percent of isolates	48	52	60	40	64	36	10	90

The physiological characteristics of 37 coagulase-positive Staphylococcus species are presented in Table 4. Of the 37 isolates,

Table 4. Physiological characteristics of coagulase-positive *Staphylococcus* species isolated from raw milk

Isolate number	Coagulase	Gelatin	Mannitol	Pigment	Hemolysis	
					Alpha	Beta
4C1	3+	+	+	Golden		+
8C1	3+	+	+	Golden	+	+
17C1	2+	+	+	Golden	+	+
24C1	3+	+	+	White	+	+
33C2	4+	+	+	Golden	+	+
37C2	4+	+	+	Golden	+	+
39C2	4+	+	+	Golden	+	+
45C2	4+	+	+	Golden	+	+
48C2	4+	+	+	Golden	+	+
49C2	4+	+	+	Golden	+	+
50C2	2+	+	+	Golden	+	+
54C2	4+	+	+	Golden		+
60B	4+	+	+	Golden		+
61B	4+	+	+	Golden	+	+
62B	4+	+	+	Golden		+
63B	4+	+	+	Golden		+
64B	4+	+	+	Golden		+
65B	4+	+	+	Golden	+	+
67B	2+	+	-	White		+
68B	4+	+	+	Golden		+
69B	4+	+	+	Golden		+
70W	2+	-	-	Golden		
71W	2+	+	+	Golden		+
76W	2+	-	-	Golden	+	+
79W	2+	+	+	White	+	+
80W	2+	+	+	White	+	+
81W	2+	+	+	White	+	+
82W	2+	-	+	Golden	+	+
84W	2+	+	-	White		+
85W	2+	-	+	White	+	+
86W	2+	+	-	White		+
87W	2+	+	+	Golden		+
88W	2+	+	+	Golden	+	+
90W	4+	+	+	Golden		+
91W	2+	+	+	White	+	+
92W	2+	+	+	White	+	
93W	3+	+	+	White	+	+

17 showed definite visible clotting for the coagulase-positive test after two hours incubation at 37°C. Of the remaining 20 isolates, four showed clotting after 3 hours incubation and 16 demonstrated a definite clot after 4 hours.

The results shown in Table 5 indicate that of the 37 coagulase-positive isolates 89% produced gelatinase, however, 11% failed to hydrolyze gelatin even though they were coagulase-positive. The mannitol fermentation showed 87% capable of fermenting the carbohydrate within 48 hours. Negative results were obtained with 13% of the isolates. Pigment formation was variable, ranging from 70% which produced a golden color to 30% that produced a white color.

With regard to hemolysin production, 97% of the isolates showed definite hemolysis, with only 3% failing to produce hemolysin. Of the 36 coagulase-positive isolates shown in Table 4 which produced hemolysin, 3% of the strains exhibited the alpha pattern, 39% showed a beta pattern and 58% elaborated an alpha and beta pattern.

Table 5. Summary of physiological characteristics of the coagulase-positive Staphylococcus species isolated from raw milk

	Gelatin		Mannitol		Pigment		Hemolysis	
	+	-	+	-	Golden	White	+	-
Number of isolates	33	4	32	5	26	11	36	1
Percent of isolates	89	11	87	13	70	30	97	3

In comparing Table 5 with Table 3 it is indicated that a more consistent relationship appeared to exist among the physiological characteristics and activities in Table 5 than existed in Table 3. It is apparent that the gelatin, mannitol and pigment activities are more important in interpreting the physiological tests for isolation of the coagulase-positive strains than for the coagulase-negative strains. This is particularly true when positive tests for hemolysin and coagulase are first employed for isolation and detection. Similar views can be expressed concerning the value of the coagulase-positive and the hemolysis tests. The value of the other physiological tests can be considered important when the coagulase and hemolysis characteristics are determined first.

Chapman et al. (5) (6) pointed out that for the serologic recognition of toxic types of Staphylococcus species it is necessary to use a combination of the results from the hemolysis reaction, the coagulase activity and a careful determination of chromogenesis. With the reactions from these three tests it is possible to estimate the toxicity of a certain strain. Hussemann and Tanner (24) pointed out that chromogenesis alone is of no value in determining the enterotoxigenic coagulase-positive strains.

The occurrence of antibiotic resistant Staphylococcus species in dairy products has been considered a great possibility. The antibiotic sensitivity discs used in this study are the following:

A-Penicillin - - - - - - - - - - 10 units
B-Chlortetracycline - - - - - - - 30 μg
C-Oxytetracycline - - - - - - - - 30 μg
D-Bacitracin - - - - - - - - - - 20 units
E-Chloromycetin - - - - - - - - - 30 μg
F-Dihydrostreptomycin - - - - - - 100 μg

The sensitivity discs were read and classified by measuring the zone of inhibition. The limits set for this classification are as follows:

Highly sensitive - - - - - - - - 11 to 17 millimeters
Sensitive - - - - - - - - - - - 6 to 10 millimeters
Slightly sensitive - - - - - - - 2 to 5 millimeters
Resistant - - - - - - - - - - - less than 2 millimeters

The results in Table 6 indicate wide variation of antibiotic sensitivity by the coagulase-positive strains. Most of the isolates of Staphylococcus species studied were sensitive to antibiotics of higher concentrations.

Results shown in Table 7 indicate that 46% of the isolates were resistant to penicillin, 27% resistant to chlortetracycline, 35% resistant to oxytetracycline, 43% resistant to bacitracin, 27% resistant to chloromycetin and 32% resistant to dihydrostreptomycin.

It is important to note that the percentage of penicillin and bacitracin resistant Staphylococcus species was relatively high. Elek (13) pointed out that several factors may be involved in the increase of antibiotic resistant Staphylococcus species. The increased incidence of strains resistant to a given antibiotic is related to the extent to which that antibiotic is used. Antibiotics, such as penicillin and bacitracin, which have been used therapeutically in cattle,

Table 6. The effect of antibiotics on coagulase-positive Staphylococcus species isolated from raw milk

Isolate number	Antibiotic used					
	A	B	C	D	E	F
4C1	R	S	S	HS	HS	SS
8C1	HS	HS	HS	HS	HS	R
17C1	R	R	R	SS	SS	R
24C1	SS	HS	HS	HS	HS	R
33C2	HS	SS	R	R	SS	SS
37C2	HS	R	R	S	SS	SS
39C2	HS	SS	S	S	R	HS
45C2	SS	HS	SS	S	R	HS
48C2	SS	HS	SS	R	R	HS
49C2	HS	HS	R	R	SS	R
50C2	SS	HS	R	SS	HS	HS
54C2	HS	R	SS	R	HS	HS
60B	R	HS	HS	R	R	R
61B	HS	HS	HS	S	HS	S
62B	S	HS	HS	HS	S	SS
63B	R	R	HS	SS	HS	SS
64B	HS	HS	S	S	R	HS
65B	HS	HS	HS	S	R	SS
67B	SS	HS	S	R	HS	S
68B	R	HS	R	HS	HS	SS
69B	R	HS	HS	R	R	HS
70W	SS	SS	SS	SS	SS	R
71W	R	R	HS	SS	HS	R
76W	SS	R	R	R	HS	HS
79W	S	R	R	S	HS	R
80W	R	R	HS	R	HS	R
81W	R	R	HS	R	S	R
82W	R	HS	R	R	HS	HS
84W	R	HS	SS	S	SS	HS
85W	SS	HS	SS	S	HS	SS
86W	R	S	HS	HS	R	HS
87W	SS	S	S	S	R	R
88W	R	SS	R	R	R	R
90W	R	SS	R	R	SS	HS
91W	R	R	R	R	HS	HS
92W	R	S	R	R	SS	HS
93W	R	S	S	R	SS	HS

HS - Highly sensitive
S - Sensitive
SS - Slightly sensitive
R - Resistant

Table 7. Summary of antibiotic resistance of coagulase-positive Staphylococcus species isolated from raw milk

	Isolates resistant to antibiotics					
	A	B	C	D	E	F
Number of isolates	17	10	13	16	10	12
Percent of isolates	46	27	35	43	27	32

may have increased the resistance of the staphylococci isolated. Elek (13) also recorded that some penicillin resistant staphylococcus organisms owe their antibiotic resistance to the formation of penicillinase. This is an enzyme capable of destroying penicillin.

The results in Table 8 show that 24% of the isolates were highly sensitive to penicillin, 46% highly sensitive to chlortetracycline, 32% highly sensitive to oxytetracycline, 16% highly sensitive to bacitracin, 43% highly sensitive to chloromycetin and 41% highly sensitive to dihydrostreptomycin.

The coagulase-positive isolates in Table 8 showed variable response to being sensitive to the antibiotics used. Three of the antibiotics (chlortetracycline, chloromycetin and dihydrostreptomycin) proved to be quite effective by exhibiting wide zones of inhibition.

Table 8. Summary of coagulase-positive Staphylococcus species highly sensitive to antibiotics

	Isolates highly sensitive to antibiotics					
	A	B	C	D	E	F
Number of isolates	9	17	12	6	16	15
Percent of isolates	24	46	32	16	43	41

Bacteriophage patterns of the 37 coagulase-positive cultures are shown in Table 9. Twenty-one isolates were sensitive to bacteriophage types considered to be of human origin while 16 of the isolates were not lysed. Three of these 21 isolates were lysed by a single bacteriophage strain, three were lysed by two bacteriophage strains, and one was lysed by three bacteriophage strains. The remainder of the 21 isolates were lysed by more than three bacteriophage strains. Bacteriophage strains 54, 42E and 81 were the most active strains in this study. Fourteen strains of coagulase-positive staphylococci were lysed by bacteriophage 54, 12 strains were lysed by bacteriophage 42E and 11 strains were lysed by bacteriophage 81.

Blair (1) reported that a relationship existed between the hemolysin test and bacteriophage typing on the origin of staphylococcus strains. In the present investigation, a relationship between lysis by bacteriophage strains and the hemolysis pattern was not found. Hendricks et al. (22), and Hobbs (23) reported that

Table 9. Bacteriophage sensitivity patterns of coagulase-positive Staphylococcus species isolated from raw milk

Isolate number	Bacteriophage sensitivity
4C1	No lysis
8C1	42E/54/80W/81
17C1	3A/3B/3C/29/52A
24C1	3A/42E/54/71/79/80/81
33C2	42E/52/52A/54/79/80/81
37C2	42E/52/54/71/80/81
39C2	42E/54/81
45C2	3A/42E/54/79/80/81
48C2	3A/3C/54/80
49C2	52A/54/79/80
50C2	55/71
54C2	3A/3C/52/52A/54/55/71/79/80/81
60B	No lysis
61B	No lysis
62B	No lysis
63B	No lysis
64B	42E
65B	No lysis
67B	No lysis
68B	No lysis
69B	No lysis
70W	42E
71W	No lysis
76W	No lysis
79W	3A/3B/3C/54/79
80W	No lysis
81W	3A/54
82W	42E
84W	25/54/80/81
85W	No lysis
86W	42E/54/80/81
87W	No lysis
88W	42E/81
90W	No lysis
91W	No lysis
92W	6/7/42D/42E/47/75/81
93W	52/52A/54/81

coagulase-positive staphylococci lysed by bacteriophage strains 6, 42D, 47, 53, 54, 73, 75, 77 and 83 have been implicated in staphylococcus food poisonings by various workers. However, Clark et al. (7) pointed out that bacteriophage typing proved to be an unreliable basis for differentiating enterotoxigenic strains.

SUMMARY AND CONCLUSIONS

Eighty-seven raw milk samples were examined from which 50 isolates of coagulase-negative and 37 isolates of coagulase-positive staphylococci were obtained.

The 87 isolates were tested for various physiological tests, such as coagulase production, gelatin hydrolysis, mannitol fermentation, pigment formation, hemolysis, antibiotic sensitivity and bacteriophage typing.

The results showed that a large number of these isolates varied in one or more of the physiological characteristics measured. Of the 87 isolates tested 37 or 43% were coagulase-positive and 50 or 57% were coagulase-negative.

Representative strains of the 37 coagulase-positive Staphylococcus species have been studied. Their physiological activities showed a high level of comparison. The coagulase, gelatin, mannitol, pigment and hemolysis activities played an important part in interpreting the physiological tests for isolation of the coagulase-positive strains.

The coagulase activity and hemolysis tests yielded the only important information in determining the ability of the culture isolates to be coagulase-positive and possibly pathogenic.

Gelatin hydrolysis, mannitol fermentation and pigment formation were more useful for isolation than for differentiation, when they are not combined with the coagulase and hemolysis tests.

Apparently all the coagulase-positive isolates studied were sensitive to antibiotics of higher concentrations.

Culture isolates of coagulase-positive staphylococci were typed by human staphylococcal bacteriophages. The comparison of bacteriophage patterns was discussed. A relationship between the bacteriophage pattern and hemolysis of the coagulase-positive isolates of staphylococci could not be shown in this study.

Raw milk may be a reservoir of staphylococcus types. Many of these types showed by their physiological characteristics that they are frequently associated with human origin.

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